On the Neurobiology of Physical Activity in Mice and Human

In Search of Eating Disorders Determinants

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On the Neurobiology of Physical Activity in Mice and Human

In Search of Eating Disorders Determinants

Over de Neurobiologie van Lichaamsbeweging in Muizen en Mens

Op Zoek naar Determinanten van Eetstoornissen

(met een samenvatting in het Nederlands)

Badania nad neurobiologicznymi podstawami aktywności fizycznej myszy i ludzi

W poszukiwaniu determinantów zaburzeń odżywiania

(ze streszczeniem w języku polskim)

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General Introduction

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Physical Activity

Definitions

Physical activity (PA) may be defined as 'bodily movement produced by contraction of skeletal muscles' (Dishman 2008) and be further subdivided into spontaneous physical activity (SPA) and voluntary exercise (VE). The VE is defined as 'locomotor activity that is not directly required for survival or homeostasis and not directly motivated by any external factor' (Garland, Jr. et al., 2011b) which could be understood as sports and fitness-related activities. All remaining PA such as activities of daily life, non-specific ambulatory behavior and maintaining posture may be considered SPA (Garland, Jr. et al., 2011b; Levine et al., 1999). It is not always easy to classify a particular type of PA into one of the two categories; however, this division enables some conceptualization and research on effects of PA and voluntary exercise in particular on health.

Operationalization of physical activity in humans

Voluntary exercise

Various methods of assessment may be used to study PA levels in humans e.g. direct observation, questionnaires (diaries and retrospective recall), surveys, calorimetry, heart rate monitors and motion sensors (Garland, Jr. et al., 2011b; Westerterp 2009). Choice between the methods involves a trade-off between feasibility and reliability. As a result of practical difficulties related to the use of objective methods, many studies involve the self-reported PA assessment (Dishman 2008) which may have limited reliability and validity (Shephard 2003). Attempts to validate the self-reported measurement methods show that the correlations between objective and subjective measurement methods are rather low (de Vilhena e Santos DM et al., 2012; Dishman 2008; Garland, Jr. et al., 2011b). Furthermore, various PA assessment methods lead to discrepant results regarding genetic associations and heritability estimates (Butte et al., 2006; Cai et al., 2006; Choh et al., 2009; de Vilhena e Santos DM et al., 2012; Seabra et al., 2008; Simonen et al., 2003a). These observations suggest that various PA assessment methods measure, in fact, partially different phenotypes or theoretical constructs (de Vilhena e Santos DM et al., 2012). A possible solution to this problem would be to involve both objective and subjective PA assessment methods in one study. It would enable comparison of the methods within one

population and give detailed assessment of different features of PA such as duration, type, context, motivation and energy expenditure (Dishman 2008).

Spontaneous physical activity

In humans, there is a partial overlap between methods that can be used to assess VE and SPA. Generally, pedometers, accelerometer and observation (direct or video recording) may be used to estimate the levels of SPA (Garland, Jr. et al., 2011b).

Animal models of physical activity

Animal models are often used to overcome some of the limitations hampering human studies. They enable standardization of the complex conditions influencing the levels of PA and methods of PA measurement. Therefore, it is hypothesized that they may be effectively used to assess biological factors influencing PA levels. The VE and SPA in humans may be best modeled by distinct animal models (Eikelboom 1999; Garland, Jr. et al., 2011b; Kelly et al., 2010; Rezende et al., 2009). In general, (voluntary) running wheel activity (RWA) is proposed as a model of voluntary exercise (Kelly and Pomp 2013; Rezende et al., 2009) while spontaneous locomotor activity, especially in the home cage (HC) environment, may be considered a model of SPA (Garland, Jr. et al., 2011b). The expressions of the two forms of energy expenditure (RWA and HC activity) are not independent. When animals are given access to a running wheel they reduce their HC activity (Koteja et al., 1999) while their total time spent on activity increases (de Visser L. et al., 2005; Kas and Edgar 1998). Furthermore, mice bred for high running wheel activity show also a reduction in the total home cage activity (Careau et al., 2012; Malisch et al., 2009) and the RWA and HC activity weakly correlate in the outbred strain used in these selection experiments (Zombeck et al., 2011).

Voluntary exercise

If voluntary exercise is defined as locomotor activity 'that is not directly required for survival or homeostasis and not directly motivated by any external factor' (Garland, Jr. et al., 2011b), then voluntary RWA may be considered the most suitable rodent behavior to model human voluntary exercise (Eikelboom 1999; Garland, Jr. et al., 2011b; Kelly et al., 2010; Rezende et al., 2009). There are several arguments to support this notion. First, RWA in rodents, similarly to human VE, is performed without any obvious immediate goals (Eikelboom, 1999). It may be considered a classic example of self-rewarding behavior (Brene et al., 2007; Garland, Jr. et al., 2011b; Kagan and Berkum 1954; Novak et al., 2012; Sherwin 1998). Indeed, rodents show conditioned

place preference to the location associated with wheel running (Lett et al., 2000) and are willing to perform an instrumental reaction in order to obtain access to the running wheel (Belke and Garland, Jr. 2007; Kagan and Berkum 1954). Secondly, age-related decline in PA is observed in both, humans and rodents (Ingram 1983; Sallis 2000) (see also further in this chapter). Thirdly, both VE and RWA may in some situations involve changes in brain reward systems that are comparable to the ones present in addictive behavior (Belke and Garland, Jr. 2007; Garland, Jr. et al., 2011b). Finally, voluntary wheel running and VE occur in environments which per se require low energy expenditure (laboratory housing in case of mice or modern industrialized Western culture) (Booth et al., 2002; Eikelboom 1999; Garland, Jr. et al., 2011b).

It is of note that there are also some arguments against the validity of RWA as a model of VE. First, gender differences in PA are observed in both humans and mice, however, in an opposite direction suggesting important differences in biological determinants of the behavior (see further in this thesis). Second, some researchers believe that wheel running is merely an artifact resulting from poor laboratory housing conditions (Sherwin 1998). Third, it was estimated that laboratory mice went through selection for more than 400 generations, which resulted in diminished PA levels. In contrast, humans from Western cultures are said to go through similar environmental pressure for no more than 25 generations (Booth et al., 2002; Garland, Jr. et al., 2011b). As a consequence, it is possible that the natural state for the laboratory mouse is to maintain low PA levels while for human it is to continue to exercise (Booth et al., 2002; Garland, Jr. et al., 2011b). This raises questions regarding the validity of using mouse models as a model of normal human behavior and physiology (Garland, Jr. et al., 2011b). Finally, some researchers point to the 'behavioral common path' as an argument against possibility to use RWA as a model of VE (Sherwin 1998). According to this notion, various internal and external factors lead to the occurrence of a given animal behavior because the repertoire of behaviors is limited. Therefore, it is not possible to deduct underlying mechanisms and motivations just on the basis of the observed phenotype (Sherwin 1998). In summary, substantial arguments support validity of RWA as a model of VE, however, caution needs to be taken as far as the translational value of the model is concerned due to some arguments against its use. It is possible that although VE and RWA are artifacts occurring in particular environments, they result from evolutionarily conserved ability and motivation to stay active in order to obtain food (despite pain, effort and potential danger).

Spontaneous physical activity

Most of the energy expenditure of a laboratory animal may be considered an analogue to human SPA e.g. locomotor activity, rearing and grooming (Garland, Jr. et al., 2011b). In an absence of a running wheel and mating partners, any activity not associated with food intake could be considered SPA. SPA in rodents may be measured using various methodologies, for example, video analysis, force plates or infrared beams (Garland, Jr. et al., 2011b), which can be used to detect a movement in a novel or familiar environment. One should note that the behavior of an animal in the novel environment (such as in the OFT) is influenced by novelty-induced anxiety. Furthermore, the readout often includes only locomotor activity, although SPA includes also other behaviors such as grooming. Therefore, the most adequate tool to measure SPA in mice is HC environment and preferably includes locomotor activity and total motility of an animal. HC phenotypes can be assessed under baseline conditions, without human interference, and across the circadian cycle (Kas and Van Ree 2004).

Argument in favor of biological basis of PA

The significant role of PA in energy homeostasis constitutes another strong argument in favor of the notion that voluntary PA is under strong biological control (Rowland 1998). According to the energy balance equation, all the energy taken in by an organism must be expended or transformed by this organism. Thus, the total energy intake will be counterbalanced by the energy expended, needed for reproduction, growth, stored as fat or glycogen and dispersed due to fecal and urinary loss. As far as the first component of the equation is concerned, the energy is expended on basal metabolic rate, digestion and processing of food, thermoregulation and PA (Garland, Jr. et al., 2011b). It is of note that PA may constitute a substantial portion of daily energy expenditure of an organism depending on the species, environment and age (Rezende et al., 2009).This function of PA in maintaining energy homeostasis caused researchers to speculate that there must be a mechanism controlling this form of energy expenditure, so called 'activity-stat' (Rowland 1998). Various arguments support this notion, including heritability of PA, age dependent-decrease of activity (Sallis 2000), sex differences in activity levels, biorhythmicity (Rowland 1998; Wade et al., 1973) of PA and finally compensatory increases or decreases of PA when other elements of the energy balance equation are changed (Epstein and Wing 1980; Goran and Poehlman 1992). Some of these arguments are discussed further in this introduction.

Heritability of PA in humans and mice

Human

Genetic contribution to physical exercise was partly supported by human family studies which showed that broad-sense heritability ranges from 0 to 60% depending on the population tested, the exact phenotype measured and the method of measurement. The highest heritability estimates come from studies using objective measurement of PA such as accelerometer instead of subjective measurements such as questionnaires (de Vilhena e Santos DM et al., 2012). This may point to the fact that the genetic basis of PA is more adequately estimated when more precise phenotyping methods are used. Further support of heritability of PA comes from various twin studies which estimated the broad-sense heritability of PA in adults to fall between 16 and 80% (Beunen and Thomis 1999; De Moor et al., 2011; Eriksson et al., 2006; Kujala et al., 2002; Lauderdale et al., 1997; Simonen et al., 2002; Stubbe et al., 2006) and in case of children and adolescent to stay in a range from 0% (Stubbe et al., 2005) to 92% (Wood et al., 2008) (for an extensive review see (de Vilhena e Santos DM et al., 2012)).

Mouse

Finally, the heritability of PA, measured as RWA and HC activity, was estimated for various mouse populations. Heritability estimates for RWA in 13 mouse inbred strains varied between 12 to 66 percent (Lightfoot et al., 2004; Lightfoot et al., 2008). This was further supported in a study using a panel of 41 inbred strains which showed similar (but higher) broad-sense heritability for distance (55%), duration (57%) and speed (60%) (Lightfoot et al., 2010). Furthermore, narrow-sense heritability of mean revolutions run per day on a RW in an outbred strain (Hsd:ICR) estimated by midparent-offspring regression was 18% (Swallow et al., 1998). Finally, the realized heritability (adjusted for within-family selection) was estimated to be on average 28% for lines selected for high running wheel activity (Swallow et al., 1998).

Estimation of heritability for a total PA in HC delivered divergent results ranging from low value of 14% (Toth and Williams 1999) to a relatively high value of 62% (Umemori et al., 2009). Interestingly, it seems that only activity in the dark, thus the active period of the day, has strong genetic component (with estimated heritability of 18%) while heritability of PA during the light phase is neglectable (5%) (Toth and Williams 1999).

Age effects on heritability and secular trends for physical activity

Human

Despite the strong genetic component, PA is not stable over the life-span. Multiple studies have shown age effects on PA participation and intensity (Sallis 2000). The reasons for this phenomenon are not well understood, however, this finding is very consistent in various human populations examined (Sallis 2000). In general, childhood is a life period with highest PA levels, followed by a strong decline during adolescence and early adulthood, relative stability of PA in middle aged people and further decline in elderly (Anderssen et al., 1996; Bijnen et al., 1998; Nelson et al., 2006; Sallis 2000; Schoenborn 1986; Telama et al., 1996).

As a consequence, the strength of influence of the genes on levels of PA is modulated by age. Stubbe and colleagues showed, using a sample of more than 1600 twin pairs, that the heritability of sport participation increased from 0% in adolescents to 85% in young adults (Stubbe et al., 2005). In the group with low genetic contribution to PA, the most profound was the effect of shared environmental factors (78-84%) (Stubbe et al., 2005). However, this observation was not supported by a follow-up study on a Finish cohort in which the genetic influence on PA seemed to decrease with age (Aaltonen et al., 2010). It is possible that some genetic or environmental factors are responsible for this discrepancy.

Mouse

Similar age-dependent decline in PA was reported multiple times for various species (Ingram 2000) including mice for both RWA (Ingram 1983) and HC activity (Goodrick 1975; Ingram 1983; Ingram and Reynolds 1986). It is of note that genetic background influences the effect of age on PA. Mouse inbred strains differ in the temporal pattern and extent of age-dependent decline for RWA and locomotor activity in the OFT (Lhotellier and Cohen-Salmon 1989).

Influence of sex on heritability and secular trends for physical activity

Human

Not only age but also sex have been shown to influence PA participation and intensity as well as on the genetic architecture of PA (Beunen and Thomis 1999; Boomsma et al., 1989; De Moor et al., 2007a; Maia et al., 2002). It was repeatedly shown that genetic factors are more relevant in determining the levels of PA in boys than in girls (Beunen and Thomis 1999; Boomsma et al., 1989; De Moor et al., 2007a).

For example, in a Portuguese twin population, in case of boys, genetic factors had a major influence on participation in sports while for girls genetic, common and unique environmental factors contributed evenly to the phenotype (Maia et al., 2002). This points out to the fact that shared environment (family and peers) and unique environmental factors have much stronger influence on exercise behavior in case of females than in males. The observed differences in genetic and environmental determinants are reflected on the level of observed PA phenotype. There are clear gender differences in the amount of sport participation with males being more active than girls at all age groups (Schoenborn 1986). Furthermore, the age-dependent decrease in PA is more pronounced in women than in men (Sallis 2000).

Mouse

In case of mice, the estimations of heritability of PA also differ between sexes. Analogous to observations from human studies, male mice are characterized by stronger influence of genetic factors for the measured aspects of RWA. Studies on a panel of 13 inbred strains showed that the estimates of heritability are as follows: for distance moved, male 31-48% and female 12-22%; for duration, male 44-61% and female 12-21%; for velocity, male 49-66% and female 44-61% (Lightfoot et al., 2004). Furthermore, in some studies, sex-specific QTLs for RWA were found (Leamy et al., 2010; Nehrenberg et al., 2010).

The sex effects regarding heritability of PA are reflected on the level of observed phenotype (Garland, Jr. et al., 2011a; Leamy et al., 2010). Contrary to observations in humans, however, female mice tend to be more active than male mice at least if the activity is measured in the running wheel (Lightfoot et al., 2004; Lightfoot et al., 2010). Various sex differences have been observed in multiple studies, for example, using a panel of 13 inbred strains Lightfoot and colleagues showed that females of F_2 population between C57L/J (high active) and C3H/HeJ (low active) strains run 49% further, 9% faster and 39% longer during a day measurement (Lightfoot et al., 2004). These results were replicated in another study using 41 inbred strains (Lightfoot et al., 2010). When data of all the strains were pooled together, there was a significant effect of strain, sex and the interaction between strain and sex on the parameters of RWA (distance, speed and duration); namely, female mice ran 24% further (24%), 11% faster and 13% longer (13%) than male mice (Lightfoot et al., 2010). However, after correction for multiple testing, the differences between genders were significant in case of only 7 strains for distance, 5 strains for speed and 5 strains for duration (Lightfoot et al., 2010). Finally, Careau and colleagues (2012) could show in an experiment involving lines selectively bred for high RWA that female mice from both

control and high running lines run longer and further than male mice. Interestingly, in these mouse lines selectively bred for high RWA, both male and female mice increased the RWA levels; however, the increase in daily energy expenditure was much higher for male mice (Rezende et al., 2009) because females reached higher PA by running faster while males run longer (Rezende et al., 2009).

Possible mechanisms that may cause the phenotypic and genetic sex differences in PA in mice may involve the endocannabinoid system and sex hormones (Keeney et al., 2008; Lightfoot 2008) as it is known that estrogens strongly modulate physical activity (Morgan et al., 2004).

Genetic basics of PA and physical inactivity

Several facts need to be considered before we can proceed with summing up the findings regarding genetic basis of PA. First, it is of a note that two components influence the levels of PA, namely the physical ability and motivation to perform PA (Dishman 2008; Garland, Jr. et al., 2011b; Kelly and Pomp 2013). Furthermore, it is likely that ability depends on physiological factors such as respiratory endurance (Rankinen et al., 2010) while motivation is regulated by central nervous system functioning (Rhodes et al., 2003). Therefore, these two components may have particular genetic basis (Garland, Jr. et al., 2011b) and work in synergy to produce the final observed PA level (Kelly and Pomp 2013).

Second, genetic architecture of PA is undoubtedly complex, as of any other complex phenotype. Numerous genetic loci with mostly minor effect size contribute to PA levels (Dishman 2008). The effect sizes of these loci are most likely influenced by individual- and species-dependent genetic background. Furthermore, many of the loci influencing PA are placed in the regulatory elements, outside of the protein coding genes, which may make it difficult to translate the findings between species. Finally, many of the loci may have pleiotropic effects (e.g. (Garland, Jr. et al., 2002; Hanson and Hakimi 2008)). Their influence on a given phenotype may be also mediated by their effect on other related phenotypes; for example, there is a 40% overlap between genes which were shown in humans to influence PA and obesity, two phenotypes that reciprocally influence their levels (Good et al., 2008).

Third, various authors pointed out that PA and physical inactivity (PI) may be considered distinct phenotypes and not two poles of one continuum of activity (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Perusse et al., 1989). This statement

may find some confirmation in a meta-analysis by Viggiano who has shown that distinct genetic manipulations or brain lesions lead to either high or low novelty-induced locomotor activity (Viggiano 2008). Furthermore, it was suggested that the brain is prone to hyperactivity, and this hyperactive tone is down-regulated by brain regions and neurotransmitter systems that decrease the PA (Rowland 1998; Viggiano 2008). It was proposed that the reticular activating system is responsible for arousal while cerebral cortex is mostly inhibitory (Rowland 1998). All in all, the interplay of the activating and inhibiting systems serves the purpose of maintaining so called 'sensoristasis' (term created by Schultz in 1965) which is an optimal level of sensory stimulation of the nervous system for each individual (Rowland 1998). An individual may regulate the level of stimulation by regulating PA levels but also by engaging in intellectually stimulating tasks that may require marginal levels of PA. Therefore, PA and PI may be two distinct behaviors which are used to maintain the balance in terms of sensory stimulation and energy equation. Each of them may have a distinct biological (including genetic) basis and a complex set of possible motivations to engage in them.

Finally, in this introduction we review only the significant genetic association with duration or intensity of PA. Reviews on genetic polymorphisms associated with performance phenotypes are reviewed elsewhere and are beyond the scope of this thesis (Bray et al., 2009; Hagberg et al., 2011; Rankinen et al., 2010; Roth et al., 2012).

Human studies

De Vilhena a Santos and colleagues, in an excellent review, gathered studies which assessed the genetic basis of PA and PI in humans (de Vilhena e Santos DM et al., 2012). The overlap between genetic regions found in these studies is somewhat small (de Vilhena e Santos DM et al., 2012). This may be explained by the following two reasons. First, cited studies used a variety of measurement methods and were conducted in various populations and age groups (de Vilhena e Santos DM et al., 2012). Second, the number of studies conducted up to date for both PA and PI is still very low (especially if one takes under consideration that PA and PI are polygenic traits). As far as PA is concerned, there are only 16 studies examining its genetic basis: four linkage studies (Cai et al., 2006; De Moor et al., 2007a; De Moor et al., 2007b; Simonen et al., 2003b), eleven association studies (Berentzen et al., 2008; Cole et al., 2010; De Moor et al., 2009; Fuentes et al., 2002; Hakanen et al., 2009; Liu et al., 2010; Loos et al., 2005; Lorentzon et al., 2001; Richert et al., 2007; Simonen et al., 2003a; Stefan et al., 2002; Winnicki et al., 2004) and one GWAS study (De Moor et al., 2009). Only a small proportion of the above mentioned studies assessed the genetic

basis of PI: two linkage studies (Cai et al., 2006; Simonen et al., 2003b) and two association studies (Loos et al., 2005; Winnicki et al., 2004).

Nevertheless, significant genetic associations were found for both phenotypes. The significant genetic association for PA was found in nine of the studies mentioned above and pointed to fifteen candidate genes or genetic regions that may be involved in the regulation of intensity or duration of PA in humans (de Vilhena e Santos DM et al., 2012). A short summary of these significant findings is shown in Table 1.1 (page 19). Interestingly, melanocortin 4 receptor (*MC4R*) gene was repeatedly associated with levels of PA, when PA was measured with the B3DPAR questionnaire (Loos et al., 2005) or accelerometer (Cole et al., 2010). Furthermore, Cai et al. have shown a significant association of the genetic region containing *MC4R* with the PA measured by accelerometer (Cai et al., 2006). As far as PI is concerned, all four cited studies showed a significant association, pointing to six candidate genes or genetic regions involved in regulation of PI (Table 1.2, page 20). Interestingly, *MC4R* gene was associated with PI measured with the B3DPAR questionnaire (Loos et al., 2005). Furthermore, the genetic region containing *MC4R* gene showed a significant association with sedentary activity measured by accelerometer (Cai et al., 2006). The overlap of the genetic basis of PA and PI points to the fact that these two phenotypes partly share the genetic and physiological basis, and are not completely unrelated as it might have been understood on the basis of the previous statements (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Perusse et al., 1989).

Mouse – QTL studies

Home cage studies

Table 1.3 (page 21) summarizes the findings from 6 different studies reporting mouse QTLs associated with HC activity. Each study was conducted using a different mouse strain, different breeding methods and different QTL estimation method. Because of methodological limitations of using recombinant inbred strains (Belknap 1992; Flint and Mott 2001), in one of the studies only provisional QTLs could be detected (Toth and Williams 1999). There were also substantial methodological differences in the way the HC experiment was conducted, for example, the age of mice used differed substantially between the studies ranging from 50 days in one study (Mayeda and Hofstetter 1999) to 4 months in another study (Kas et al., 2009a). In most cases, animals were kept in 12h/12h light/dark cycle but in one of the studies mice were housed in constant darkness (Mayeda and Hofstetter 1999). Furthermore, the time window for activity measurement ranged from the first two hours of the dark phase (Henderson et al., 2004) to 10 days of continuous data collection (Mayeda

and Hofstetter 1999). In some cases, animals were habituated to the HC (Furuse et al., 2002; Toth and Williams 1999; Umemori et al., 2009) while in others the activity in the HC was measured from the fist day (Kas et al., 2009a; Mayeda and Hofstetter 1999). Finally, various methods of activity measurement were used between studies: intraperitoneal transmitters (Toth and Williams 1999), video tracking (Kas et al., 2009a) and infrared sensors (Furuse et al., 2002; Henderson et al., 2004; Mayeda and Hofstetter 1999; Turri et al., 2004; Umemori et al., 2009).

It is of a note that HC activity is a complex phenotype in which different components may be separated. With a use of principle component analysis, de Visser and colleagues (de Visser L. et al., 2006) could differentiate between two factors within total PA in a HC: temporal and velocity components. It is very likely that different genomic regions regulate temporal pattern and intensity components of PA in a HC (Umemori et al., 2009). Furthermore, temporal patterns of activity may be under control of a central nervous system mechanism regulating circadian rhythm while the intensity of activity may depend on neuromuscular properties (Umemori et al., 2009). This hypothesis gained support from genetic studies examining various aspects of locomotor activity in a HC. Umemori and colleagues showed that the temporal and intensity components are only weakly correlated. Furthermore, QTLs contribute to these aspects of HC activity overlap only partially (Umemori et al., 2009). This partial overlap of the QTLs suggests that temporal and intensity components have partly shared and partly divergent genetic basis.

Voluntary running wheel activity studies

Table 1.4 (page 23) presents the major findings from 8 different studies reporting mouse QTLs associated with voluntary RWA. One of the studies included in this summary examined RWA only in constant darkness conditions (Shimomura et al., 2001). This difference in the light-dark cycle assessment between the studies could be a confounding factor; however, another study by Suzuki and colleagues shows that the correlation between RWA in the standard light-dark cycle and in the constant darkness strongly correlate (Suzuki et al., 2000). Furthermore, these researchers showed complete overlap of QTLs regulating RWA in these two lighting conditions (Suzuki et al., 2000). The two studies mentioned here were conducted in mice with different genetic background, age and gender; thus one cannot make direct parallels between the two studies. However, the experiment by Suzuki et al. shows that we cannot exclude findings reported by Shimomura et al. only because of the altered lighting conditions. Importantly, the experimental setup of the RWA experiments seems much more comparable between the studies than in the case of HC

experiments. However, substantial consequences may arise from the present methodological differences such as age and gender of animals, lighting conditions, size of the running wheel and the duration of the data collection.

In five of the studies, mice of both genders were used while in three studies only male mice were examined (Suzuki et al., 2000; Yang et al., 2009; Yang et al., 2012). This may have a substantial effect on the outcome of the QTL studies as sex specific QTLs for RWA were detected in, at least, one study (Lightfoot et al., 2010). Using males of a chromosome substitution strain number 13 (CSS13), Yang and colleagues unraveled a single QTL on chromosome 13 with accounted for 15% of the phenotypic variance (Yang et al., 2009; Yang et al., 2012). They could also repeatedly show a clear difference between male C57BL/6J and male CSS13 in the levels of RWA at any time point (Yang et al., 2009; Yang et al., 2012). However, female mice of CSS13 showed no difference in RWA in comparison to female C57BL/6J mice (data in preparation). It is of note that there were other significant methodological differences between these two studies, including age (Kas: 8-12 weeks, Yang: 15 weeks), duration of measurement (Kas: 1 week, Yang: 3 weeks), LD cycle (Kas: standard lighting conditions, Yang: light-dark 14h:10h). Any of these methodological differences could have contributed to the observed discrepancy.

When given access to running wheels, mice require few days to adapt before they exhibit stable RWA levels. Interestingly, this behavioral adaptation is also reflected in a complex architecture of genetic loci influencing the behavior. Kelly and colleagues showed that some of the QTLs are associated consistently across days with RWA while multiple QTLs have an effect on particular days only (Kelly et al., 2010). Furthermore, Leamy and colleagues found another profound mediating effect of time on the underlying genetic architecture of RWA (Leamy et al., 2010). They conducted a RWA experiment for 21 days and averaged the behavioral observations into seven intervals lasting 3 days each. After conducting the QTL analysis for each of these time windows, they concluded that some of the QTLs affect the behavior only at the younger or older mice while other show stable effect over time (Leamy et al., 2010). These two studies, taken together, raise questions regarding the conditions under which RWA behavior can be used to model VE in humans. One could ask whether there is a threshold for the duration of the RWA experiment after which the behavior and underlying QTLs may be considered comparable with the VE phenotype and its genetic basis. Another valid question regards whether mouse QTLs with a transient effect on RWA may be still considered candidate regions for the VE in humans. Furthermore, it is not clear whether syntenic human regions

would also have a transient effect on PA. In this case, one could wonder to what extent the age dependent architecture of PA would be comparable between species and which human and mouse age groups should be compared with each other.

Finally, distinct aspects may be used to characterize RWA, for example speed, duration of activity and distance moved (counted as a number of revolutions or derived from it physical distance). Four of the studies assessed whether these distinct aspects of RWA have a different genetic basis (Kelly et al., 2010; Lightfoot et al., 2008; Lightfoot et al., 2010; Nehrenberg et al., 2010). In an F_2 cross between C57L/J and C3H/HeJ, Lightfoot and colleagues detected one common QTL on chromosome 13 which contributed to duration, speed and distance (Lightfoot et al., 2008). Furthermore, they pointed to an additional QTL on chromosome 9 which regulated speed (Lightfoot et al., 2008) and distance moved on some of the days of testing (Leamy et al., 2010). However, the same researchers, when using a panel of 41 inbred strains, could detect one QTL regulating the duration of RWA and other three QTLs regulating the distance in both genders (Lightfoot et al., 2010). Furthermore, they detected 2 sex-specific QTLs for speed and 6 sex-specific QTLs for distance (Lightfoot et al., 2010). Also, Kelly and colleagues found no overlap between QTLs regulating speed and distance moved in an advanced intercross line (Kelly et al., 2010). Finally, Nehrenberg and colleagues could localize only QTLs regulating speed but no QTLs for the duration or distance moved (Nehrenberg et al., 2010).

Table 1.1. Genetic regions associated in humans with physical activity (PA).

Author	Country	Assessment method	Phenotype	Locus	Gene / marker
Linkage studies					
(Cai et al., 2006)	USA	Accelerometer	TPA, MPA	18q21.32-18q21.33	D18S64-D18S68
			LPA	18q12.2-18q21.2	D18S1102 - D18S474
(De Moor et al., 2007a)	The Netherlands	Questionnaire	Dichotomous: "Do you participate in exercise regularly?"	19p13.3	D19S247
(Simonen et al., 2003b)	USA	Questionnaire	TPA	13q22–q31	D13S317
		B3DPAR	MVPA	4q28.2	UCP1
		(Bouchard et al., 1983)		7p11.2	IGFBP1
				9q31.1	D9S938
				13q22–q31	D13S317
			TSPA	11p15.2	C11P15_3
				15q13.3	D15S165
Association studies					
(Cole et al., 2010)	USA	Accelerometer	TPA, MPA, VPA	18q21.32	MC4R
(Loos et al., 2005)	Canada	B3DPAR	TPA, MVPA	18q21.32	MC4R
(Lorentzon et al., 2001)	USA	Questionnaire	Past year physical activity	3q21.1	CASR
(Stefan et al., 2002)	USA	Respiratory chamber	24h energy expenditure	1p31.3	LEPR
(Winnicki et al., 2004)	Italy	Questionnaire	Type and frequency of exercise	17q23.3	ACE
GWAS					
(De Moor et al., 2009)	The Netherlands and USA	Questionnaire	Leisure time exercise behavior (type, frequency, duration)	2q33.1	DNAPTP6
				10q23.2	PAPSS2
				18p11.32	C18orf2

TPA = total physical activity; MVPA = moderate to vigorous PA; TSPA = time spent on PA; MPA = moderate PA; VPA = vigorous PA; LPA = low PA

Table 1.2. Genetic regions associated in humans with physical inactivity (PI).

Author	Country	Assessment method	Phenotype	Locus	Gene / marker
Linkage studies					
(Cai et al., 2006)	USA	Accelerometer	SA	18q12.2-18q21.2	D18S1102-D18S474
(Simonen et al., 2003b)	USA	Questionnaire	PI	2p22 – p16	D2S2347
		B3DPAR		2p22 – p16	D2S2347
		(Bouchard et al., 1983)		7p11.2	IGFBP1
				20q12	PLC1
Association studies					
(Loos et al., 2005)	Canada	B3DPAR	PI	18q21.32	MC4R
(Winnicki et al., 2004)	Italy	Questionnaire	SA	17q23.3	ACE

SA = Sedentary activity

Table 1.3. Mouse QTL studies using a home cage (HC) activity model.

article	Phenotype	Mouse strain	gender	QTL	Mouse genomic location	Mouse genomic location in bp	Homologous human region	Chromosomal position
(Furuse et al., 2002)	Spontaneous HC activity	Backcross between KJR and BLG2	females	Chr3	Between D3Mit86 and D3Mit128	Chr3:147,002,779 – 150,756,405	Chr1:80,283,988-84,313,096	1p31.1
		Backcross between NJL and BLG2	females	no locus				
(Henderson et al., 2004; Turri et al., 2001; Turri et al., 2004)	Locomotor activity in various behavioral tests of anxiety and HC	F2 intercross between DeFries lines H1, H2, L1 and L2	Male and female	Chr1 Chr4 Chr7 Chr8 Chr15 Chr18 ChrX	76cM 36cM 32cM 60cM 22cM 28cM 26cM			
	Activity in the HC during first 2 hours of the dark phase	F2 intercross between DeFries lines H1, H2, L1 and L2	Male and female	Chr4 Chr8	31-41cM 56-68cM			
(Kas et al., 2009a)	HC activity	A/JxC57BL/6J CSS panel	Male and female	Chr1	Region containing A830043J08Rik=Fam124b	Chr1:80,198,699-80,213,944	Chr2:225,243,406-225,266,790	2q36.2
(Mayeda and Hofstetter 1999)	mean amount of daily locomotor activity at HC in continuous darkness	B6.D2-Mtv7a/Ty congenic strain[1]	?	Chr1	89-106cM (approximate markers: rs107876071, D1Mit361)	chr1:170,940,982-186,261,829	chr1:219,115,791-227,644,727 chr1:240,253,165-247,125,743 chr1:158,516,903-161,661,531	1q41-42.13 1q43-44 1q23.1-q23.3
(Toth and Williams 1999)[2]	Locomotor activity over 2 days in a HC	Recombinant CXB inbred strains	males	Chr3	D3Mit120			

				Chr12	D12Mit147 (G50Kbp 9130015A21Rik) D12Nyu15	Chr12:35,723,478-35,723,626		
(Umemori et al., 2009)	Spontaneous HC activity – total activity	F2 cross between C57BL/6J and KJR/Ms	females	Chr2	*D2Mit126-D2Mit423*	Chr2:84,182,854-149,000,000	Chr11:26,296,397-57,753,949	11p14.2-q12.1
							Chr20:1,746,871-23,642,232	20p13-p11.21
							Chr15:34,933,139-51,298,144	15q14-q21.2
					D2Mit22 – D2Mit29	Chr2:151,799,021–164,277,226	Chr20:29,889,343-43,767,941	20q11.21 – q13.12
				Chr10	*D10Mit73-D10Mit180*	Chr10:111,732,321 – 118,150,744	Chr12:68,717,060-76,169,323	12q15-q21.2

[1] *This congenic strains contains a small DBA/2J genomic insert that covers previously reported region of the provisional QTL on C57BL/6J background.*

[2] *The results obtained using RI strains enable to point to provisional linkage and not to confirmed QTL.*

Table 1.4. Mouse QTL studies using running wheel activity model (RWA).

article	Phenotype	Mouse strain	gender	QTL	Mouse genomic location	Mouse genomic location in bp	Homologous human region	Chromosomal position
(Kelly et al., 2010)[6]	RWA – distance and time (day 1 through 6)	Advanced intercross line between C57BL/6J and high runner line bred from outbred Hsd:ICR line	Male and female	Chr7	99–124 Mb	Chr7:99,000,000–124,000,000	Chr11:3587645-17316591	11p15.4-p15.1
							Chr16:18515287-25839874	16p12.3-12.1
							Chr11:71304680-75216964	11q13.4
							Chr16:16764146-18232691	16p12.3
	– average speed on day 5 and 6			Chr2	81–103 Mb	Chr2:81,000,000-103,000,000	Chr11:34,845,921-57,753,949	11p13-q12.1
							Chr2:184,648,829-188,410,216	2q32.1
				Chr14	69–92 Mb	Chr14:69,000,000-92,000,000	Chr13:53,226,033-65,622,467	13q14.3-q21.31
							Chr13:41,471,003-49,799,099	13q14.1-q14.2
							Chr8:20,190,005-24,475,895	8p21.3-p21.2
				Chr12	73–81 Mb	Chr12:73,000,000-81,000,000	chr14:61,068,749-70,317,981	14q23.1-24.2
	– maximum speed, average on day 5 and 6			Chr2	80–115 Mb	Chr2:80,000,000-115,000,000	Chr11:34,845,921-57,753,949	11p13-q12.1
							Chr2:183,423,068-188,410,216	2q32.1
				Chr11	7-14 Mb	Chr11:7,000,000-14,000,000	Chr7:45,649,431-52,696,893	7p12.3-p12.1
							Chr5:130,483,100-157,964,527	5q23.3-q33.3
					45–61 Mb	Chr11:45,000,000-	Chr5:177,530,538-	5q35.3

						61,000,000	180,682,008	
(Lightfoot et al., 2008)[5]	Daily RWA – duration	F2 intercross between C57L/J and C3H/HeJ	Male and female	Chr13	rs6329684 (Chrm3)	Chr13:10,065,818 Chr13:9,876,613-10,360,803	Chr1:239,792,373-240,072,720	1q43
	– distance			Chr9	rs13480073			
				Chr13	rs6329684	Chr13:10,065,818	Chr1: 239,792,373-240,072,720	1q43
	– speed			Chr9	rs13480073			
				Chr13	rs6329684	Chr13: 9,876,613-10,360,803	chr1:239,550,008-240,073,138	1q43
(Lightfoot et al., 2010)	RWA – distance	41 inbred lines (haplotype association mapping)	Male and female	Chr12	89.35-89.46 Mbp (Nrxn3)	Chr12:89,350,000-89,460,000	Chr14:79,271,856-79,377,115	14q24.3-q31.1
				Chr18	11.57-11.74 Mbp (Rbbp8)	Chr18:11,570,000-11,740,000	Chr18:20,410,696-20,599,048	18q11.2
				Chr19	15.77-16.19 Mbp	Chr19:15,770,000-16,190,000	Chr9:80,574,663-81,084,208	9q21.2
	– distance		Male	Chr5	115.03-118.12 Mbp	Chr5:115,030,000-118,120,000	chr12:117,394,310-121,287,075	12q24.2-q24.31
				Chr6	145.45-145.46 Mbp (Ifltd1)	Chr6:145,450,000-145,460,000	Chr12:25,742,744-25,771,753	12p12.1
				Chr8	61.37-85.40 Mbp	Chr8:61,370,000-85,400,000	Chr4:141,255,401-170,039,614	4q31.1-q32.3
							Chr8:17,992,087-20,177,976	8p22-p21.3
							Chr22:33,659,180-35,953,121	22q12.3
							Chr19:12,757,314-14,683,008	19p13.2-p13.12
							Chr19:17,970,477-19,774,937	19p13.11
				Chr13	96.25-96.63 Mbp	Chr13:96,250,000-96,630,000	Chr5:74,681,475-75,242,696	5q13.3
	– speed			Chr6	119.46-119.81 Mbp	Chr6:119,460,000-119,810,000	Chr12:1,157,035-1,720,652	12p13.33

	– distance		Female	Chr8	96.96-97.22 Mbp	Chr8:96,960,000-97,220,000	Chr8:97,029,176-97,289,176	8q22.1
				Chr11	84.20-86.67 Mbp	Chr11:84,200,000-86,670,000	Chr17:58,204,722-60,306,122	17q23.1-q23.2
							Chr17:34,838,583-35,675,858	17q12
							Chr17:57,802,952-58,046,693	17q23.1
	– speed			Chr11	83.71-86.23 Mbp	Chr11:83,710,000-86,230,000	Chr17:34,838,583-36,200,511	17q12
							Chr17:58,204,722-59,962,753	17q23.1-q23.2
	– duration			ChrX	106.30-108.65 Mbp	ChrX:106,300,000-108,650,000	ChrX:77,421,042-79,711,308	Xq21.1
(Nehrenberg et al., 2010)	Daily RWA – average running speed	Back-cross [1]	Male and female	Chr7	42.75–60.75 cM			
	– maximum running speed			Chr6 Chr7	39.72–73.00 cM 35.74–60.74 cM			
(Shimomura et al., 2001)	RWA in constant darkness – daily activity level (average running wheel revolutions over 15 cycles)	F2 cross between BALB/cJ and C57BL/6J	Female	Chr16 and ChrX interaction	D16Mit106(activity 1 (Act1) locus) (Prdm15) x DXMit27(Act2 locus) (Gm14483)	Chr16:97,830,150-97,830,295 Chrx:11,247,634-11,299,757	Chr21:43,267,661-43,267,711 Chrx:39,164,446-39,221,777 ChrX:39,164,446-39,221,777	21q22.3 Xp11.4
(Suzuki et al., 2000)[2]	RWA – normal light/dark cycle and constant darkness (average running wheel revolutions)	23 SMXA recombinant inbread strains of SM/J and A/J progenitor strains	males	Chr1 Chr17	D1Rik136[3] D17Rik98			
(Yang et al., 2009)	RWA – mean activity over 3	F2 population between	males	Chr13	peak: D13Mit254 21-95Mbp			

	weeks of recording in habituated mice	C57BL/6J and CSS13 (BxA) strain			(narrowed down in Yang et al., 2012)			
(Yang et al., 2012)	RWA – mean activity over 3 weeks of recording in habituated mice	ISCS[4] between C57BL/6J and CSS13 (BxA) strain	males	Chr13	38.84–42.60 Mbp	Chr13:38,840,000-42,600,000[7]	chr6:8,317,673-12,616,944	6p24.3-p24.1

[1] *Backcross mice selectively bred for high RWA and a strain with a recessive mutation resulting in triceps muscle size reduction.*
[2] *Suggestive QTLs*
[3] *According to authors this QTL overlaps with QTL associated by Gershenfeld and colleagues with the open field activity (Gershenfeld et al., 1997).*
[4] *ISCS = interval-specific congenic strains.*
[5] *Further refined by subsequent analysis on the same data by (Leamy et al., 2010; Leamy et al., 2011)*
[6] *There are additional QTLs detected for RWA on specific days of testing. For details, please see the original article.*
[7] *The region contains only 10 genes. Authors proposed a candidate gene: Tcfap2a.*

Eating Disorders

Definitions

According to Klein and Walsh 'An eating disorder (ED) may be defined as a persistent disturbance of eating behavior or a behavior intended to control weight, which significantly impairs physical health or psychosocial functioning, and is not secondary to a general medical condition or another psychiatric disorder' (Klein and Walsh 2004). EDs are mental disorders which are most prevalent among young females (between 15 and 25 years of age) (Lucas et al., 1999). They are characterized by the highest mortality rate among psychiatric disorders (10-15%) (Hoek 2006) due to extreme emaciation and subsequent neuroendocrine adaptations (Haas et al., 2005). Two main types of ED will be further characterized in this introduction, namely anorexia nervosa (AN) and bulimia nervosa (BN). Some researchers suggest that AN and BN are two phases of the same disorder because one fifth of AN patients has episodes of BN with a majority of BN starting with a short episode of AN (Sodersten et al., 2006). Indeed, there is a high rate of cross-over between the AN and BN diagnosis (Eddy et al., 2008; Klump et al., 2001a; Peat et al., 2009). Nevertheless, the subdivision within ED is clinically relevant as the subtypes differ in treatments, their effectiveness, medical complications and prognosis.

Anorexia nervosa

AN patients are best characterized by a 'refusal to maintain minimally normal body weight' for their age, sex and height (Klein and Walsh 2004), and the experience of intense fear of gaining weight or becoming fat. This extremely low body weight is accompanied by a lack of insight into the emaciation of ones own body. The full DSM-V diagnostic criteria for AN are shown in Table 1.5. AN is significantly more common among women than men, with lifetime prevalence estimates of AN being 0.9% for women, and 0.3% for men according to DSM-IV (Hudson et al., 2007).

Bulimia nervosa

BN patients are best characterized by 'recurrent episodes of binge eating followed by inappropriate behaviors aimed to avoid weight gain such as self-induced vomiting' (Klein and Walsh 2004). The full DSM-V diagnostic criteria for BN are shown in Table 1.6. The majority of people seeking medical help because of BN are females, with lifetime prevalence estimates of BN being 1.5% for women, and 0.5% for men according to DSM-IV (Hudson et al., 2007).

Table 1.5. DSM-V diagnostic criteria for AN.

A. Restriction of energy intake relative to requirements leading to a significantly low body weight in the context of age, sex, developmental trajectory, and physical health. Significantly low weight is defined as a weight that is less than minimally normal, or, for children and adolescents, less than that minimally expected.
B. Intense fear of gaining weight or becoming fat, or persistent behavior that interferes with weight gain, even though at a significantly low weight.
C. Disturbance in the way in which one's body weight or shape is experienced, undue influence of body weight or shape on self-evaluation, or persistent lack of recognition of the seriousness of the current low body weight.

Table 1.6. DSM-V diagnostic criteria for BN

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following:
(1) Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than most people would eat during a similar period of time and under similar circumstances (2) A sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating).
B. Recurrent inappropriate compensatory behavior in order to prevent weight gain, such as self-induced vomiting; misuse of laxatives, or diuretics.
C. The binge eating and inappropriate compensatory behaviors both occur, on average, at least once a week for 3 months.
D. Self-evaluation is unduly influenced by body shape and weight.
E. The disturbance does not occur exclusively during episodes of anorexia nervosa.

The genetic basis of ED

ED have complex (and mostly unknown) etiologies, which means that both environmental and genetic risk factors contribute to the development of the disorder (Klein and Walsh 2004). Furthermore, the relationship between these two groups of risk factors is complex due to gene-environment correlation and interaction effects (Klump et al., 2001b; Mazzeo and Bulik 2009; Wade et al., 1998). Multiple genetic factors contribute to a variety of personal (psychological and physiological) traits, which are expressed under specific environmental conditions, and in combination with these environmental conditions contribute to the development of ED (Klein and Walsh 2004). As a consequence, cultural settings, age, gender and other factors may modify the way that the genetic variability manifests itself (Klein and Walsh 2004).

Heritability

Various lines of evidence suggest that ED have a strong genetic basis. Family studies have consistently shown that relatives of ED probands have a higher lifetime risk of developing an ED than controls (Hudson et al., 1987; Lilenfeld et al., 1998; Strober et al., 2000). The fact that AN and BN occur even more frequently within the same family suggests that there is a substantial overlap for genetic or shared environmental factors that predispose to AN and BN (Strober et al., 2000). Further evidence for the heritability of ED comes from twin studies, which have shown higher rates of ED between monozygotic than dizygotic twins (Bulik et al., 1998). As far as AN is concerned, it was estimated that genetic effects are responsible for 58% of the observed variance while the remaining variance (38%) depends mostly on unique environmental factors (Bulik et al., 2006). In the case of BN, 47% of variance can be attributed to genetic effects, 30% to shared environmental factors and 23% to unique environmental factors.

Genes and genetic regions associated with AN and BN

Despite high levels of heritability, the detection of causal genes for ED is difficult, as a large number of genetic loci and gene-environment interactions may contribute to the development of this mental illness (Uher 2009). Furthermore, studies investigating the genetic basis of ED have so far mostly been underpowered (Sullivan et al., 2012). Nevertheless, various attempts have been made to detect genetic variants that increase the risk of developing ED. Candidate genes and regions, which were examined up to now, included those previously associated with appetite and energy regulation, food motivation and reward systems, neuroendocrine signaling (with emphasis on sex hormones), body weight regulation and the immune system (Boraska et al., 2012; Clarke et al., 2012; Pinheiro et al., 2009; Pinheiro et al., 2010; Rask-Andersen et al., 2010; Scherag et al., 2010; Slof-Op 't Landt et al., 2011; Wang et al., 2010). Although significant associations are rare, some genes or genetic regions were associated with an increased risk for ED, for example, the *TPH2* gene (Slof-Op 't Landt et al., 2011), rare copy-number variants at 3p25 and 13q12 (Wang et al., 2010) and single nucleotide polymorphisms (SNPs) at 1q41 and 11q22 (Nakabayashi et al., 2009). Additionally, a number of genetic associations were replicated in independent populations, for example, the association with brain-derived neurotrophic factor (*BDNF)* gene Val-66-Met polymorphism (Ribases et al., 2004; Ribases et al., 2005) - although this was not confirmed in all studies (Brandys et al., 2011; Scherag et al., 2010)-, the *COMT* gene Val-158-Met polymorphism (Frieling et al., 2006; Frisch et al., 2001; Mikolajczyk et al., 2006), agouti-related peptide (*AGRP)* gene (Dardennes et al., 2007; Vink et al., 2001) and serotonin transporter (*SERT*) gene,

as was shown in a meta-analysis by Gorwood (2004). Interestingly, despite a clear role of leptin signaling in appetite an energy expenditure regulation, no associations were reported for genes encoding leptin or the leptin receptor (Hinney et al., 1998; Quinton et al., 2004).

The role of leptin in the development and course of ED

Leptin is a 16-kDa hormone secreted mostly by adipose tissue. Its levels in healthy, normal weight subjects correlate strongly with body fat percentage and BMI (Ehrlich et al., 2009; Hebebrand et al., 2007). After being released by adipocytes, leptin travels via the circulation to the central nervous system, where it binds to leptin receptors, which are present in various brain areas including several hypothalamic nuclei (arcuate nucleus, paraventricular nucleus, dorsomedial hypothalamus, ventromedial hypothalamus and lateral hypothalamus), the hippocampus (dentate gyrus and CA fields), and the ventral tegmental area. Leptin acts as a lipostatic signal by decreasing energy intake and simultaneously increasing energy expenditure, an effect that is mediated by the sympathetic nervous system (Polito et al., 2000). The increase in energy expenditure is achieved by an increase in physical activity and thermogenesis (Hwa et al., 1997; Scarpace et al., 1997; van Dijk 2001; van Elburg et al., 2007). In summary, high leptin levels act as a signal for a high body fat percentage, which leads to degradation of fat stores (Farooqi et al., 2002; Heymsfield et al., 1999; van Dijk 2001). Leptin exerts its effects by down-regulating orexigenic signals (exerted by neuropeptide-Y *(NPY)* neurons) and up-regulating anorexigenic signaling (exerted by *POMC* neurons) (Schwartz et al., 2000). Indeed, deletion of genes encoding leptin (ob/ob mice) or its receptor (db/db mice) results in hyperphagia and obesity (Ribeiro et al., 2011; Trayhurn 1984), whereas the administration of leptin causes a reduction of adipose tissue levels (Halaas et al., 1995).

Based on the role of leptin in energy balance regulation, it was hypothesized that changes in leptin levels or signaling might mediate the decreased basal metabolic rate observed in subjects with AN (Polito et al., 2000). Indeed, plasma leptin levels in ED patients differed from those of healthy controls during the starvation and body weight restoration phases of the illness. Plasma levels of leptin are significantly lower in acutely ill AN and BN patients than in healthy controls (Frederich et al., 2002; Klein and Walsh 2004; Monteleone et al., 2002). This effect prevails even when leptin levels are corrected for body fat percentage (Polito et al., 2000). During the body weight recovery phase, leptin levels increase rapidly and often exceed levels that would be typical for the age and gender of ED patients (Eckert et al., 1998;

Hebebrand et al., 1997; Mantzoros et al., 1997; Wabitsch et al., 2001). This apparent 'rebound effect' of leptin levels occurs despite the persistence of a very low BMI and body fat percentage in newly recovered patients (Hebebrand et al., 1997; Hebebrand et al., 2007). Therefore, the correlation of plasma leptin levels with body fat percentage decreases during ED treatment and body weight restoration (Hebebrand et al., 1997).

It remains to be determined whether altered plasma leptin levels act as a causal factor underlying some of the symptoms of ED or that they occur solely as a consequence of starvation. Either way, altered leptin levels may negatively influence the course of ED. On the one hand, in the acute phase of ED, leptin levels were inversely correlated with physical activity levels and restlessness (Holtkamp et al., 2006). On the other hand, during recovery, high leptin levels (higher than expected on the basis of restored body fat percentage) may predispose to relapse of ED (Hebebrand et al., 1997; Holtkamp et al., 2003a; Holtkamp 2004) by facilitating a reduction in food intake, a degradation of fat storages and an increase in physical activity (Farooqi et al., 2002; Heymsfield et al., 1999; Hwa et al., 1997; Scarpace et al., 1997; van Dijk 2001; van Elburg et al., 2007). Indeed, leptin injections decrease running wheel activity in an animal model of ED (ABA model, see below) (Exner et al., 2000; Hillebrand et al., 2005). These discrepant results suggest that leptin has different functions in states of energy deficit and energy balance (Prentice et al., 2002).

Leptin levels alterations are only one of many changes in hormonal and neurotransmitter systems found in ED patients. Levels of various other hormones (including thyroid hormones, sex hormones (Klein and Walsh 2004), neurotransmitters such as serotonin (Kaye et al., 2009) and NPY (Sodersten et al., 2006) are all affected in patients with ED or hypothesized to play a role in the development of the illness. Therefore, changes in plasma leptin levels alone, cannot be considered a sufficient explanation for complex symptoms of ED. Nevertheless, the role of leptin in energy intake and expenditure combined with the alterations of leptin levels observed in ED patients at different stages of the illness make this hormone an interesting candidate for therapeutic interventions.

Hyperactivity in ED

In the first descriptions of ED, hyperactivity (or overactivity) was already mentioned as an important symptom of the condition (Gull 1997; Pearce 2004). The majority of clinical specialists (85%) considers it to be an important factor in both the

development and maintenance of the ED (Hechler et al., 2005). Some even suggest that high levels of physical activity (PA) play the most important role in the etiology of ED (Epling and Pierce 1992; Pierce et al., 1994), whereas the psychological factors may be viewed as an adaptive response to starvation (Sodersten et al., 2006). Indeed, high levels of PA counteract the therapeutic efforts of re-establishing a healthy body weight (Shroff et al., 2006) and are associated with a reduced recovery rate (Bratland-Sanda et al., 2010; Carter et al., 2004; Kaye et al., 1988; Solenberger 2001) and poor outcome in ED patients (Casper 1996; Casper and Jabine 1996; Shroff et al., 2006; Strober et al., 1997).

Unfortunately, physical activity is often only assessed at the first clinical interview, and not during the course of the illness (Hechler et al., 2005). Furthermore, there is no clear definition of hyperactivity in ED (Hechler et al., 2005), partly due to the multifactorial character of this phenotype. As a consequence, hyperactivity is often not assessed in studies investigating ED etiology and risk factors (Gutierrez 2013). One may distinguish three types of hyperactivity in ED patients: 1) excessive exercise (i.e. exercising at least 6 hours per week (Davis et al., 1994) or exercise that, when postponed, evokes negative emotional states in the ED patient (Hubbard et al., 1998; Mond et al., 2006)); 2) high commitment to exercise despite its adverse consequences for ones health or social contacts (Davis et al., 1993); or, 3) restlessness which may express itself in a constantly active posture, fidgeting or an inability to sit still (Alberti et al., 2013; Beumont et al., 1994). Finally, empirical research does not always confirm that ED patients are more active than the general population. Some researchers reported a clear difference in activity levels between ED and a control group (Blinder et al., 1970; Bratland-Sanda et al., 2010; Davis et al., 2005) while others failed to confirm this difference (Davis 1995; Hechler et al., 2008).

The reasons for increasing levels of physical activity differ between patients and may create a complicated array of interconnected factors in individual cases. Reasons for hyperactivity or high levels of physical exercise may include: further catabolism and reduction of body weight (Klein and Walsh 2004; Kron et al., 1978), maintaining control (Fairburn et al., 1999; Slade 1982), or regulation of negative emotional states, including anxiety (Bratland-Sanda et al., 2010; Carrera et al., 2012; Holtkamp 2004; Pike et al., 2008). Other findings suggest that the rewarding properties of high levels of physical activity may contribute to maintaining some forms of the behavior (Schebendach et al., 2007).

Interestingly, it has not been resolved whether hyperactivity is a premorbid characteristic of ED patients or whether it is evoked by extreme food restriction. It

has also been proposed to view hyperactivity in ED patients as a response to starvation in stead of a cause (Epling and Pierce 1992; Holtkamp et al., 2004; Sodersten et al., 2006). Indeed, the extent of hyperactivity correlates with levels of food restriction (Holtkamp 2004) and 75% of AN patients report that their level of exercise increases during periods of stricter dieting (Davis et al., 1994). Finally, it is possible that high levels of physical activity may be pre-existent in ED patients and may worsen the outcome, but that they are not symptoms of the disease *per se* (Davis et al., 1994; Kron et al., 1978).

Due to the issues mentioned above, there are large differences between studies in the reported prevalence of hyperactivity in ED patients, with numbers ranging from 30 to 80% in AN (Hebebrand et al., 2003) and 22 to 57% in BN (Brewerton et al., 1995; Davis 1997). Furthermore, it is uncertain whether physical activity should be expected to decrease or remain unaltered during treatment for AN (Bratland-Sanda et al., 2010; Inoko et al., 2005; Kron et al., 1978).

Treatment of ED

Different lines of treatment are appropriate for people suffering from AN or BN, due to different clinical profiles of AN and BN. The primary goal in the treatment of AN is body weight restoration, as the severe psychological and physiological adaptation to starvation would result in failure of additional treatment efforts (Klein and Walsh 2004). Body weight gain is achieved by simultaneous refeeding and curtailment of exercise. Secondary aims of treatment include modification of cognitive biases regarding body image and food intake as well as preventing relapse. This is usually achieved by a combination of behavioral therapy and pharmacological intervention, however, only partial successes are observed (Fairburn 2005; Steinhausen 2002; Treasure et al., 2010). Behavioral therapy, both individual and group, may include cognitive behavioral therapy, interpersonal psychotherapy, cognitive analytical therapy and family based therapy (Treasure et al., 2010). Pharmacological treatment of AN patients may involve antidepressants (tricyclics and selective serotonin reuptake inhibitors (SSRIs)), antipsychotics (typical and atypical), antihistamines, zinc, Lithium, naltrexone, human growth hormone, cannabis, clonidine and tube feeding (Aigner et al., 2011). Although the use of none of the compounds is fully supported by controlled clinical trials, olanzapine and zinc supplementation delivered promising results (Aigner et al., 2011). Despite these treatment efforts, less than half of AN patients will experience a full remission and less than a third will go through a partial remission. As far as the remaining patients are concerned, in 20% of

cases AN will develop into a chronic condition and between 5 to 10% of patients will ultimately die of complications of AN (Steinhausen 2002).

There seems to be more consistency in the results regarding BN treatment, as two strategies have proved to be effective. First, cognitive behavioral therapy targets the distorted body image, attitudes and behaviors of BN patients, which contribute to the vicious circle of binging and purging (Fairburn et al., 1995). Second, antidepressants (tricyclics and SSRIs) are successful in diminishing the frequency of binging, even in subjects without a comorbid mood disorder (Aigner et al., 2011; Zhu and Walsh 2002). Unfortunately, despite the short-term effectiveness of these treatment strategies, the long term effects are limited (Keel and Mitchell 1997). When assessed 5-10 years after onset, full recovery is observed in 50% of patients, relapse in 30% and treatment resistant BN in 20% of cases (Keel and Mitchell 1997).

Animal models of ED

The progress in the treatment of ED requires deepened understanding of their etiology and symptoms. Undoubtedly, psychological and environmental factors play a pivotal role in the development of ED. However, this does not imply that biological factors should be neglected. A better understanding of the biological basis underlying the etiology and symptomatology of ED may help create better pharmacological and psychological treatments. As the investigation of these aspects of ED can be difficult or impossible in humans, one may attempt to use animal models to study certain aspects of ED in a controlled genetic and environmental manner.

Various approaches can be used to create an animal model of complex neuropsychiatric disorders such as ED. The created animal models may be grouped into four categories (Smith 1989):

- etiological models - these are based on the same cause as the disease modeled;
- isomorphic models - these are based on the similarity of an animal phenotype to a human phenotype associated with the disease (e.g. hyperactivity);
- mechanistic models - in which the human disorder and the animal model share a common mechanism of disease;

- predictive models – these can be used to predict the therapeutic effectiveness of new compounds.

The process of developing an animal model for ED is complicated by the fact that the etiology of ED is most likely multifactorial with environmental, social, cultural and biological factors together creating a combination of circumstances that ultimately lead to the development of the ED. Efforts to model ED are further complicated by the lack of knowledge regarding the etiology of these disorders (Siegfried et al., 2003; Smith 1989). Currently, no predictive models of AN are available (Siegfried et al., 2003). Furthermore, due to different pharmacological responses between humans and rodent in BN models, testing of new drugs for BN concentrates on human subjects (Casper et al., 2008). Under certain circumstances, genetically modified organisms may be considered a mechanistic model of a disease; however, their use conveys a risk of both false positive and negative conclusions (see below) and their validity is established based on isomorphic behavioral models. Furthermore, there is a lack of genes associated with ED in multiple independent populations (Casper et al., 2008). As a consequence, researchers mostly use isomorphic models (Casper et al., 2008; Siegfried et al., 2003; Smith 1989). The aim of this approach is to discover the biological basis of a symptom or a phenotype related to ED. Below we will give examples of genetic and isomorphic models of ED. It is noteworthy that all the available genetic models target questions related to AN, whereas isomorphic models exist for both AN and BN (Casper et al., 2008).

Genetic manipulation

Genes that encode proteins of multiple neurotransmitter systems have been examined as candidates for phenotypes broadly related to ED pathology (Siegfried et al., 2003). These studies used various techniques to modify gene expression and thereby elucidate their function of the gene. Genes involved in energy balance regulation that have been examined in the context of ED included tyrosine hydroxylase (and dopamine deficiency resulting from deletion of the gene) (Szczypka et al., 1999), M3 muscarinic receptor (Yamada et al., 2001), melanin concentrating hormone (Shimada et al., 1998), corticotropin releasing hormone receptor 2 (Bale et al., 2000), cannabinoid receptor 1 (Di Marzo et al., 2001), serotonin 1B receptor (Lucas et al., 1998), serotonin 4 receptor (Jean et al., 2007) and others.

It is of note that studies using genetic modification techniques in mice may produce both false positive and false negative findings in relation to AN research. For example, although AGRP and NPY play a role in feeding behavior (Schwartz et al., 2000), mice with a double knockout of these genes do not show any feeding or body

weight alterations in comparison to control mice (Qian et al., 2002). Interestingly, the opposite occurred when the BDNF gene was considered as a candidate gene for AN (Casper et al., 2008). This gene was tested in populations of AN patients because of the altered feeding behavior and increased body weight observed in *Bdnf* knockout mice (Casper et al., 2008). Although initially a significant association was found between the BDNF gene and AN (Ribases et al., 2003) and later replicated by the same group (Ribases et al., 2004; Ribases et al., 2005), a meta-analysis of subsequent studies showed that there was no significant correlation between BDNF gene variants and the risk of developing AN (Brandys et al., 2011).

Spontaneous genetic mutations – anx/anx mice

A certain mouse line, so called anx/anx mice, is characterized by a spontaneous recessive mutation on chromosome 2. Homozygotes for this mutation have low food intake despite the preserved ability to eat (Maltais et al., 1984). Furthermore, these mutants are hyperactive, have tremors and their gait is uncoordinated (Maltais et al., 1984). Reduced food intake and growth in these mice result in peri-weaning lethality, approximately 20-30 days after birth, depending on the genetic background. The observed phenotypes may be partially explained by abnormalities discovered in dopaminergic (Johansen et al., 2001), serotonergic (Jahng et al., 1998), noradrenergic systems and signaling of neuropeptide Y in hypothalamus (Broberger et al., 1999). Johansen and colleagues (2003) proposed that anx/anx mice provide a suitable model to study the changes in food intake and energy expenditure observed in AN patients. However, considering the lack of motor disturbances and maintained appetite in AN patients, anx/anx mice 'bear little resemblance to the human condition' (Casper et al., 2008).

Isomorphic models of AN

In their review, Casper and colleagues (2008) pointed out that, as far as AN is concerned, 'characteristics such as female sex, puberty, decreased food intake associated with significant weight loss, and neuroendocrine adaptations would seem fundamental to modeling the syndrome in animals'. Smith (1989) proposed a longer list of AN phenotypes which may be modeled isomorphically and additionally included: decreased body weight, increased activity, abnormal sleep, and acquired taste aversions. Some of these phenotypes are targeted by currently available animal models of AN. Four models will be discussed briefly: 1) the dietary restriction model; 2) stress-induced appetite loss; 3) the stress-induced hyperactivity model and 4) the activity-based anorexia model.

The dietary restriction model. This model serves to evoke neuroendocrine changes in a starved animal that are comparable to those found in AN patients (Siegfried et al., 2003). There are two ways to achieve food restriction: 1) animals may be given access to a fixed percentage of the food that they would normally consume during 24-hours; or, 2) food may be provided ad libitum for a fixed amount of time (Siegfried et al., 2003). As a consequence, the physiological adaptations to starvation and their reversibility can be studied in this model (Casper et al., 2008).

Stress induced appetite loss. Various acute and chronic stressors may be used to evoke a loss of appetite in experimental animals, including tail pinch, cold swimming, direct brain stimulation or separation stress. Exposure to these stressors evokes a decrease in food intake, which is ascribed to a loss of appetite (Casper et al., 2008). Models classified to this category, however, are not suitable as models of AN because AN patients limit their food intake despite preserved high levels of hunger (Casper et al., 2008).

The remaining two activity-based models (stress-induced hyperactivity and activity-based anorexia) target the combination of two AN symptoms: 1) restricted feeding and 2) high levels of physical activity. Both models are based on the observation that access to a running wheel causes a transient decrease in food intake in rats and mice (Premack and Premack 1963). Furthermore, the effect on energy expenditure is paradoxically potentiated when running wheel access is combined with dietary restriction, which leads to a further increase in activity levels (Finger 1951; Hall and Hanford 1954; Reid and Finger 1955). Rodents exposed to this combination of factors are not capable to compensate for their increased energy expenditure by increasing caloric intake and may eventually starve themselves to death (Casper et al., 2008; Gutierrez 2013; Routtenberg and Kuznesof 1967).

In both activity-based models for AN, food restriction not only causes increased physical activity levels, but also results in the cessation of the estrous cycle, generalized endocrine disorders (e.g. the HPA axis), decreased leptin and increased ghrelin concentrations, hypothermia, and alterations in the circadian sleep-wake cycle (Gutierrez 2013). Furthermore, stomach ulcers may occur if body weight loss exceeds 30% in rats and 20% in mouse models (Gutierrez 2013). Each of these phenotypes mimics symptoms of AN observed in starving patients (Gutierrez 2013). There are, however, also differences between the two models, which we will briefly explain below.

The stress-induced hyperactivity (SIH) model. In this model, rodents have unlimited access to running wheels and receive a fixed percentage of the amount of food they would ingest per day under *ad libitum* conditions. There is no time limit on the consumption of the fixed amount of food. Animals receive the same amount of food over the whole experimental period, which prevents the self-starvation observed in the activity-based anorexia model. The primary focus of the SIH model is the increase of physical activity levels evoked by food restriction (Gutierrez 2013).

The activity-based anorexia (ABA) model. In this model, animals are given access to food for a limited amount of time per day (e.g. 2 hours per day for mice and 1 hour per day for rats). This time is too short for animals to consume the amount of food they would ingest under *ad libitum* conditions. Moreover, in the ABA model, rats exposed to a running wheel will consume even less food than controls (rats without running wheel access) given an equal duration of food access (Gutierrez 2013). Interestingly, this is observed only if food availability is limited to once per day (Routtenberg 1968) and the difference in food intake disappears if the number of periods of food availability is increased, even when the total time of food availability remains the same (e.g. twice daily for 30 min in rats) (Kanarek and Collier 1983). It is noteworthy that there are interspecies differences in the ABA model between mice and rats, as well as differences between different inbred mouse strains. The ABA model originally emphasized the influence of the running wheel activity on food intake (Gutierrez 2013). However, it is of note that, in the ABA model, total running wheel activity levels may also increase as a response to restricted feeding, depending on the genetic background (Gelegen et al., 2006). Therefore, ABA is not the opposite of the SIH model but rather it incorporates the mutual influence of limited food access and increased running wheel activity.

One of the measures of increased running wheel activity in the ABA model is the increase of activity in the hours preceding the period of food availability (Mistlberger 1994; Richter 1922). This phenomenon, known as food anticipatory activity (FAA), is considered an equivalent to the search for food. It is, therefore, interpreted as motivated foraging behavior, triggered by the limitation of food availability. According to this view, it is beneficial from an evolutionary perspective if some of the members of a given species are capable of high energy expenditure in times of food scarcity, leading to either the discovery of new food sources or to migration and limitation of competition over limited resources (Epling and Pierce 1992; Fessler 2002). It is of note, however, that there are only anecdotal descriptions of an increase

in physical activity levels of AN patients before meals (Scheurink et al., 2010) and this issue needs to be addressed by empirical studies (Carrera et al., 2012).

With regard to translational value, most researchers in the field agree that ABA is the best isomorphic model for AN (Casper et al., 2008; Gutierrez 2013). However, it is important to note that the ABA model cannot be considered an etiological model of ED (Gutierrez 2013). Furthermore, the value of the ABA model as a predictive model can be questioned. Although various compounds (targeting various brain systems such as dopaminergic, serotonergic, melanocortinergic and opioidergic systems) decrease activity levels of rats in ABA, they were not effective in the treatment of AN patients (Gutierrez 2013). Furthermore, although leptin levels correlate with physical activity in AN patients (Holtkamp et al., 2006) and leptin injections diminish running wheel activity in the ABA model in rats (Exner et al., 2000), these injections also result in a further decrease in food intake which counteracts restoration of body weight (Hillebrand et al., 2005; van Elburg et al., 2007). Therefore, one may conclude that the usefulness of the ABA model is limited to the testing of compounds that could target symptoms of AN, (mostly evoked by starvation) and could be used for symptomatic treatment of AN (Gutierrez 2013).

Isomorphic models of BN

As far as isomorphic animal models of BN are concerned, characteristics such as female sex, puberty and high physical activity overlap with characteristics essential for AN research. Nevertheless, available animal models of BN all focus on environmental factors influencing binge eating (Casper et al., 2008). Two of these models will be briefly described here: the stress-induced hyperphagia model and the sham-feeding model.

The stress-induced hyperphagia model. This model evokes hyperphagia of palatable food as a result of alternate exposure to periods of food restriction and unlimited food access in combination with acute stress (Casper et al., 2008; Hagan et al., 2002; Inoue et al., 2006). The stress-induced hyperphagia model was proposed to model the occurrence of binge eating due to negative emotional states.

The sham-feeding model. This model aims to simulate the disturbances in satiety observed in BN patients and further potentiated by purging episodes (Davis and Campbell 1973). Meals consisting of liquid food ingested by rats increase dramatically due to the draining of consumed food using a stomach cannula. The increase in a meal size is associated with defective satiation signals. As a consequence, with time rats learn to eat even more. This model is considered the

most suitable of all available BN models to simultaneously mimic overeating, postprandial vomiting and impaired satiety (Casper et al., 2008).

Translational Approach in Genetic Studies

Monogenic traits (Mendelian traits; simple traits) are regulated by a single gene and, for simplicity, we may say that their occurrence is binary (present or not present). In contrast, quantitative genetic traits are phenotypes with polygenetic etiology. They show a continuum of possible levels of expression with approximately normal distribution in a population (Flint 2003; Flint and Mott 2001). Each of the genes influencing the variance of the complex trait will most likely have a small contribution to this variance (Flint 2003). There are various approaches to detect genes modulating a level of the complex trait. In this chapter, we will briefly discuss methods which were used in the current thesis, and we will explain the genetic synteny between mouse and human.

Human studies

Gene association study is a methodology which enables discovery of genetic variants with a small effect size for the complex diseases (Cardon and Bell 2001). In this approach DNA variants over the whole genome or in the candidate genetic region are tested one by one for their association with the disease phenotype. A statistical correction for the number of conducted statistical comparisons is of pivotal importance for obtaining reliable results. Most commonly, single nucleotide polymorphisms (SNPs) are used for this purpose. The most commonly used study design is the case-control study in which individuals with a known disease are compared with the general population. Unfortunately, this approach often delivers false positive results diseases most likely due to the poorly matched control group (Cardon and Bell 2001). Any genetic difference between the cases and the control group may be attributed to the disease even if it is actually associated with other independent factors such as migratory history, gender and mating practices (Cardon and Bell 2001). These limitations may be addressed using a prospective cohort study design in which a population is chosen and its members assessed over time for the development of a disease of interest. Another advantage of the cohort study is the substantial sample size which increases the power to robustly detect the genetic association (Cardon and Bell 2001). In general, gene association studies are limited by various factors including small effect sizes of detected associations, population stratification, multiplicity of functional genetic variants within one gene, genetic heterogeneity of disorder, role of novel mutations in the etiology of the disease and even positive publication bias (Cardon and Bell 2001; Ropers 2007). Furthermore,

many of the significant associations are found in intergenic regions or in genes of unknown function (Ropers 2007).

Mouse studies

The genetic basis of complex phenotypes is difficult to unravel in heterogeneous human populations. Animal models may enable the study of the genetic architecture of complex phenotypes in a controlled environmental and genetic background conditions (Schughart et al., 2012). Mouse is a particularly interesting species due to the richness of gene manipulation techniques and breeding strategies which enable studies not possible in other organisms (even in rats). The types of mouse lines (inbred, knock-out (KO) and chromosome substitution strains panel) and the genetic method (quantitative trait loci analysis) used in this thesis will be briefly described.

Classical inbred mice

Inbred mouse line may be seen as a line consisting of only monozygotic twins. This type of line is obtained by successive breeding between brothers and sisters for 20 generations (so called inbreeding) until the number of homozygous loci is greater than 99.4% (Wade and Daly 2005). Use of inbred strains in laboratory research enables conducting experiments in extremely controlled genetic and environmental conditions. This leads to decrease in within population variance and increases the effect sizes of detected differences. For this reasons, currently available 450 inbred strains were generated in the 20th century. Each of the inbred strains has an unique genetic variation of genetic loci coming from a limited number of founding parental strains. Therefore, comparison between inbred strains may be useful for detecting genes associated with complex phenotypes (Kas et al., 2009c).

Knock-out (KO) mouse lines

One of the possible strategies to discover a function of a gene is to examine functional consequences of this gene deletion. In the mouse knockout line, a fragment of a selected gene is substituted by a marker sequence. As a consequence, the function of this gene is disturbed. It is assumed that biochemical, physiological and behavioral changes observed in this knockout mouse line may elucidate the function of that gene when it is present. However, caution needs to be taken when interpreting the consequence of the gene knockout as due to the developmental challenges some compensatory mechanisms may take place and obscure the conclusions regarding the gene function. Although KO lines may be very useful for research, 15% of knockout lines is lethal, and that hampers research efforts regarding

some of the genes (http://www.genome.gov/12514551) (Guan et al., 2010). The National Institute of Health estimates even higher numbers suggesting that almost 30% of all generated KO lines will be embryonic or perinatal lethal (compare: http://grants.nih.gov/grants/guide/pa-files/PAR-13-231.html).

Chromosome Substitution Strains (CSS) panel

In a chromosome substitution strains (CSS) panel, two progenitor inbred strains (for example A/J and C57BL/6J mice) are crossed for 10 generations to obtain lines with a desired genetic makeup. The crossing is done in such a way that, in each of the obtained lines, only one chromosome pair of the host strain is substituted by a chromosome pair of the donor strain (Nadeau et al., 2000; Singer et al., 2004). As a consequence, the whole CSS panel consists of 21 strains (19 autosomes, 2 sex chromosomes and one mitochondrial chromosome). Each strain is designated by a symbol C57BL/6J-Chr #A/J/NaJ (CSS# in short), where # is the number of the substituted chromosome. The CSS panels are used in order to map genetic loci for complex traits (QTLs).

Quantitative Trait Loci (QTL) analysis

A genomic region with a genetic variance which modifies the level of a quantitative trait is called a quantitative trait locus (QTL). By definition, each QTL contributes to a small portion of a variance of a complex trait (Flint 2003). The QTLs may be discovered with a use of various linkage and mapping methods (so called QTL analysis) (Flint 2003; Mackay 2009). The fundamental assumption of these methods is that we may track down QTLs using a limited number of marker loci (Mackay 2009). This type of analysis enables investigation of the genetic basis of complex traits without 'a priori assumptions about causative genes or pathways' (Nathan et al., 2006).

The process of mapping novel QTLs for a complex phenotype is facilitated by the use of CSS panel due to two factors: 1) obtained QTLs explain higher portion of the variance of the complex trait because the background genetic variance is smaller, 2) fewer animals need to be used to map a significant QTL because of the reduction of the marker number (Singer et al., 2004). The process of discovering new QTL consists of four stages. First, both progenitor lines and the whole CSS panel are characterized for a phenotype of interest. The CSS lines, which significantly differ from the progenitor lines for this phenotype, point to which of the chromosomes (e.g. Chr#) might contain a at least one QTL for this phenotype (Singer et al., 2005). Second, the CSS# strain is crossed with the host strain. The F_1 population obtained by this cross is heterozygous for loci on Chr# and homozygous for all remaining loci. Animals of

this F_1 population are crossed to obtain an F_2 population which, due to genetic recombination, consists of genetically unique animals – their Chr# consisting of random and unique combination of loci from progenitor lines while the remaining chromosomes are homologous for the host strain (see Figure 1.1a). Three, F_2 population animals are phenotyped and genotyped for the markers on Chr# which differ between the two progenitor lines. At each genetic locus three genotypes are possible (AA, AB and BB, where A is a genetic variant of the host strain and B is a genetic variant of the donor strain; Figure 1.1b). Four, QTL analysis is performed by examining the average level of the phenotype for the genetic variants (AA, AB and BB) of each genetic locus on Chr# (Mackay et al., 2009). In this analysis, the level of the phenotype examined is compared for each of the three genotypes present at this locus (Flint 2003). As a result of this analysis, a genetic region is pointed which contains a gene or regulatory element which contribute to the variance of the measured phenotype (Flint and Mott 2001). More detailed description of QTL methodology and its statistic basis have been reviewed extensively elsewhere (Broman 2001; Doerge 2002; Kearsey 1998; Mackay 2009).

Comparative genomic approaches – human mouse synteny

Comparative genomics is a branch of science which focuses on studying the functional and structural genome similarities and differences between various species or strains. This approach enables, on the one hand, studying the process of mammalian evolution and, on the other hand, translational studies using model organisms of complex human phenotypes. Detection of regions conserved between distant species points to high functional importance of these fragments of the DNA sequence.

Human and mouse developmental lines diverged about 75 million years ago, and ever since evolutionary forces shaped the two genotypes in a different manner (Waterston et al., 2002). Nevertheless, the extent of the changes is, however, small enough for conservation of local gene order (Waterston et al., 2002). Consequently one may identify syntenic regions between the two species and even homologous genes based on the evolutionary conservation of functionally relevant fragments. Although mouse genome is about 14% smaller than the human genome, more than 90% of the human genome can be considered syntenic (literally ‘same thread’),

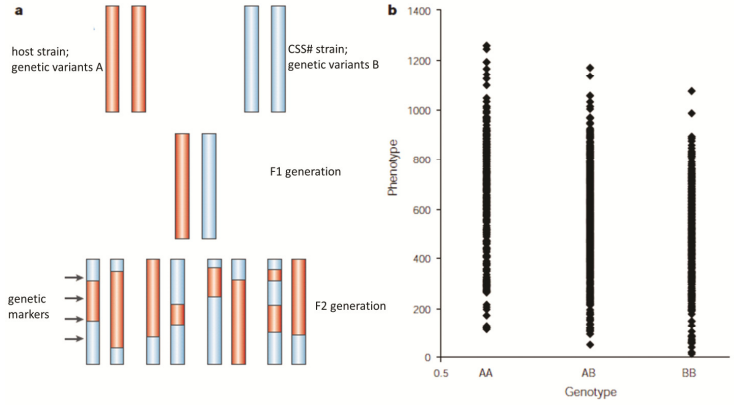

Figure 1.1. Quantitative trait loci detection using a CSS panel. a) Two mouse strains are crossed: 1) the host strain; and, 2) CSS# strain which significantly differs from the host strain in terms of a phenotype tested. Mice of the obtained F_1 population are heterozygous for every genetic loci on the chromosome #. These mice of the F_1 population are intercrossed to obtain F_2 population. Each of these F_2 population animals possesses a unique combination of genetic variants originating from the host and the CSS# strain. As a consequence each genetic loci in any given animal of the F_2 population may be either AA, AB or BB (or in other words red-red, red-blue, blue-blue). b) The phenotype is assessed and the levels of the phenotype are plotted for each of the tested genetic markers (AA, AB or BB) of the chosen loci. In this example genetic variant A possesses an additive genetic effect (each additional copy of the variant A increases the level of expression of the phenotype). This figure is adapted from a review by Flint and Moot (2001).

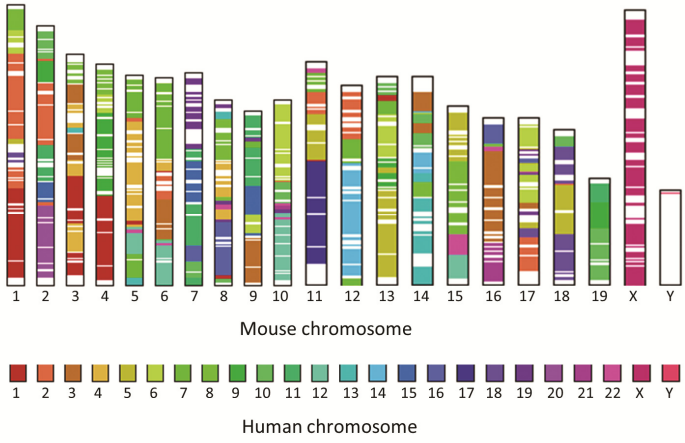

Figure 1.2. Genetic synteny between mouse and human. 342 syntenic segments of more than 300kbp from the human genome (23 chromosomes coded with distinctive colors) are superimposed on the mouse genome (20 chromosomes). This figure is re-printed from Mouse Genome Sequencing Consortium review (Waterston et al., 2002).

meaning that portions of the human genome may be superimposed on the mouse genome (Figure 1.2). Forty percent of the human genome may be aligned precisely with the mouse genome and remaining 50% may be matched based on satisfactory resemblance. 80% of the mouse genes have an orthologue gene in human (Brudno et al., 2004; Waterston et al., 2002).

Scope of the Thesis

Background and the general aims

The appropriate levels of physical activity (PA) are essential for the maintenance of good health. On the one hand, there are increasing concerns regarding adverse effects of low levels of PA on health because low levels of PA are said to contribute to the obesity epidemic, cardiovascular diseases and other health problems (Casazza et al., 2013; Haskell et al., 2007; Manson et al., 2004; Mokdad et al., 2004; Myers et al., 2002). On the other hand, excessively high levels of PA may have a negative effect on psychological and physical well-being of an individual as is observed in exercise addiction or excessive exercise present in some eating disorder patients.

The high levels of PA have a strong and manifold influence on the course of the eating disorders (ED) (Hechler et al., 2005). However, this symptom is often not assessed in the clinical (Hechler et al., 2005) and scientific practice (Gutierrez 2013). Consequently, questions regarding the etiology of high levels of PA in ED patients remain unanswered. Furthermore, it is not clear whether one should expect a decrease in PA after complete or partial recovery from ED.

It is hoped that increased understanding of the factors which influence the voluntary PA levels could lead to more accurate treatments and prevention programs (Rowland 1998). However, these efforts are complicated by the fact that numerous psychological, cultural and environmental factors have an influence on the levels of PA. Furthermore, the habitual PA is strongly regulated by evolutionary conserved genetic factors (Rowland 1998). It may be possible to investigate the biological pathways involved in the regulation of PA by unraveling the genetic architecture of this heritable phenotype (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Kelly and Pomp 2013; Stubbe et al., 2006). However, as any other complex phenotypes, the PA levels are under the influence of multiple genetic loci, which influence on PA levels is further modified by gender, age and environmental factors.

Taking under consideration the complex basis of PA, as well as the potential relevance of PA for ED pathology, we aimed in the current thesis:

1) to elucidate some of the biological basis of PA,
2) to understand the role of PA in the course of ED pathology.

As far as the first aim is concerned, we applied various methodologies to assess novel candidate genes and genetic regions for PA in humans (candidate genetic region association studies) and mice (candidate gene knockout and knockdown studies; genome wide scan for novel candidate genetic regions). We also made an effort to integrate the findings originating from the two species.

In order to increase our understanding of the role of PA in the course of ED pathology, we used a general population based sample (TwinsUK) to assess whether PA is a risk factor for the development of ED. Furthermore, we investigated the consequences of high levels of PA at the acute stage of ED as well as at the recovery in the population of anorexia nervosa adolescent patients.

Outline of the thesis

Chapter 1: General introduction

Chapter 2: Translational approach was applied to identify a possible candidate genetic region associated with voluntary exercise in humans. In order to do so, we first pointed to a possible candidate gene region for voluntary exercise by using a mouse model of this phenotype and a specific mouse panel (so called chromosome substitution strain panel) suitable to conduct quantitative trait loci analysis. We identified a genetic region on mouse chromosome 2 which contributed to voluntary exercise in mice. We regarded this significant finding to be a source of hypothesis that syntenic genetic region in humans would also contain a genetic variant contributing to the levels of voluntary exercise in humans. We used the knowledge of the synteny between species to establish which region of the human genome is syntenic with the detected candidate region in mice. We tested this hypothesis in two independent human populations in which physical activity was measured using two established methods of measuring voluntary exercise in humans: questionnaire data and accelerometer. Finally, we found a genetic association in both populations tested.

Chapter 3: As in Chapter 2, the translational approach was applied in order to identify a possible candidate genetic region associated with voluntary exercise in humans. We discovered a significant QTL which explains 26% of variance of the running wheel activity in the mouse. However, we did not detect a

significant association between genetic variation in a syntenic human genetic region (HSA10) and the level of voluntary exercise in human population.

Chapter 4: Phenotypic consequences of the *Nfatc2* gene knockout in mice were established in relation to physical activity. We could show that knockout of the *Nfatc2* gene causes a decrease in running wheel activity that cannot be attributed to disturbed motor coordination or changes in reward sensitivity.

Chapter 5: We tested the hypothesis that the expression of protein tyrosine non-receptor type 1 (*Ptpn1*) gene in the mouse hippocampus has an influence on behavioral adaptation to restricted feeding as measured by a behavioral readout called food anticipatory activity. It was done in mice subjected to a restricted feeding schedule with access to the running wheels. The expression of *Ptpn1* gene was knocked-down with the use of shRNA delivered to mice hippocampus in adeno-associated virus vector.

Chapter 6: In this chapter we addressed the question whether excessive exercise is a risk factor for the development of eating disorders (ED) in the general population. In order to do so, we used a Saint Thomas Twins Register population of adult women for whom the following phenotypic data were available: 1) the diagnosis of ED; and, 2) the amount of time per week spent on physical exercise.

Chapter 7: The longitudinal data of the population of adolescent anorexia nervosa (AN) patients were examined to establish whether high level of physical activity (excessive exercise) diminishes after partial body weight recovery. Furthermore, we assessed whether patients who exercise excessively in the acute phase of AN are characterized by different profile of body fat percentage restoration and plasma leptin levels than patients who do not exercise excessively.

Chapter 8: Using the longitudinal data of the population of adolescent AN patients we tested whether plasma leptin levels of recovered AN patients depend on the magnitude of the relative body weight loss during the progression of the disease.

Chapter 9: The discovery of genetic variants that underlie a complex phenotype is challenging. One possible approach to facilitate this endeavor is to identify quantitative trait loci (QTLs) that contribute to the phenotype and

consequently unravel the candidate genes within these loci. Each proposed candidate locus contains multiple genes and, therefore, further analysis is required to choose plausible candidate genes. One of such methods is to use comparative genomics in order to narrow down the QTL to a region containing only few genes. Here, we illustrated this strategy by applying it to genetic findings regarding physical activity (PA) in mice and human. We provided points for caution regarding the translatability of this phenotype between species. Based on a large variety of studies in mice and human, statistical analysis revealed that the currently available overlap between the studies is below chance level.

Chapter 10: General discussion

A Candidate Syntenic Genetic Locus is Associated with Physical Activity Levels in Mice and Humans

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PLOS ONE (major revision, in progress)

2

Abstract

Individual levels of physical activity, and especially of voluntary physical exercise, highly contribute to the susceptibility for developing metabolic and cardiovascular diseases. Here, we applied a cross-species approach to explore a candidate genetic region for voluntary exercise levels. First, a panel of mouse chromosome substitution strains was used to map a genomic region on mouse chromosome 2 that contributes to voluntary wheel running levels - a behavioral readout considered a model of voluntary exercise in humans. Subsequently, we tested the syntenic region (HSA20: 51,212,545–55,212,986) in a human sample (Saint Thomas Twin Register; n=3038) and found a significant association between voluntary exercise levels (categorized into excessive and non-excessive exercise) and an intergenic SNP *rs459465* (adjusted *P*-value of 0.001). Taking under consideration the methodological challenges embedded in this translational approach in the research of complex phenotypes, we wanted to further test the validity of this finding. Therefore, we repeated the analysis in an independent human population (ALSPAC data set; n=2557). We found a significant association of excessive exercise with two SNPs in the same genomic region (*rs6022999*, adjusted *P*-value of *P*=0.011 and *rs6092090*, adjusted *P*-value of 0.012). We explored the locus for possible candidate genes by means of literature search and bioinformatics analysis of gene function and of trans-regulatory elements. We propose three potential candidate genes for voluntary physical exercise levels (*MC3R*, *CYP24A1*, and *GRM8*). To conclude, the identified genetic variance in the human locus 20q13.2 may affect voluntary exercise levels.

Introduction

Low level of voluntary exercise (one of types of physical activity) in modern societies is considered to be one of the most profound risk factors for the development of obesity and cardiovascular diseases, among other illnesses (Casazza et al., 2013; Mokdad et al., 2004; Myers et al., 2002). Conversely, appropriate level of exercise is believed to have broad beneficial effect on human health. Despite the accumulating evidence and common knowledge regarding benefits of physical activity, only a minority of the Western population engages in sufficient physical activity to experience its benefits (Kelly and Pomp 2013; Marcus and Forsyth 1999). Broadened understanding of the mechanisms that influence levels of physical activity could help in improving the existing health programs. However, participation in physical activity is influenced by an array of factors including biological, psychological,

cultural and environmental ones. Furthermore, many of these factors influence various complex phenotypes which may be correlated (e.g. obesity and levels of physical activity). This complexity hampers efforts to understand biological processes underlying physical activity. One of the possible ways to unravel the biological pathways involved in physical activity is to investigate the genetic basis of this highly heritable phenotype (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Kelly and Pomp 2013; Stubbe et al., 2006) under controlled genetic and environmental conditions (Schughart et al., 2012).

Voluntary exercise is a complex phenotype and various definitions (de Vilhena e Santos DM et al., 2012; Garland, Jr. et al., 2011b) and operationalizations may be used in studies to examine it, e.g. direct observation, questionnaires (diaries and retrospective recall), surveys, calorimetry, heart rate monitors and motion sensors(Garland, Jr. et al., 2011b; Westerterp 2009). These assessment methods differ in terms of feasibility and reliability, therefore, the results obtained by the use of various methods do not necessarily correlate(de Vilhena e Santos DM et al., 2012; Garland, Jr. et al., 2011b). Use of animal models may, to some extent, help to standardize the complex conditions influencing the levels of physical activity and methods of measurement. In rodent studies, voluntary running wheel activity (RWA) in a home cage was proposed to be the most appropriate model for voluntary exercise in humans (Kelly and Pomp 2013; Rezende et al., 2009). Indeed, if one defines voluntary exercise as locomotor activity 'that is not directly required for survival or homeostasis and not directly motivated by any external factor' (Garland, Jr. et al., 2011b), than voluntary RWA is indeed the most suitable rodent behavior to model human voluntary exercise (although some researchers would not agree(Sherwin 1998)). Studying RWA in various mouse lines which differ in expression of this phenotype allows systematic genetic studies on this complex trait.

Previous studies proved that genetic factors have an influence on the levels of physical activity. Heritability of physical activity in humans was assessed repeatedly (de Vilhena e Santos DM et al., 2012). Multiple studies pointed to genes associated with physical activity levels in humans (Bray et al., 2009; Hagberg et al., 2011; Rankinen et al., 2010; Roth et al., 2012), and various linkage and GWAS studies in humans were able to point to genetic regions associated with physical activity levels (de Vilhena e Santos DM et al., 2012). Furthermore, it is possible to selectively breed lines of mice based on their high or low physical activity (Swallow et al., 1998). Finally, numerous studies using RWA in mice as an animal model for physical activity, pointed to promising candidate genes and genetic regions. Nevertheless,

despite the relative (in comparison to other complex phenotypes) ease of operationalization of physical activity in humans and translation of this phenotype to an animal model, there is virtually no overlap between the results obtained from rodent and human genetic studies for voluntary activity (de Vilhena e Santos DM et al., 2012). Therefore, there is a need for research aiming at the translation of genetic findings from animal to human studies.

In the current study, we aimed at identifying a narrow candidate genetic region contributing to physical activity levels. For this purpose we used a cross-species approach. First, we used a panel of mouse chromosome substitution strains (CSS, also called consomic strains or lines) (Singer et al., 2004) that enable identification of candidate genetic regions for complex traits, such as voluntary RWA. Later, we tested this discovered candidate region for mouse RWA in two independent human population. Finally, we propose new candidate genes potentially contributing to the individual levels of physical activity.

Methods

Ethical statement

All animal experiments were approved by Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act'; 1996) and on the Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision'; 1996). Every effort was made to minimize animal suffering.

The relevant institutional review boards or ethics committees approved the research protocol of the individual population based studies used in the current analysis. The study involving participants enrolled to TwinsUK was approved by the St Thomas' Hospital research ethics committee. Ethical approval for the study involving ALSPAC dataset was obtained from the ALSPAC Ethics and Law Committee and the Local Research Ethics Committees. All human participants and also, in case of the minors, their first kin gave written informed consent.

Mice

Initial breeding pairs for CSS and their progenitors A/J (A) and C57BL/6J (B6) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The advantages of

using CSS strains as well as the steps required to generate a CSS panel has been described previously (Nadeau et al., 2000). All mice were bred in the Rudolf Magnus Institute of Neuroscience animal facility and were 2-3 months old at the start of the experiment (lights on at 2:00 a.m. off at 2:00 p.m., temperature 22.0 ± 2.0 °C, fed ad libitum). In total, 384 female mice were tested in the experimental procedure; B6 strain (n = 36), A strain (n = 23) and of the 19 tested CS strains (n = 257; median of 12 mice per CS strain), CSS2F2 (F_2-intercross between C57BL/6J-Chr 2^A/NaJ (CSS2) and B6) (n = 68). Mice from CSS16 were not tested due to low availability. The low number of mice in the F_2 population was adequate for proper analysis due to the used methodology. The consomic F_2 cross approach is a specifically designed to identify single QTLs on a single chromosome as there is a strong reduction in the amount of epistatic interactions with loci from the other 20 chromosomes. Because of this sensitivity, significant less F_2 mice are necessary to identify these QTLs when compared to the traditional whole genome intercross mapping approach (this has been indicated elegantly by the laboratory that generated the consomic mice (Singer et al., 2004). In addition QTL analysis was performed by MQM-mapping (multiple-QTL-model or marker-QTL-marker) which is more powerful than the traditional interval mapping approach (Jansen 1994). The levels for physical activity, such as voluntary RWA, are known to be very different in males and females. As we are interested in the genetics of physical activity levels in relation to eating disorders with a gender pre-dominance in females (such as in anorexia nervosa) (Gelegen et al., 2010), we decided to perform this genetic screen in females.

Running wheel activity measurement in mice

Mice were maintained in running wheel cages (wheel circumference: 43.96 cm; surface made of metal rods) for a week. We did not observe coasting of mice in the running wheels. Individual wheel running revolutions were automatically registered. The average of the RWA for the days 6 and 7 was used in the QTL analysis. This was done because mice require time to adapt to the running wheel cages and to develop stable RWA pattern. Patterns of the RWA for CSS2, A, B6 mice as well as for mice from the F_2 populations are present on Figure 2.1.

DNA samples, genetic marker analysis and map construction

Genomic DNA was isolated from spleen and/or tail from F_1-hybrids, F_2-intercross mice and CSS2 and B6 mice, using a phenol/chlorophorm/iso-amylalcohol protocol (Laird et al., 1991). A total of 14 microsatellite markers (D2Mit117, D2Mit417, D2Mit370, D2Mit458, D2Mit156, D2Mit380, D2Mit94, D2Mit66, D2Mit206, D2Mit525, D2Mit493, D2Mit51, D2Mit113, D2Mit148), dispersed throughout mouse

chromosome 2 (Chr2), was used to map a region on Chr2 associated with RWA in the F_2 population. In addition nine single nucleotide polymorphisms (SNPs) were genotyped (Taqman Assay by Design, Applied Biosciences, Foster City, CA, USA): *rs27524348*, *rs27498297*, *rs27434812*, *rs28277299*, *rs13476894*, *rs27292002*, *rs27289000*, *rs27619825*, *rs27289254*. SNP analysis was performed as described in (Kas et al., 2009b). Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit-test. None of the markers showed segregation distortion ($P < 0.05$).

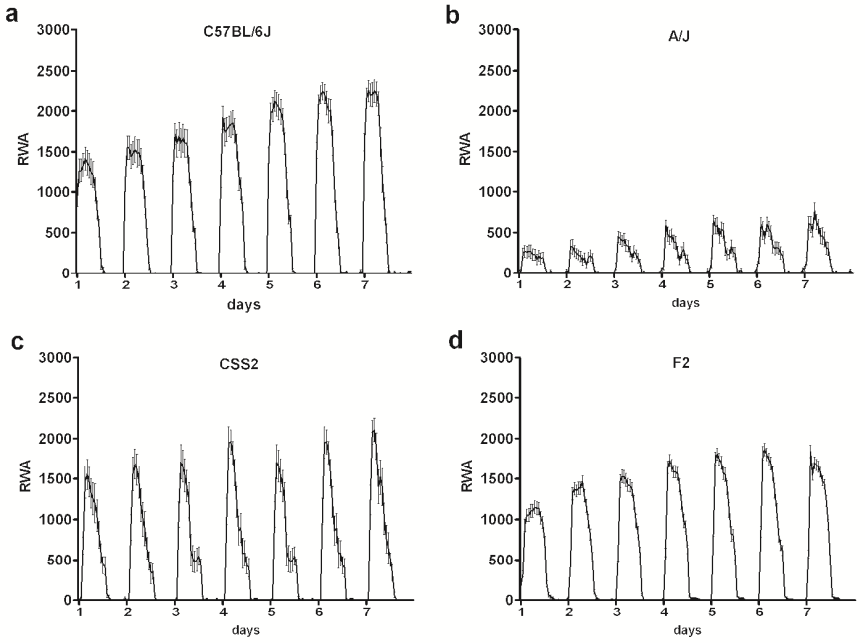

Figure 2.1. Running wheel activity (RWA) averaged for each hour of the day over a 7-day period in B6 (panel A), A (panel B), CSS2 (panel C) and F_2 population (panel D). The figure shows stability of RWA on days 6 and 7 of the measurement after initial 5 days of adaptation.

QTL analysis

The quantitative trait loci (QTL) analysis was conducted on the females from CSS2 x B6 F_2 population. The purpose of this analysis was to fine-map genetic regions regulating levels of voluntary wheel running. This method was chosen because the consomic-F_2-cross approach is specifically designed to identify single QTLs on a single chromosome as there is a strong reduction in the amount of epistatic

interactions with loci from the other 20 chromosomes. Because of this sensitivity, significant less F_2 mice are necessary to identify these QTLs when compared to the traditional whole genome intercross mapping approach (Singer et al., 2004).

The location of the QTLs affecting the measured quantitative trait and the variance explained by each locus were determined using the MapQTL® software package (version 4.0) (Van Ooijen et al., 2002). RWA was normally distributed in the F_2 population (Figure 2.2). QTL analysis was performed by MQM-mapping (multiple-QTL- model or marker-QTL-marker) (Jansen and Stam 1994). Results were expressed as LOD scores. Permutation tests (10,000 permutations) were done to assess the statistical significance of a QTL (5% significance level: LOD score ≥ 2.62).

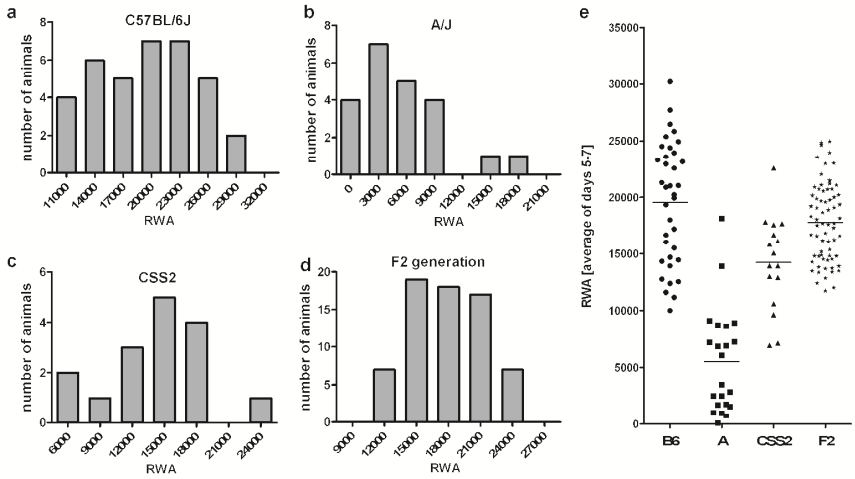

Figure 2.2. Distribution of the running wheel activity (RWA) measurement in B6 (panel A), A (panel B), CSS2 (panel C) and F_2 population (panel D). Panel E indicates the relative RWA levels in the B6, A, CSS2 and F_2 population. Figure show normal distribution of the RWA in B6 mice and in F_2 population.

Genetic association study in human participants

The coordinates of a QTL found in the mouse linkage study have been converted to a syntenic region in humans via the UCSC LiftOver tool (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Mouse region on Chr2: 169,276,294–172,557,293 bp, according to 37.2 mouse genome build, was syntenic to a region on human chromosome 20 (HSA20; 51,212,545 - 55,212,986 bp), according to hg18 genome build.

TwinsUK- sample description and physical activity measurements

The participants were 3038 females enrolled to the St. Thomas' UK Adult Twin Registry (TwinsUK) between 1994 and 2007. These participants were a portion of the total TwinsUK sample who provided data for physical activity. The composition of the TwinsUK was described in detail elsewhere (Spector and MacGregor 2002; Spector and Williams 2006). The analysis was conducted in the age group between 30 and 55 years of age (mean age=44.16, SD=7.49) at the time of examination, which was considered the most representative age group with respect to the self-reported, voluntary physical activity. Participants were genotyped on Illumina 370k or Illumina 610k platform.

Physical activity of participants from TwinsUK study was assessed on the basis of self-reported questionnaires (so called Q10 and Q17D). The questionnaire Q10 included the following questions: 1): *Currently, how many minutes per week do you spend in weight bearing activity? e.g. aerobics, running, dance, football, basketball, racquet sports etc. (do not include walking or gardening)*; 2) Questionnaire Q10: *Currently, how many minutes per week do you spend in non-weight bearing activity? e.g. swimming, cycling, yoga, aqua aerobics etc.* The questionnaire Q17D included the following questions: 1) *During the last week, how many hours did you spend on each of the following physical activities? / Physical exercise such as swimming, jogging, aerobics, football, tennis, gym workout, power walking etc.*; 2) *During the last week, how many hours did you spend on each of the following physical activities? / Cycling, including to work and during leisure time*; 3) *During the last week, how many hours did you spend on each of the following physical activities? / Gentler exercise, Yoga and Pilates.* Data of questionnaire Q10 were used preferentially as they were available for 3233 participants (in comparison to only 2683 in case of Q17D questionnaire). If data from questionnaire Q10 for a given participant were not available, the score from questionnaire Q17D was used. Finally, the amount of sport was categorized as excessive exercise (EE) if a participant spent 5 or more hours per week to exercise as proposed by Davis (1995).

TwinsUK data - quality control

As mentioned, the TwinsUK individuals were genotyped on Illumina 370k or Illumina 610k platform. The data were quality controlled per each platform separately (Table 2.1). The merging of the data (variants genotyped by both platforms; no imputed SNPs included) was performed using Plink v1.07 (Purcell et al., 2007). After merging of both datasets, there were 496 males, 5150 females and 7422 SNPs. Further analyses were performed on the merged dataset, of 3038 females who had non-missing EE phenotype data. After quality control, there were 2766

SNPs in the HSA20 region of interest. The QQ-plots (observed *P*-values plotted against the *P*-values expected under the null hypothesis) in the analyses did not suggest inflation of the *P*-values (Figure 2.3a, Lambda < 1).

Table 2.1. Quality control steps per each genotyping platform in the TwinsUK data. *99,9% total genotyping rate.

STEP \ Platform	Illumina 370k	Illumina 610k
Total number of individuals	2040	3614
Removed individuals with more than 5% missing genotypes	0 *	0 *
Removed for increased heterozygosity (+/- 3SD)	5	3
Total nr of SNPs	4158	7760
SNPs removed for more than 5% missed calls	170	157
SNPs removed for violation of Hardy-Weinberg equilibrium at p<0.001	9	32
Removed SNPs which had minor allele frequency of less than 1%	2	275

ALSPAC - sample description and physical activity measurements

ALSPAC (Avon Longitudinal Study of Parents and Children) recruited 14,541 pregnant women resident in Avon, UK with expected dates of delivery 1st April 1991 to 31st December 1992. Of these *initial* pregnancies, there were a total of 14,676 fetuses, resulting in 14,062 live births and 13,988 children who were alive at 1 year of age. The enrolment was extended to include additional children eligible using the original enrolment definition up to the age of 18 years. This increased the number of pregnancies included to 15,247. Of this total sample of 15,458 fetuses, 14,775 were live births and 14,701 were alive at 1 year of age. The cohort profile describes the index children of these pregnancies. Follow-up includes 59 questionnaires (4 weeks–18 years of age) and 9 clinical assessment visits (7–17 years of age) (Boyd et al., 2012). Please note, that the study website contains details of all the data that is available through a fully searchable data dictionary (http://www.bristol.ac.uk/alspac/researchers/data-access/data-dictionary/). We obtained data for 7220 girls from the total cohort. Out of those 6759 were not related, 2557 had data on physical activity measured by the questionnaire and 1312 had activity data measured by accelerometer. The assessment of activity levels took place at the age of 15. For 969 girls we had the data for both the questionnaire and the accelerometer measurements of physical activity. All participants were genotyped on Illumina Human Hap 550-quad.

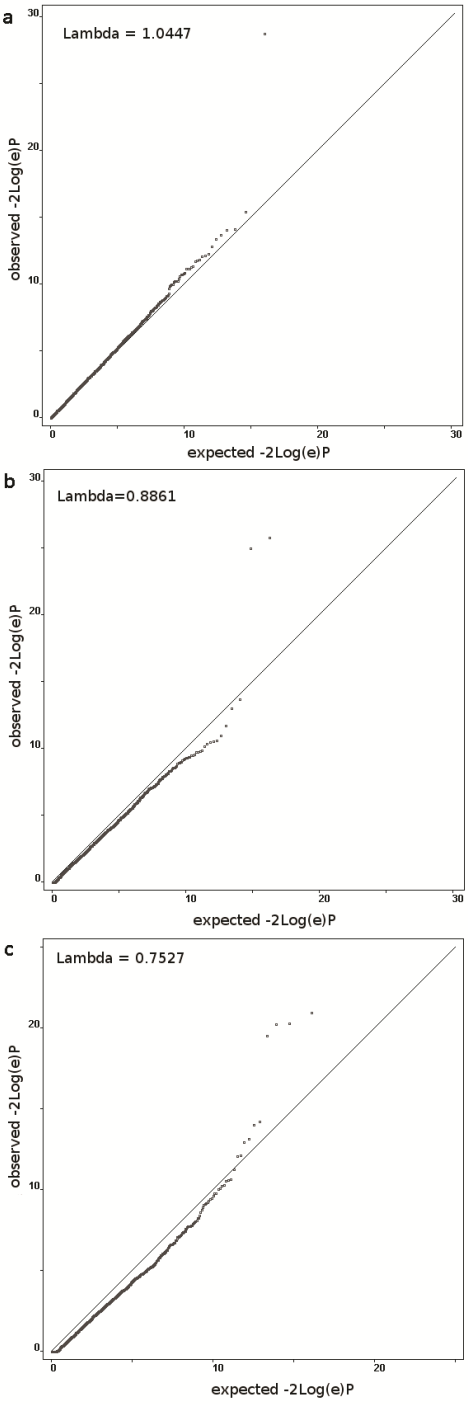

Figure 2.3. A quantile-quantile (QQ) plots of observed *P* values for the SNPs tested versus the values expected under the null hypothesis. QQ plots for A) TwinsUK data, B) ALSPAC questionnaire data and C) ALSPAC accelerometer data, were created to assess whether populations tested are genetically homogenous or whether there is a population substructure (which could potentially cause detection of false positive results in the association analysis). Red squares represent an observed -2log(e)*P* values for the SNPs tested plotted against the -2log(e)*P* values expected under the null hypothesis. In each case Lambda (test statistic) is smaller than 1 indicating a lack of *P*-value inflation. Therefore, we concluded that the TwinsUK and ALSPAC populations are genetically homogenous.

Physical activity was assessed using: 1) self reported questionnaire and 2) accelerometers. For the research involving self-reported questionnaire, participants answered a following question: *Average number of times respondent participated in vigorous activity in the past month: 1) none, 2) less than once per week, 3) 1 to 3 times per week, 4) 4 to 6 times per week, 5) every day*. The threshold for EE was set for answer 4 or higher. For the research involving objective measurement of activity using accelerometer, participants wore an accelerometer for seven consecutive days. Participants were categorized as participating in EE if the total time spent on sporting was equal or higher than 5 hours during 7 days of measurement.

ALSPAC - Quality control

We excluded the subjects with increased number of heterozygous genotypes (more or less than 3SD from the mean). Exclusion of outliers with increased or decreased number of heterozygous genotypes is a part of quality control procedures done in a number of genome-wide and smaller studies (Anderson et al., 2010). because a largely increased number of heterozygotes may suggest a contamination of a sample (e.g. mixing of two samples) or inbreeding.

Furthermore, we excluded the subjects with more than 3% missing genotypes. This resulted in 3846 females remaining for the further analysis. The genetic data were available for 8393 SNPs located in two chromosomal regions, including the HSA20 syntenic region of interest. We removed 12 SNPs due to more than 3% unsuccessful calls and 16 SNPs based on violation of the Hardy-Weinberg equilibrium test at $P<0.001$. No SNPs had minor allele frequency of less than 1%.

After quality control, there were 3162 SNPs in the HSA20 region of interest. The total genotyping rate in the remaining individuals was 99.9%. The QQ-plots (observed *P*-values plotted against the *P*-values expected under the null hypothesis) in the analyses did not suggest inflation of the *P*-values (Figure 2.3, panels b and c, Lambda < 1 in both cases). Of the 3846 females who passed the QC, there were 1396 with non-missing physical activity questionnaire data and 987 with non-missing accelerometer data. Those individuals were included in the logistic regression analyses of the association between the genotypes and two phenotypes.

Statistical analysis

Mouse running wheel activity. Differences in RWA in the panel of CS-strains were assessed by a Kruskal-Wallis test (due to the heteroscedasticity of the data), with post hoc (unpaired Student's t-test) comparing all CSS with the B6 control strain (corrected α =.003) (Laarakker et al., 2008).

QTL analysis. The Kolmogorov-Smirnov one-sample test was used to check normality of the data. All data within genotype groups were found to be normally distributed.

TwinsUK data. Genetic markers were tested for association with a dichotomous phenotype representing excessive vs. non-excessive exercise by means of the DFAM procedure implemented in Plink v1.07 (Purcell et al., 2007). The *P*–values were corrected for multiple testing by means of Bonferroni correction. These adjusted *P*-values are reported through the manuscript. The significance threshold for the adjusted *P*-values was set at α =.05. This procedure uses a clustered analysis (with the Cochran-Mantel-Haenszel test), which allows for inclusion of non-independent individuals (such as siblings). DFAM procedure in Plink does not allow the use of covariates (such as age). Therefore, we replicated the analysis using the GWAF procedure implemented in R-package (function gee.batch), and using age as a covariate. The results corroborated the findings from Plink.

ALSPAC data. The phenotype (excessive vs. non-excessive exercise) was used as an outcome variable in the logistic regression models, with genotypes as the predictor variables and age as well as BMI as covariates. The *P*–values were corrected for multiple testing by means of Bonferroni correction. These adjusted *P*-values are reported through the manuscript.

The significance threshold for the adjusted *P*-values was set at α =.05. The analyses of the human genetic data assumed an additive genetic model. Data were handled and analyzed with SPSS v20 and Plink v1.07 (Purcell et al., 2007).

Results

Running wheel activity in CSS panel

In order to determine which mouse chromosome contains genetic loci regulating a RWA, we tested baseline RWA of a panel of CSS (except CSS16 due to low availability) and both progenitor strains which are known to be low active (A/J, abbreviated to A) and high active (C57BL/6J, abbreviated to B6) (Gelegen et al., 2010) (Figure 2.4a). The Kruskall-Wallis test showed a significant difference between the strains ($\chi^2(20) = 106.055$, $P < 0.001$). Post-hoc analysis showed that progenitor lines were significantly different in respect to the RWA, with A mice being less active than

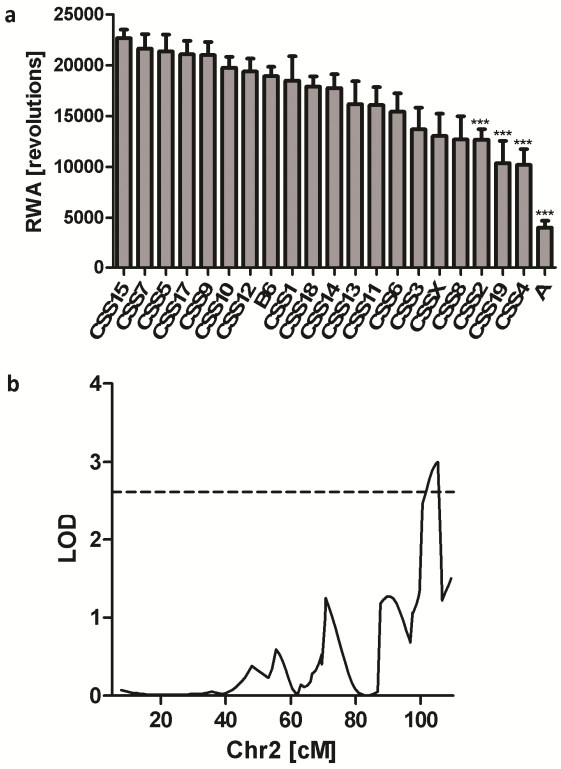

Figure 2.4. Mouse chromosome 2 contains QTL for voluntary running wheel activity. A) Running wheel activity (RWA) as average count of wheel revolutions on days 5, 6 and 7 for the CSS mouse panel and two progenitor lines (A and B6). Data are expressed as means with SEM. Kruskall-Wallis test with post-hoc test: ***P < 0.001. B) Chromosomal mapping of locus associated with RWA for F_2 population between CSS2 and B6 strains. Dotted line indicates the significance cutoff for the LOD score (= 2.62).

B6 strain (P < 0.001). Three consomic lines run significantly less than the host strain (B6) (CSS2 (P < 0.001), CSS4 (P < 0.001) and CSS19 (P < 0.001)). These results suggested that QTLs for voluntary RWA could be found on one of the three chromosomes (Chr2, Chr4, Chr19). In the initial effort to find a chromosomal region associated with RWA we chose to further proceed with line CSS2 due to two reasons. First, CSS4 strain has severely impaired maternal care and therefore, any phenotype observed in adult animals may be contributed to the improper care and nutritional status during this important developmental period (Hessel et al., 2009). Second, females from CSS2 line do not differ from females of B6 host line for the latency to fall from accelerating rotarod (data presented on the Mouse Phenome

Database: http://phenome.jax.org/db/q?rtn=projects/details&sym=Lake3; experiments conducted by Jeffrey Lake, Leah Rae Donahue and Muriel T Davisson from The Jackson Laboratory, Bar Harbor, ME USA and published as data set Lake3). This indicates that the differences in the RWA observed between B6 and CSS2 mice are not caused by sensory-motor coordination of CSS2 mice. Therefore, we considered CSS strain to be the most appropriate strain to search for candidate genes contributing to the voluntary exercise. Finally, mice from CSS19 line perform worse on the rotarod test than mice from CSS2 line, which may influence their ability to run in the running wheel (due to impaired sensory-motor coordination).

QTL mapping

To fine-map genetic regions regulating levels of voluntary wheel running, we conducted a quantitative trait loci (QTL) analysis using RWA and polymorphic DNA markers of females (n = 68) from a CSS2 x B6 F_2 population. The distributions of the measured RWA in the progenitor lines (A and B6), CSS2 parental lines as well as the F_2 population are shown in (Figure 2.2). QTL analysis showed a locus on mouse Chr2 (Figure 2.4b) associated with voluntary wheel running (MQM-MapQTL procedure). The significant peak of the QTL region (169,276,294 to 172,557,293 bp; estimated minus 1 LOD support confidence interval using linear interpolation calculations (Lynch and Walsh 1998)) was positioned at SNP *rs27289254* (LOD score = 3.00) which is relatively close to *Mc3r* (MGI:96929), a gene known to influence RWA levels in mice (Butler et al., 2000). The *rs27289254* is placed in the intergenic region between *Dok5* (MGI:1924079) and *Cbnl4* (MGI:2154433) genes. The QTL explained 16.6% of the variance in RWA in the F_2 population. The total QTL region contains 47 genes (according to Ensembl Database; Table 2.2 shows all the transcripts) out of which only 16 are validated protein-coding genes: *Tshz2*(MGI:2153084), *Zfp217* (MGI:2685408), *Bcas1* (MGI:1924210), *Cyp24a1* (MGI:88593), *Pfdn4* (MGI:1923512), *Dok5*, *Cbln4*, *Mc3r*, *Fam210b* (MGI:1914267), *Aurka* (MGI:894678), *Cstf1* (MGI:1914587), *Cass4* (MGI:2444482), *Rtdc1* (MGI:1913654), *Gcnt7* (MGI:3606143), *Fam209* (MGI:1923676) and *Tfap2c* (MGI:106032). Using Mouse SNP Query (http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=snpQF) we assessed that, out of these 16 genes, only six (*Bcas1*, *Pfdn4*, *Fam210b*, *Cass4*, *Rtdc1*, *Fam209*) have a coding non-synonymous SNP between B6 and A mice.

Table 2.2. Genes in the QTL on mouse Chr2. According to Ensembl database the indicated region on Chr2 contains 47 genes coding for 89 transcripts.

Gene symbol	Ensembl name 1	Ensembl name2	strand	cdsStart	cdsEnd
1700007M16Rik	ENSMUST00000129075	ENSMUSG00000086613	+	171238991	171238991
1700028P15Rik	ENSMUST00000139894	ENSMUSG00000086823	-	171962799	171962799
1700028P15Rik	ENSMUST00000151904	ENSMUSG00000086823	-	171962131	171962131
1700028P15Rik	ENSMUST00000125465	ENSMUSG00000086823	-	171962824	171962824
1700040N02Rik	ENSMUST00000131931	ENSMUSG00000086999	+	170380435	170380435
1700101G07Rik	ENSMUST00000077067	ENSMUSG00000074571	+	169582513	169583020
4930470P17Rik	ENSMUST00000062355	ENSMUSG00000043583	-	170579490	170601163
4930529I22Rik	ENSMUST00000131509	ENSMUSG00000085350	-	169405450	169405450
AL731822.1	ENSMUST00000180895	ENSMUSG00000097065	-	170070671	170070671
AL844576.1	ENSMUST00000180625	ENSMUSG00000097514	-	170194053	170194053
AL929563.1	ENSMUST00000181446	ENSMUSG00000096972	-	169888503	169888503
AL929563.1	ENSMUST00000180945	ENSMUSG00000096972	-	169888503	169888503
Aurka	ENSMUST00000109140	ENSMUSG00000027496	-	172356795	172369027
Aurka	ENSMUST00000028997	ENSMUSG00000027496	-	172356795	172370455
Aurka	ENSMUST00000109139	ENSMUSG00000027496	-	172356795	172369027
Aurka	ENSMUST00000128004	ENSMUSG00000027496	-	172363131	172363131
Aurka	ENSMUST00000126107	ENSMUSG00000027496	-	172370521	172370521
AY702102	ENSMUST00000087964	ENSMUSG00000067581	-	169962337	169962691
Bcas1	ENSMUST00000109152	ENSMUSG00000013523	-	170348073	170420662
Bcas1	ENSMUST00000154650	ENSMUSG00000013523	-	170348073	170406509
Bcas1	ENSMUST00000068137	ENSMUSG00000013523	-	170348073	170420662
Bcas1	ENSMUST00000013667	ENSMUSG00000013523	-	170348073	170420662
Bcas1	ENSMUST00000156657	ENSMUSG00000013523	-	170349740	170349740
Bcas1	ENSMUST00000133673	ENSMUSG00000013523	-	170357113	170357113
Bcas1	ENSMUST00000152461	ENSMUSG00000013523	-	170349795	170349795
Bcas1	ENSMUST00000147577	ENSMUSG00000013523	-	170388003	170388003
Bcas1	ENSMUST00000145920	ENSMUSG00000013523	-	170427845	170427845
Cass4	ENSMUST00000099061	ENSMUSG00000074570	+	172393969	172428057
Cass4	ENSMUST00000103073	ENSMUSG00000074570	+	172393969	172432643
Cass4	ENSMUST00000109136	ENSMUSG00000074570	+	172393969	172432643
Cbln4	ENSMUST00000087950	ENSMUSG00000067578	-	172037369	172042298
Cstf1	ENSMUST00000151511	ENSMUSG00000027498	+	172372960	172376070
Cstf1	ENSMUST00000116375	ENSMUSG00000027498	+	172372960	172380619
Cyp24a1	ENSMUST00000038824	ENSMUSG00000038567	-	170485676	170496774
Dok5	ENSMUST00000029075	ENSMUSG00000027560	+	170732122	170879238
Fam209	ENSMUST00000029007	ENSMUSG00000027505	+	172472592	172474219
Fam210b	ENSMUST00000028995	ENSMUSG00000027495	+	172345661	172352802
Gcnt7	ENSMUST00000099060	ENSMUSG00000074569	-	172451063	172454902
Gcnt7	ENSMUST00000161334	ENSMUSG00000074569	-	172451063	172454742
Gm11011	ENSMUST00000109160	ENSMUSG00000078912	-	169582844	169587745
Gm14249	ENSMUST00000156239	ENSMUSG00000087074	+	169504843	169504843
Gm14250	ENSMUST00000148458	ENSMUSG00000086840	+	169395524	169395524
Gm14259	ENSMUST00000133586	ENSMUSG00000086970	+	170407175	170407175
Gm14263	ENSMUST00000117955	ENSMUSG00000080955	-	170954824	170954824

Gm14264	ENSMUST00000147141	ENSMUSG00000085158	-	170662465	170662465
Gm14266	ENSMUST00000125631	ENSMUSG00000084829	+	171269200	171269200
Gm14266	ENSMUST00000146542	ENSMUSG00000084829	+	171271350	171271350
Gm14269	ENSMUST00000120455	ENSMUSG00000084067	-	170463819	170463819
Gm14270	ENSMUST00000118388	ENSMUSG00000084013	+	170285880	170285880
Gm14271	ENSMUST00000137886	ENSMUSG00000087409	+	172050134	172050134
Gm14272	ENSMUST00000133525	ENSMUSG00000085426	-	171989195	171989195
Gm14273	ENSMUST00000136315	ENSMUSG00000085905	+	172338663	172338663
Gm14274	ENSMUST00000117182	ENSMUSG00000083793	+	172101235	172101235
Gm14275	ENSMUST00000152112	ENSMUSG00000085949	+	172290520	172290520
Gm14303	ENSMUST00000180124	ENSMUSG00000081344	-	172509697	172509697
Gm14303	ENSMUST00000121320	ENSMUSG00000081344	-	172509691	172509691
Gm14455	ENSMUST00000138288	ENSMUSG00000087633	-	172440560	172440560
Gm14637	ENSMUST00000156516	ENSMUSG00000085225	+	170578424	170578424
Gm14640	ENSMUST00000145800	ENSMUSG00000084818	+	171217947	171217947
Gm14641	ENSMUST00000136486	ENSMUSG00000087100	-	172003995	172003995
Gm16796	ENSMUST00000126086	ENSMUSG00000085495	-	170511927	170511927
Gm26489	ENSMUST00000157880	ENSMUSG00000088505	-	171306431	171306431
Mc3r	ENSMUST00000038532	ENSMUSG00000038537	+	172248859	172249831
Pfdn4	ENSMUST00000075087	ENSMUSG00000052033	+	170516588	170518804
Pfdn4	ENSMUST00000063682	ENSMUSG00000052033	+	170511445	170518804
Pfdn4	ENSMUST00000136839	ENSMUSG00000052033	+	170511445	170518804
Pfdn4	ENSMUST00000109148	ENSMUSG00000052033	+	170516588	170518804
Pfdn4	ENSMUST00000170167	ENSMUSG00000052033	+	170516588	170518711
Pfdn4	ENSMUST00000109147	ENSMUSG00000052033	+	170516588	170518718
Pfdn4	ENSMUST00000139328	ENSMUSG00000052033	+	170516588	170516633
Rtfdc1	ENSMUST00000029005	ENSMUSG00000027502	+	172440670	172468774
Rtfdc1	ENSMUST00000109132	ENSMUSG00000027502	+	172466654	172466654
Rtfdc1	ENSMUST00000140048	ENSMUSG00000027502	+	172469908	172469908
Tfap2c	ENSMUST00000030391	ENSMUSG00000028640	+	172549834	172557376
Tfap2c	ENSMUST00000128406	ENSMUSG00000028640	+	172558607	172558607
Tfap2c	ENSMUST00000122982	ENSMUSG00000028640	+	172557826	172557826
Tfap2c	ENSMUST00000099058	ENSMUSG00000028640	+	172550981	172557376
Tfap2c	ENSMUST00000170744	ENSMUSG00000028640	+	172551056	172557376
Tfap2c	ENSMUST00000142633	ENSMUSG00000028640	+	172553518	172553518
Tshz2	ENSMUST00000109159	ENSMUSG00000047907	+	169633739	169886578
Tshz2	ENSMUST00000109157	ENSMUSG00000047907	+	169633739	169886578
Tshz2	ENSMUST00000123300	ENSMUSG00000047907	+	169885156	170070269
Tshz2	ENSMUST00000140699	ENSMUSG00000047907	+	169885864	169912609
Tshz2	ENSMUST00000087966	ENSMUSG00000047907	+	169886119	169886578
Zfp217	ENSMUST00000063710	ENSMUSG00000052056	-	170112504	170120405
Zfp217	ENSMUST00000109155	ENSMUSG00000052056	-	170112504	170120405
Zfp217	ENSMUST00000149088	ENSMUSG00000052056	-	170131220	170131220
Zfp217	ENSMUST00000125162	ENSMUSG00000052056	-	170133945	170133945
Zfp217	ENSMUST00000149318	ENSMUSG00000052056	-	170148103	170148103

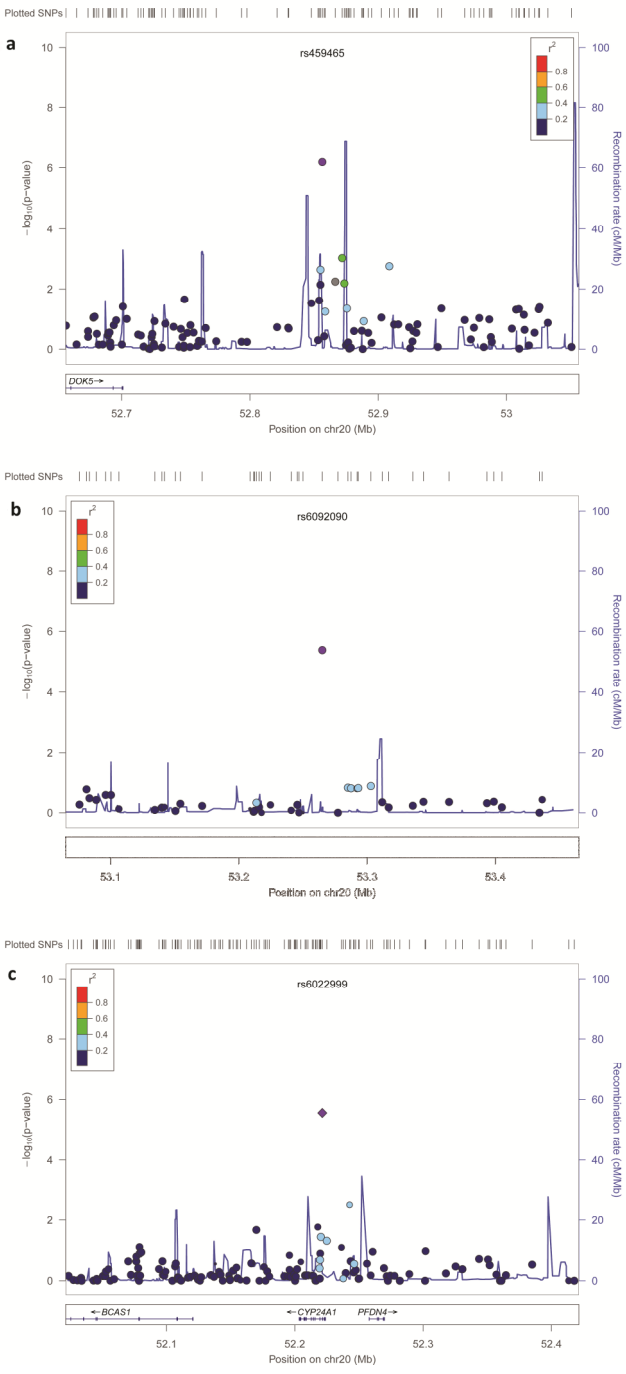

Figure 2.5. Genetic context of SNPs associated with high physical activity in humans. The SNPs which were significantly associated with physical activity levels (marked in purple) measured in the human populations included in this paper are shown in the context of their neighboring SNPs and genes. Circles represent annotated SNPs. Colors depict the squared correlation (r^2) of each SNP with the most associated SNP (marked in purple). Gray circles represent SNP for which r^2 information was missing. A) association of *rs459465* in TwinsUK sample (adjusted *P*-value = 0.001). B) association of *rs6022999* with EE assessed using questionnaire in ALSPAC sample (adjusted *P*-value = 0.011). C) association of *rs6092090* with EE assessed using accelerometer in ALSPAC sample (adjusted *P*-value = 0.012). Plots were produced using the LocusZoom program (Pruim et al., 2010).

Testing of genetic associations in syntenic human regions

In order to test the hypothesis that the QTL for physical activity found on mouse Chr2, contains a candidate gene or a regulatory element regulating levels of physical activity in humans, we translated the mouse QTL region and tested the associations in two different human populations (initial analysis: TwinsUK (Spector and Williams 2006); replication: ALSPAC (Boyd et al., 2013)). The region on mouse Chr2 between 169,276,294 and 172,557,293 bp is syntenic to the human region on chromosome 20 (HSA20:51,212,545–55,212,986).

Basic characteristics of the TwinsUK sample

Basic characteristics of the TwinsUK sample, divided into EE and nonEE groups, are shown in Table 2.3. There were no significant differences between the groups in terms of age, BMI and ethnicity.

Table 2.3. Basic characteristics of the TwinsUK sample divided in excessive exercise (EE) and non-excessive exercise (nonEE) groups.

	nonEE			**EE**		
	Mean	N (%)	SD	Mean	N (%)	SD
Age	51.3	2979	8.54	50.32	137	8.75
BMI	26.68	2398	5.17	24.48	113	3.94
Sport [h]	1.04	2979	1.34	7.14	137	2.04
Ethnicity						
Asian		6 (0.2)			0	
Black		18 (0.6)			2 (1.5)	
Chinese		2 (0.1)			0	
Mixed		17 (0.6)			0	
Other		4 (0.1)			0	
White		2664 (89.5)			126 (92.0)	

Mapping of the QTL region in TwinsUK sample

In the adult population of females from TwinsUK we assessed if there is an association between participation in EE and the candidate genetic region. Gene association analysis of Chr20 region syntenic to the candidate region on mouse Chr2 revealed a significant association of EE with an SNP *rs459465* (unadjusted *P*-value= 5.23E-07; adjusted *P*-value of 0.001). *Rs459465* is located in an intergenic region, and a gene in the closest proximity to it is *DOK5*. The gene and the SNP are not in linkage disequilibrium. Figure 2.5 panel a shows the SNP in the genetic context.

Basic characteristics of the ALSPAC sample

Basic characteristics of the ALSPAC sample divided in EE and nonEE groups are shown in Table 2.4. The division was made twice: 1) based on the questionnaire

outcome; and, 2) based on accelerometer readouts. There were no significant differences between the groups in terms of age, BMI and ethnicity.

Table 2.4. Basic characteristics of the ALSPAC sample divided in excessive exercise (EE) and non-excessive exercise (nonEE) groups. The division was made twice: 1) based on the questionnaire outcome; and, 2) based on accelerometer readouts.

	questionnaire					
	nonEE			EE		
	Mean	N (%)	SD	Mean	N (%)	SD
Age (months)	185.69	965	3.49	186.00	174	4.08
BMI	21.99	1696	3.75	21.41	306	2.99
Time spent on PA						
< 1 per week		808 (37.1)			-	
1 – 3 per week		1000 (45.9)			-	
4 – 6 per week		-			258 (67.9)	
daily		-			122 (32.1)	
Ethnicity						
Black		89 (4.21)			9 (2.4)	
White		1901 (87.3)			336 (88.4)	
	accelerometer					
Age	185.88	1209	3.77	185.88	103	3.38
BMI	21.75	714	3.37	21.82	52	2.96
Sport [h]	1.78	1209	1.23	6.52	103	1.51
Ethnicity						
Black		35 (2.9)			6 (5.8)	
White		1075 (88.9)			85 (82.5)	

Mapping of the QTL region in ALSPAC sample

In order to replicate the finding from TwinsUK population, we repeated the analysis in adolescent population from ALSPAC longitudinal study. We conducted two association analyses between genotype and the EE measured with the use of: 1) the questionnaire, 2) accelerometer. For the analysis of EE defined on the basis of the questionnaire, gene association analysis of HSA20 region revealed a significant association of EE with two SNPs: 1) *rs6022999* (unadjusted *P*-value= 3.63E-03; adjusted *P*-value of *P*=0.011) and 2) *rs6092090* (unadjusted *P*-value= 3.84E-03; adjusted *P*-value of 0.012). *Rs6022999* is placed in the intron region of *CYP24A1* gene whereas *rs6092090* is between the *DOK5* gene and the SNP found in the analysis of the TwinsUK sample. None of the SNPs in LD with the two reported SNPs appeared to be associated or trend-associated with the phenotype (Figure 2.5 panel b and c). None of the SNPs in LD with the two reported SNPs appeared to be associated or trend-associated with the phenotype.

For the analysis of EE defined on the basis of the accelerometer measurement, there was no significant genetic association.

Accordance between physical activity measurement methods

We assessed is the extent of the overlap for EE classification based on the self-reported questionnaire and objectively measured with accelerometer. In the total sample 203 participants were classified into EE group according to one of the methods of EE assessment. However, only 16 participants were classified into EE group according to both physical activity assessment methods (Table 2.5). Chi-square test confirmed that there is no significant overlap between the two assessment methods ($\chi^2(1)=2.90$, $P > 0.05$).

Table 2.5. The overlap between two methods of classification into EE group: 1) the subjective (questionnaire based); and, 2) the objective (based on accelerometer data). Chi-square test confirmed that there is no significant overlap between the two assessment methods ($\chi^2(1)=2.90$, $P > 0.05$).

	Accelerometer – nonEE	**Accelerometer - EE**
Questionnaire - nonEE	766	57
Questionnaire - EE	130	16

Candidate gene analysis

In order to point possible candidate genes which could be associated with the levels of PA, we explored the candidate locus indicated in the current study to be associated with PA in mice and humans.

Gene ontology analysis

We conducted a gene ontology (GO) analysis using a list of genes present in the genetic region pointed out by QTL analysis. Using DAVID Gene Ontology database (Huang et al., 2009a; Huang et al., 2009b), we assessed if any of the genes found in the QTL region on Chr2 was previously associated with locomotor activity or energy expenditure. None of the transcripts was previously associated with these phenotypes according to GO classification. However, it has previously been shown that homozygous *Mc3r* KO mice are characterized by obesity and reduced locomotor activity on the running wheel (Butler et al., 2000) suggesting that *Mc3r* could potentially be an interesting candidate gene in the identified region.

Regulatory element analysis

In order to broaden the search of potential candidate genes from the region, we assessed if there are any known trans-regulatory elements in the region tested. To estimate if there are any eQTLs located within the region on chromosome 20 we used the GTEx (Genotype-Tissue Expression) eQTL Browser (http://www.ncbi.nlm.

nih.gov/gtex/GTEX2/gtex.cgi#) however, no eQTL association was found for any of the SNPs within the region.

We went back to mouse data and assessed what genes have trans regulatory elements within the region on Chr2 using the resource of GeneNetwork – WebQTL database (http://www.genenetwork.org/webqtl/main.py). We used Hippocampus Consortium M430v2 (Jun06) PDNN Database, which contains data for mRNA expression levels assessed in the adult hippocampus of 99 genetically diverse strains of mice including 67 BXD recombinant inbred strains, 13 CXB recombinant inbred strains, a diverse set of common inbred strains (including A and B6 lines), and two reciprocal F_1 hybrids. WebQTL searched the Hippocampus Consortium M430v2 (Jun06) PDNN Database for all records with a trans-QTL having an LRS between 9.2 and 1000 using a 20 Mb exclusion buffer and with LRS between 9.2 and 1000 on mouse Chr2 within the range of 169 to 173 Mbp. The search pointed to 15 genes having regulatory elements within the tested region of Chr2. One of those genes, *Grm8* (glutamate receptor, metabotropic 8) is described further in the Discussion section.

Discussion

By using a panel of mouse CS-strains we identified a significant QTL for RWA located on Chr2. This genetic region was considered a candidate region for voluntary exercise in humans. To test this hypothesis, we conducted a gene association analysis of a human genetic region, syntenic to this mouse candidate gene region, in two independent human populations. Firstly, using a population of adult women (average age 44.16 years) we could confirm that there is a genetic association of the region with high levels of voluntary physical activity in humans. Secondly, taking under consideration the methodological challenges embedded in this translational approach, we validated this finding in an independent human sample of adolescent girls (assessed at the age of 17).

We further explored the candidate region for potential protein-coding candidate genes which could be associated with the levels of PA in mice or human. We based our exploratory analysis on known functional data and on regulatory elements analysis. At this stage, it is not possible to determine whether any of the proposed candidate genes is the causal factor for the observed phenotypes in mice and humans. The proposed genes should be treated as a hypothesis and tested using

functional assays. Furthermore, it is plausible that other genetic elements, such as miRNA, may be in fact responsible for the observed decrease in RWA. Nevertheless, here we would like to propose three genes which could be the primary candidates for the future research efforts. First, KO mice of melanocortin 3 receptor (*Mc3r*, KO mice MGI:5302394) gene express reduced voluntary RWA levels (Butler et al., 2000). Second, the protein product of the *CYP24A1* (cytochrome P450, family 24, subfamily A, polypeptide 1; HGNC:2602) gene is responsible for degradation of vitamin D into a physiologically inactive form. Vitamin D was shown to be essential for proper muscle functioning (Endo et al., 2003; Pfeifer et al., 2002) and polymorphisms in the vitamin D receptor are associated in humans with changed muscle strength in both genders (Windelinckx et al., 2007); these changes are likely to influence the levels of physical activity. However, neither *Mc3r* nor *Cyp24a1* contain a known non-synonymous coding SNP between the progenitor strains, A and B6. Last but not least, out of the fifteen genes that have trans-regulatory elements within the mouse QTL region *GRM8* (glutamate receptor, metabotropic 8; HGNC:4600) could be considered a potential candidate. *Grm8* KO mice are characterized by decreased locomotor activity in the home-cage during the first 3 days of testing; however, this phenotype is accompanied by increased body weight, slight insulin intolerance as well as reduced speed of swimming in the Morris water maze (Duvoisin et al., 2005). Furthermore, the home-cage activity and the RWA should be treated as distinct phenotypes (Garland, Jr. et al., 2011b) and therefore, the Grm8 defficient mice should be tested for their RWA

The comparative interspecies behavioral genetics approach is complicated by question regarding the translational value of animal models of human behavior. However, in case of voluntary exercise, the translational value of voluntary RWA is well documented in literature (Eikelboom 1999; Garland, Jr. et al., 2011b; Kelly et al., 2010; Rezende et al., 2009). The translational approach is further complicated by difficulty in creating a precise definition and operationalization of human phenotypes. However, numerous validated methodologies exists for assessing voluntary exercise in humans. Accelerometer data as well as questionnaire data are established and most commonly used methods of measuring voluntary exercise in humans (Garland, Jr. et al., 2011b; Westerterp 2009). Although they both have limitations, they may be chosen for answering specific research questions after informed analysis of feasibility and reliability of these measurement methods (Dishman 2008; Garland, Jr. et al., 2011b). In the current analysis, there was a clear discrepancy between categorization to EE group based on the objective (accelerometer) and subjective (questionnaire) assessment method. This stays in

agreement with previous findings which showed that the heritability estimates of physical activity are higher when physical activity is assessed using accelerometers (Butte et al., 2006; Cai et al., 2006; Choh et al., 2009; de Vilhena e Santos DM et al., 2012; Seabra et al., 2008). Furthermore, various physical activity assessment methods lead to discrepant results regarding genetic associations (de Vilhena e Santos DM et al., 2012; Simonen et al., 2003a). These observations suggest that various physical activity assessment methods may be, in fact, measuring different constructs (de Vilhena e Santos DM et al., 2012). This should be considered a limitation of the current study.

There are several other limitations of the current study which should be taken under consideration. First, the logistic regression model for the genetic association in ALSPAC sample was corrected for BMI; however, we did not correct the analysis for other potentially relevant variables such as socio-economic status, urban/rural living, education. Second, in case of the genetic association analysis in the ALSPAC sample, none of the SNPs in linkage disequilibrium with the two reported SNPs appeared to be trend-associated with the EE. This suggest that the results obtained in this analysis could be obtained by chance.

Taken together, this study identified a QTL for voluntary RWA in mice. As this phenotype is considered to be a model of voluntary exercise levels in humans, we tested association of voluntary exercise in humans with a genetic human region syntenic with this candidate mouse locus. The association of genetic variants within 20q13.2 region with voluntary exercise was demonstrated in two independent human cohorts. The analysis (based on bioinformatics and a search for trans-regulatory elements) suggested some possible candidate genes which could contribute to the voluntary physical exercise levels. However, further studies are needed to test their functional significance for this complex phenotype.

A QTL on Chromosome 19 is Associated with Voluntary Running Wheel Activity Levels in Mice

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Manuscript in preparation

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Abstract

Unraveling the neurobiological processes underlying the motivation and ability to participate in a sufficient amount of voluntary physical activity may facilitate the creation of better disease prevention programs. The understanding of the genetic basis of physical activity may aid in this process. Animal studies are used to facilitate the discovery of genetic predisposition to complex phenotypes such as the voluntary physical activity. In the current study we used running wheel activity as a mouse model for voluntary physical activity in humans. Using a mouse chromosome substitution strain panel we genetically mapped a candidate genetic region (QTL) on mouse chromosome 19 which explained 25% of the phenotypic variance. Subsequently, we tested the hypothesis that a genetic region in humans, syntenic with the discovered mouse QTL, is also associated with the levels of voluntary physical activity in a human population. However, no significant association was discovered in the human sample. To conclude, a genetic region on mouse chromosome 19 significantly and robustly contributes to the levels of running wheel activity in mice.

Introduction

Voluntary exercise may be defined as any locomotor activity 'that is not directly required for survival or homeostasis and not directly motivated by any external factor' (Garland, Jr. et al., 2011b). Although appropriate levels of voluntary exercise may be essential for health maintenance (Casazza et al., 2013; Mokdad et al., 2004; Myers et al., 2002) only a minority of the Western population engages in sufficient physical activity (Kelly and Pomp 2013; Marcus and Forsyth 1999). Knowledge of the factors which influence exercise participation could help to create better disease prevention programs. To facilitate this process, animal studies are used to investigate the genetic basis of a given heritable phenotype (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Kelly and Pomp 2013; Stubbe et al., 2006) under controlled genetic and environmental conditions (Schughart et al., 2012). In studies regarding voluntary exercise in humans, voluntary running wheel activity may be considered the most appropriate to fulfill this research aim in rodent species (Kelly and Pomp 2013; Rezende et al., 2009).

Numerous studies confirmed that voluntary exercise in humans as well as voluntary running wheel activity in mice have a genetic basis (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Hagberg et al., 2011; Rankinen et al., 2010; Roth et al., 2012; Swallow et al., 1998). Despite this fact and relatively high levels of synteny between mouse and human genomes (Brudno et al., 2004; Waterston et al., 2002), there is virtually no overlap between the results coming from rodent and human genetic studies for voluntary physical activity (de Vilhena e Santos DM et al., 2012). However, the use animal models of human phenotypes facilitates a systematic genetic studies of complex traits. Therefore, more efforts are needed to bridge the gap between animal to human studies regarding the genetic basis of voluntary physical activity.

In the current paper, we used a cross-species approach in order to identify a novel candidate region for physical activity in humans. First, we screened a panel of mouse chromosome substitution strains (CSS) (Singer et al., 2004) in a mouse model of voluntary exercise (a running wheel activity model). We discovered quantitative trait loci (QTLs) which significantly influenced the variance in the running wheel activity in mice. Second, we hypothesized that this candidate region found in a mouse study will contain genetic variance contributing to the voluntary exercise in humans. We tested this hypothesis in a human population for which the genetic data and phenotypic information on the levels of physical activity (self-reported questionnaires) was available.

Methods

Ethical statement

All animal experiments were approved by Animal Experiments Committee of the Academic Biomedical Centre in Utrech, The Netherlands. The Animal Experiments Committee based its decision on 'De Wet op de Dierproeven' (The Dutch 'Experiments on Animals Act'; 1996) and on the Dierproevenbesluit' (The Dutch 'Experiments on Animals Decision'; 1996).

The study involving participants of the St. Thomas' UK Adult Twin Registry (TwinsUK) was approved by the St Thomas' Hospital research ethics committee. All human participants and also, in case of the minors, their first kin gave written informed consent.

Finding a candidate region for voluntary running wheel activity

Mice

Initial breeding pairs of the CSS and their progenitor lines (A/J (A) and C57BL/6J (B6)) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Generation of the mouse CSS panel has been described previously (Nadeau et al., 2000). All mice were bred at the Brain Center Rudolf Magnus animal facility and were 2-3 months old at the start of the experiment (lights on at 2:00 a.m. off at 2:00 p.m., temperature 22.0 ± 2.0 °C, fed ad libitum). In total, 425 female mice were tested in the experimental procedure; B6 strain (n = 36), A strain (n = 23) and of the 19 tested CS strains (n = 298; median of 12 mice per CS strain), CSS19F2 (F_2-intercross between C57BL/6J-Chr 19^A/NaJ (CSS19) and B6) (n = 85). Mice from CSS16 were not tested due to low availability. The consomic F_2 cross approach is a specifically designed to identify single QTL on a single chromosome using a relatively low number of F2-individuals (Singer et al., 2004). By using this methodology, one reaches a strong reduction in the amount of epistatic interactions between the QTL and loci from the other 20 chromosomes. Because of this sensitivity, significantly less F_2 mice are necessary to identify QTLs when compared to the traditional whole genome intercross mapping approach. This has been indicated elegantly by Singer and colleagues (2004). In addition, QTL analysis was performed by MQM-mapping (multiple-QTL- model or marker-QTL-marker) which is more powerful than the traditional interval mapping approach (Jansen 1994). The levels of physical activity, such as voluntary running wheel activity, are known to be very different in males and females. As we are interested in the genetics of physical activity levels in relation to eating disorders with a gender pre-dominance in females (such as in anorexia nervosa) (Gelegen et al., 2010), we decided to perform this genetic screen in female mice only.

Running wheel activity measurement

Mice were maintained in running wheel cages (wheel circumference: 43.96 cm) for a week. Body weight and food intake were measured daily just before the beginning of the dark phase. Individual wheel running revolutions were continuously registered using Cage Registration Software (Department of Biomedical Engineering, UMC Utrecht, The Netherlands).

DNA samples, genetic marker analysis and map construction

Genomic DNA was isolated from spleen and/or tail from F_1-hybrids, F_2-intercross mice and CSS19 and B6 mice, using a phenol/chlorophorm/iso-amylalcohol protocol

(Laird et al., 1991). A total of 16 microsatellite markers (D19Mit59, D19Mit109, D19Mit61, D19Mit16, D19Mit106, D19Mit86, D19Mit46, D19Mit65, D19Mit19, D19Mit119, D19Mit10, D19Mit123, D19Mit36, D19Mit1, D19Mit34, D19Mit137), dispersed throughout the mouse chromosome 19, was used to map a region on chromosome 19 associated with running wheel activity in the F_2 population. Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit-test. None of the markers showed segregation distortion (when $P<0.05$).

Quantitative trait loci analysis

The QTL analysis was conducted on the females from CSS19 x B6 F_2 population. The location of the QTLs affecting the measured quantitative trait and the variance explained by each locus were determined using the MapQTL® software package (version 4.0) (Van Ooijen et al., 2002). Running wheel activity was normally distributed in the F_2 population. QTL analysis was performed by MQM-mapping (multiple-QTL- model or marker-QTL-marker) (Jansen and Stam 1994). Results were expressed as LOD scores. Permutation tests (10,000 permutations) were done to assess the statistical significance of a QTL (5% significance level: LOD score ≥ 2.50).

Genetic association study on human participants

Establishing the syntenic region

The coordinates of a QTL found in the mouse linkage study have been converted to a syntenic region in humans via the UCSC LiftOver tool (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Mouse region on chromosome 19 (33,068,914-40,118,011 bp, according to 37.2 mouse genome build) was syntenic to a region on human chromosome 10 (HSA10:89,978,389-96,829,399, according to hg18 genome build).

TwinsUK- sample description and physical activity measurements

The participants were 3038 females enrolled to the TwinsUK study between 1994 and 2007. This was only a portion of the total TwinsUK participants who provided data for physical activity. The composition of the TwinsUK was described in detail elsewhere (Spector and MacGregor 2002; Spector and Williams 2006). The analysis was conducted in the age group between 30 and 55 years of age (mean age=44.16, SD=7.49) at the time of examination, which was considered the most representative age group with respect to the self-reported, voluntary physical activity. Participants were genotyped on Illumina 370k or Illumina 610k platform.

Physical activity of participants from TwinsUK study was assessed on the basis of self-reported questionnaires (so called Q10 and Q17D). The questionnaire Q10 included the following questions: 1): *Currently, how many minutes per week do you spend in weight bearing activity? e.g. aerobics, running, dance, football, basketball, racquet sports etc. (do not include walking or gardening)*; 2) Questionnaire Q10: *Currently, how many minutes per week do you spend in non-weight bearing activity? e.g. swimming, cycling, yoga, aqua aerobics etc.* The questionnaire Q10 included the following questions: 1) Questionnaire Q17D: *During the last week, how many hours did you spend on each of the following physical activities? / Physical exercise such as swimming, jogging, aerobics, football, tennis, gym workout, power walking etc.*; 2) Questionnaire Q17D: *During the last week, how many hours did you spend on each of the following physical activities? / Cycling, including to work and during leisure time*; 3) Questionnaire Q17D: *During the last week, how many hours did you spend on each of the following physical activities? / Gentler exercise, Yoga and Pilates.* Data of questionnaire Q10 were used preferentially as they were available for 3233 participants (in comparison to only 2683 in case of Q17D questionnaire). If data from questionnaire Q10 for a given participant were not available, the score from questionnaire Q17D was used. Finally, the amount of sport was categorized as excessive exercise (EE) if a participant spent 5 or more hours per week on exercise, as proposed by Davis (1995)(Davis 1995).

Statistical analysis

Mouse running wheel activity

Differences in running wheel activity in the panel of CS-strains were assessed by means of the Kruskal-Wallis test (due to the heteroscedasticity of the data), with a post hoc (unpaired Student's t-test) in which all the CSS were compered with the B6 control strain (corrected α =.003) (Laarakker et al., 2008).

QTL analysis

The Kolmogorov-Smirnov one-sample test was used to check normality of the data. All data within genotype groups were found to be normally distributed.

TwinsUK data

Genetic markers were tested for their association with a dichotomous phenotype, namely excessive vs. non-excessive exercise. The analysis was conducted by means of the DFAM procedure implemented in Plink v1.07 (Purcell et al., 2007) with a Bonferroni correction for multiple testing. This procedure uses a clustered analysis (with the Cochran-Mantel-Haenszel test), which allows for inclusion of non-independent individuals (such as siblings).

Results

Running wheel activity in CSS panel

In order to determine which mouse chromosome contains genetic loci regulating the running wheel activity, we tested baseline running wheel activity of a panel of CSS (except CSS16 due to low availability) and the progenitor strains which are known to be low active (A/J, abbreviated to A) and high active (C57BL/6J, abbreviated to B6) (Gelegen et al., 2010) (Figure 3.1a). The Kruskall-Wallis test showed a significant difference between the strains (χ2 (20) = 106.055, P<0.001). Post-hoc analysis showed that progenitor lines were significantly different in respect to the running wheel activity, with A mice being less active than B6 strain (P<0.001). Three consomic lines run significantly less than the host strain (B6) (CSS2 (P<0.001), CSS4 (P<0.001) and CSS19 (P<0.001)). This statistical analysis suggested that QTLs for voluntary running wheel activity could be found on one of the three chromosomes (Chr2, Chr4, Chr19).

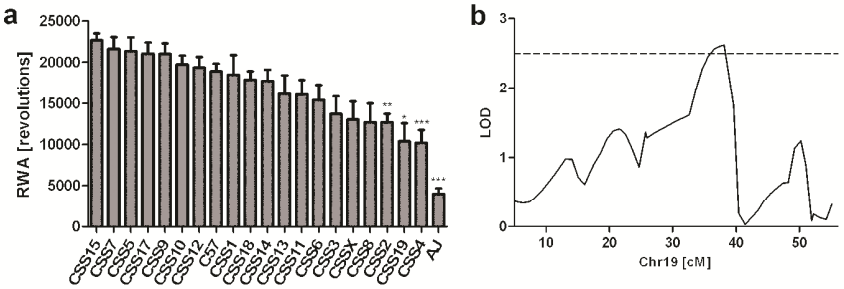

Figure 3.1. Mouse chromosome 19 contains QTL for voluntary running wheel activity. (a) Running wheel activity (RWA) as an average count of wheel revolutions on days 5, 6 and 7 of mice from the CSS panel and two progenitor lines (A and B6). Data are expressed as a mean ± SEM. The Kruskall-Wallis test with a post-hoc test: *** P<0.001. (b) Chromosomal mapping of the locus associated with running wheel activity in an F_2 population between CSS19 and B6 strains. Dotted line shows the significance cutoff for the LOD score (= 2.50).

QTL mapping

To fine-map genetic regions regulating levels of voluntary wheel running, we conducted a QTL analysis for the running wheel activity mouse model using

polymorphic DNA markers. We tasted females from a CSS19 x B6 F_2 population (n = 85). QTL analysis showed a locus on mouse chromosome 19 (Chr19) (Figure 3.1b) associated with the voluntary wheel running (MQM-MapQTL procedure). The QTL spanned between 33,068,914bp and 40,118,011bp as estimated on the basis of minus 1 LOD support confidence interval (linear interpolation calculations (Lynch and Walsh 1998)). The significant peak of the QTL region (was positioned at the marker D19Mit65 (38,910,391-38,910,568bp; LOD score = 2.62) which is placed within TBC1 domain family, member 12 gene (*Tbc1d12*; chr19:38,836,579-38,919,923bp). The QTL explained 25.8% of the variance in running wheel activity in the F_2 population and it contained 104 confirmed genes.

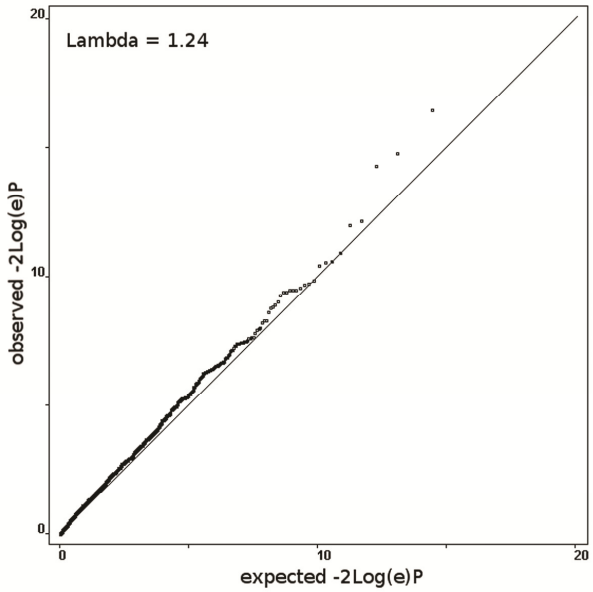

Figure 3.2. A quantile-quantile (QQ) plots of observed *P*-values for the SNPs tested versus the values expected under the null hypothesis. QQ plots for TwinsUK data were created to assess whether the tested population is genetically homogenous or whether there is a population substructure (which could potentially cause detection of false positive results in the association analysis). Black squares represent an observed -2log (e)*P* values for the SNPs tested plotted against the -2log (e)*P*-values expected under the null hypothesis. Lambda (test statistic) is higher than 1 indicating a *P*-value inflation.

Testing of genetic associations in a syntenic human region

In order to test the hypothesis that the QTL for the running wheel activity found on a mouse chromosome 19 contains a candidate gene or a regulatory element influencing

the levels of the voluntary physical activity in humans, we translated the mouse QTL region to a human genetic region. The region on mouse Chr19 between 33,068,914 and 40,118,011bp is syntenic to human region on chromosome 10 (HSA10:89,978,389-96,829,399bp).

Data - quality control

The TwinsUK individuals were genotyped on Illumina 370k or Illumina 610k platform. The data were quality controlled per each platform separately as described previously (Chapter 2 of this thesis). After merging of both datasets, there were 496 males, 5150 females and 1337 SNPs. Further analyses were performed on the merged dataset, of 3038 females who had non-missing EE phenotype data. The QQ-plots (observed *P*-values plotted against the *P*-values expected under the null hypothesis) in the analyses suggest slight inflation of the *P*-values (Figure 3.2, Lambda=1.24). This can be accounted for by the fact that the chosen region was hypothesized to contain a significant association.

Mapping of the QTL region in the TwinsUK sample

We assessed whether there is an association between participation in EE and the candidate genetic region by using the adult population of females from TwinsUK . Gene association analysis of HSA10 region syntenic to the candidate region on mouse Chr19 revealed no significant associations of EE with any of the single nucleotide polymorphisms (SNPs). Three of the tested SNPs with the lowest unadjusted *P*-values are in a linkeage disequilibrium (Figure 3.3): *rs11202812* (unadjusted P=0.0003; panel a), *rs7912222* (unadjusted P=0.0007; panel b), *rs12357879* (unadjusted P<0.0009; panel c).

Candidate gene analysis

Gene ontology analysis

We conducted a gene ontology analysis using the DAVID Gene Ontology database (Huang et al., 2009a; Huang et al., 2009b) and a list of 104 genes from the QTL found on mouse chromosome 19. The analysis showed three functional clusters of genes in the region: 1) 12 genes belonging to the cytochrome 450 family, 2) 5 of the genes being lipases, 3) 3 of the genes classified as interferon-induced proteins with tetratricopeptide repeats.

Using the GeneNetwork - WebQTL database (http://www.genenetwork.org/webqtl/main.py) we searched the Hippocampus Consortium M430v2 (Jun06) PDNN

Database in order to find genes within the QTL which were previously associated with locomotor activity or energy homeostasis. However, the search delivered a negative result.

Discussion

With the use of a panel of mouse CS-strains we could identify a novel QTL on mouse chromosome 19 which explains 26% of the variance of the running wheel activity. We hypothesized that a syntenic human genetic region contributes significantly to the voluntary exercise in humans. We tested this hypothesis by using data of a large population based human study. However, we did not detect a significant association in the human sample.

The mouse QTL identified does not contain genes previously proposed to regulate voluntary physical activity levels or energy expenditure in a broad sense. Nevertheless, few genes deserve further discussion. It is of note that the QTL region contains 5-hydroxytryptamine (serotonin) receptor 7 (*Htr7*) which plays a role in thermoregulation (Matthys et al., 2011). According to the energy balance equation, any changes in thermoregulation need to be compensated by changes in other factors in the equation such as energy intake or physical activity (Garland, Jr. et al., 2011b). However, the *Htr7* gene does not have any known polymorphism between A and B6 mice as assessed using Mouse Phenome Database (http://phenome.jax.org/).

Nineteen of the genes within the QTL have coding non-synonymous SNPs between A and B6 progenitor strains: STAM binding protein like 1 (*Stambpl1*), TNF receptor superfamily member 6 (*Fas*), interferon-induced protein with tetratricopeptide repeats 1 (*Ifit1*), solute carrier family 16 (monocarboxylic acid transporters), member 12 (*Slc16a12*), kinesin family member 20B (*Kif20b*), BTAF1 RNA polymerase II, B-TFIID transcription factor-associated (*Btaf1*), kinesin family member 11 (*Kif11*), myoferlin (*Myof*), centrosomal protein 55 (*Cep55*), phospholipase C, epsilon 1 (*Plce1*), nucleolar complex associated 3 homolog (*Noc3l*), cytochrome P450, family 2, subfamily c, polypeptide 29 (*Cyp2c29*), cytochrome P450, family 2, subfamily c, polypeptide 38 (*Cyp2c38*), cytochrome P450, family 2, subfamily c, polypeptide 39 (*Cyp2c39*), cytochrome P450, family 2, subfamily c, polypeptide 68 (*Cyp2c68*),

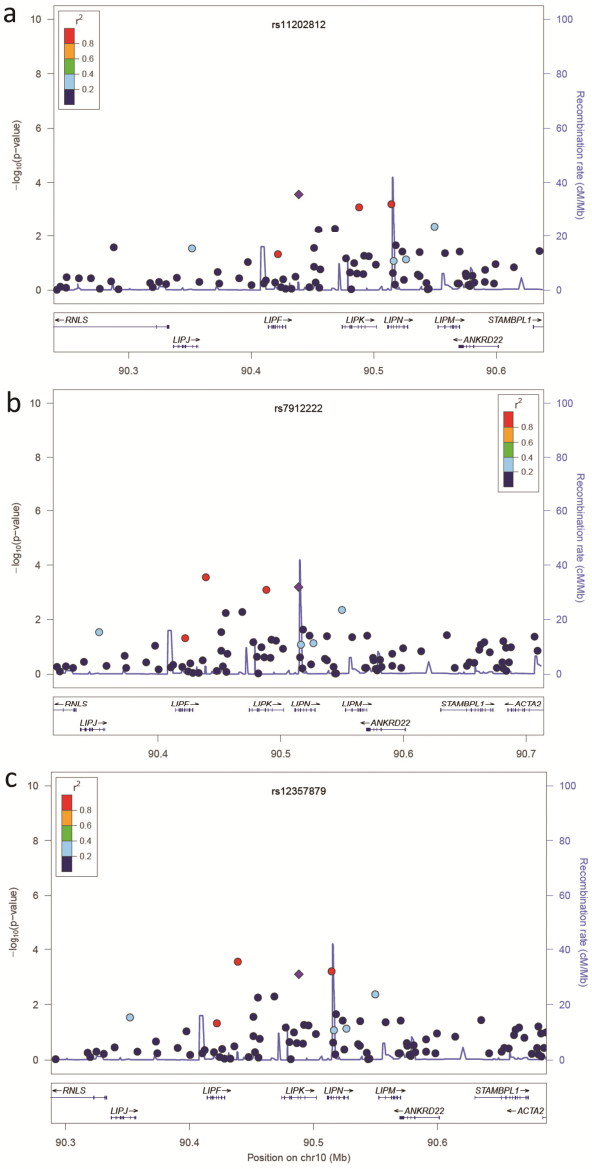

Figure 3.3. Genetic context of the most significant SNPs. The SNPs with the lowest unadjusted *P*-values when tested for association with physical activity levels are shown in the context of neighboring SNPs and genes. Circles represent unnotated SNPs. Colors depict the squared correlation (r^2) of each SNP with the most associated SNP (marked in purple). (a) association of *rs11202812* (adjusted *P*-value = 0.3678). (b) association of *rs7912222* (adjusted *P*-value = 0.8508). (c) association of *rs12357879* (adjusted *P*-value = 1.00). Plots were produced using the LocusZoom program (Pruim et al., 2010).

cytochrome P450, family 2, subfamily c, polypeptide 69 (*Cyp2c69*), cytochrome P450, family 2, subfamily c, polypeptide 37 (*Cyp2c37*), cytochrome P450, family 2, subfamily c, polypeptide 54 (*Cyp2c54*), cytochrome P450, family 2, subfamily c, polypeptide 50 (*Cyp2c50*). Interestingly, especially the genes belonging to the cytochrome P450 family have multiple polymorphisms between the two progenitor strains. The cytochrome P450 family is also a gene family with most numerous cluster of genes within the current QTL. The proteins belonging to the cytochrome P450 family are intra- and extracellular heme-containing monooxygenases which function is to catalyze a metabolism of various endogenous and exogenous compounds (Pikuleva and Waterman 2013). The Cyp2 family of the cytochrome P450 system, which is over-represented in the current QTL, takes part in a drug and steroid metabolism (Gotoh 1992; Nebert and Russell 2002).

Last but not least, myoferlin (*Myof*) is an interesting gene due to its physiological function and to the fact that it possesses two coding non-synonymous SNPs between the progenitor strains. First, the single nucleotide polymorphism (SNP, rs31052565) between A and B6 mice (ATT ⇒ ACT) causes an amino acid substitution of isoleucine (aliphatic and hydrophobic) to threonine (small, polar and hydrophobic). This substitution may have important consequences for the protein structure and function. Another SNP (rs46477910, CTG ⇒ ATG) causes a substitution of leucine (aliphatic, hydrophobic) with methionine (hydrophobic) which may have less of an impact for protein function. Second, *Myof* mediates myoblast fusion during muscle development (Davis et al., 2002; Demonbreun et al., 2010; Posey, Jr. et al., 2011). It is also activated during muscle regeneration (Demonbreun et al., 2010; Posey, Jr. et al., 2011). One can speculate that coding non-synonymous SNPs could have an effect on the *Myof* protein function and as a consequence could cause differences in muscle growth between the strains. Indeed, previous studies have shown that A mice have profoundly reduced soleus (Kilikevicius and Lionikas 2013a) and tibialis (Kilikevicius and Lionikas 2013b) muscle weight in comparison to B6 mice due to the reduction in the type 2a muscle fibers (Kilikevicius and Lionikas 2013c). Furthermore, de Mooij-van Malsen and colleagues could show that A mice have significantly reduced grip strength in comparison to the B6 progenitor strain (Kas et al., 2008). However, we cannot prove the causal relationship between the discovered genetic polymorphism and the muscle and running wheel activity phenotypes. This hypothesis requires further testing.

To conclude, the study identified a novel QTL significantly contributing to the levels of voluntary wheel running in mice – a model of voluntary physical activity in

humans. The locus explained 26% of the phenotypic variance in the CSS panel. However, the syntenic human region on chromosome 10 does not contain any SNP significantly associated with excessive voluntary exercise. Based on the current data it is not possible to propose any candidate genes for the mouse phenotype. However, some genes in the region, such as *Myof* and *Htr7* are likely candidates which could be further tested for their influence on running wheel activity levels in mice.

Nfatc2 Deficient Mice Exhibit a Specific Decrease in Voluntary Running Wheel Activity

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Manuscript submitted

4

Abstract

The nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 (*Nfatc2*) gene is a transcription factor with a broad spectrum of functions including the regulation of tissue differentiation. It may also be involved in energy balance regulation as it is activated by adipogenesis and reciprocally influences the levels of the adipose tissue hormone, leptin. Taking under consideration the potential role of the *Nfatc2* gene in energy balance, we examined the effects of targeted *Nfatc2* gene deletion on the energy intake and expenditure in mice. We could show that *Nfatc2* gene knockout mice did not show changes in body weight, food intake, and in adiposity when compared to wild type and heterozygous mice. However, we discovered that *Nfatc2* deficient mice significantly reduce their voluntary running wheel activity levels. Here we show that the reduced levels of running wheel activity cannot be explained by muscle strength, sensory-motor coordination, or the alterations in natural reward sensitivity. Concomitantly with previous reports, this suggests a role for *Nfatc2* in regulating biological pathways contributing to exercise capacity.

Introduction

Physical activity (PA) may be most broadly defined as 'bodily movement produced by contraction of skeletal muscles' (Dishman 2008). PA serves many functions including obtaining shelter, food, mating partners, and regulating body temperature. However, one of the types of PA, so called voluntary physical activity, is a type of "locomotor activity that is not directly required for survival or homeostasis and not directly motivated by any external factor" (Garland, Jr. et al., 2011b). It can be operationalized in mice as a running wheel activity (RWA), a spontaneously occurring behavior which is measured in the home cage in which the mice have voluntary access to a running wheel. Two factors, working in synergy (Kelly and Pomp 2013), will have an influence on the observable levels of voluntary PA (Dishman 2008; Garland, Jr. et al., 2011b; Kelly and Pomp 2013): 1) the physical ability, which depends on physiological factors such as muscle strength, motor coordination, and respiratory endurance (Rankinen et al., 2010); and, 2) the motivation to engage in PA, which will be mostly regulated by the central nervous system functioning (Rhodes et al., 2003). Taking under consideration that different physiological systems regulate the ability, and motivational aspect of PA, these two factors will likely have a distinct genetic basis (Garland, Jr. et al., 2011b).

The nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 (*Nfatc2*) gene is a transcription factor which was originally shown to play a role in an inducible expression of cytokine genes in activated T-cells (McCaffrey et al., 1993; Shaw et al., 1988). Various DNA sequences are recognized by the *Nfatc2* and utilized as a transcription factor binding sites (Rao et al., 1997). These recognition sequences are highly prevalent in the genome. However, the functional role of *Nfatc2* in activation of the gene transcription was confirmed for only a limited number of genes; mostly for those which are involved in the immune response: IFN-g, IL-2, IL-3, IL-4, IL-5, IL-13, TNF-a, GM-CSF, CD40L, FasL, and CTLA-4 (Rao et al., 1997; Viola et al., 1998). Nevertheless, *Nfatc2* has broad functions and regulates the development and differentiation of several tissue types (Dave et al., 2006; Horsley et al., 2001) as well as adipogenesis (Yang et al., 2006). It also reciprocally influences the levels of the adipose tissue hormone, leptin (Liefers et al., 2005; Rajapurohitam et al., 2012; Yang et al., 2006).

Taking under consideration the potential role of the *Nfatc2* gene in energy balance, we examined the effects of *Nfatc2* knockout on the energy intake and expenditure. We observed that *Nfatc2* gene knockout mice have a significant reduction in RWA in a gene-dose dependent manner. We tested whether this effect on RWA was specific for the performance on the running wheel or whether it is mediated by: 1) impaired sensorimotor coordination, muscle strength, or neurological impairments; or, 2) changes in the natural reward sensitivity between the WT, heterozygous (HE) and KO animals.

Methods

Mice

Initial mouse breeding pairs were kindly provided by Anjana Rao, The Center For Blood Research and Department of Pathology Harvard Medical School Boston, Massachusetts. The *Nfatc2* germline KO mice were generated as described by Xanthoudakis (1996). Briefly, a vector targeting for the *Nfatc2* gene has been designed that way that it allows disruption of the amino terminal exon, encoding residues conserved in DNA-binding domains, by the insertion of a PGK-neo cassette (Xanthoudakis et al., 1996). The use of this vector enabled production of germline *Nfatc2* knockout mice in a 129/SvJ strain. Positive embryonic stem cells from this strain were selected and injected into blastocysts from C57BL/6J. Heterozygous mice were established from several independent chimeras and bred to homozygosity. The

WT, HE and KO mice used in the current experiments, were generated by crossing the heterozygous animals. The genotypes of the WT, HE and KO mice were determined by polymerase chain reaction analysis conducted on the DNA extracted from ear punches. The primers used were as follows: 1) forward primer: 5'-TCCCTGTGACTGCATCCCTC-3'; 2) WT reverse primer: 5'-CACCACAGGGT GTCCTCCTGTT-3'; and, 3) KO reverse primer: 5'-CAGCGCATCGCCTTCTATCG-3'.

All mice used in the experiment were bred at the Brain Center Rudolf Magnus animal facility and were 3–4 months old at the start of the experiment. In line with the previous studies (Gelegen et al., 2008; Gelegen et al., 2010), test-naive female mice were used in the experiments. As the effects of the estrous cycle are much more subtle in mice than in rats (Kopp et al., 2006) and previous studies found no relationship between the variation in the estrous cycle and variation in mouse behavior (Laarakker et al., 2011), we did not systematically monitored the different stages of estrous during this study. Following weaning at 3–4 weeks, female and male mice were separately housed in groups (2–5 mice) in cages (Macrolon Type II, Tecniplast, Milan Italy) with sawdust bedding and 1–2 tissues per cage (Kleenex®, Kimberly-Clark B.V., Ede, The Netherlands). The housing facilities were maintained on a 12:12-h dark/light cycle with an ambient temperature of 21 ± 2°C and relative humidity of 55% ± 10%. The mice were given water and food *ad libitum* at all times (Rat and Mouse Breeder and Grower diet CRM; Special Diet Services, Essex, UK). All the procedures described were approved by the Animal Experiment Committee of the Academic Biomedical Centre, Utrecht, The Netherlands.

Fat tissue sampling

Nine animals of each genotype were sacrificed and their epididymal, perirenal, and inguinal WAT, as well as BAT from the interscapular were removed and weighed (Heine et al., 2000).

Running wheel activity

During the experiment, all mice were individually housed in cages with voluntary access to a running wheel. The wheel is made of a metal grid with a circumference of 44.5 cm, a diameter of 14 cm, and a width of 8.5 cm. The distance between the grid wires is approximately 1 cm. The mice were left in the cage with the running wheel for 7 days. Their individual running wheel activity (RWA) was continuously registered. RWA was operationalized as running wheel revolutions, which were measured by means of a magnet-activated counter, and registered using Cage Registration Software version 5.5 (Department of Biomedical Engineering, University

Medical Center Utrecht, The Netherlands). The average RWA of 3 last days (days 5-7) of the experiment was taken as the baseline RWA level.

Rotarod performance

Rotarod is a test for motor coordination and balance. The protocol is performed as in (Crawley 1999). Mice are subjected to one test on the Rotarod (Ugo-Basile, Italy) for up to 5 minutes. The rotation speed accelerates from 4 rpm to 40 rpm. The latency to fall (or rotate grasping the rotarod bar in some cases) in each test is measured as indicative of motor balance and coordination.

SHIRPA battery

The SHIRPA protocol (SmithKline/Harwell/Imperial College/Royal Hospital/ Phenotype Assessment) is a primary screen test battery which may be used to tests gross abnormalities, general health and neurological deficits (Lalonde et al., 2005; Rogers et al., 1999). We used a 10 min SHIRPA primary screen to assess basic sensorimotor functions and various reflexes in mice. Their body position, tremor, palpebral closure, coat appearance, whiskers, lacrimation, and defecation were assessed in a viewing-jar. Consequently the mice were transferred to a novel arena to observe: transfer arousal, locomotor activity, gait, tail elevation, startle response, and touch escape. Further measures included: positional passivity, skin color, trunk curl, limb grasping, pinna reflex, corneal reflex, contact righting reflex, evidence of biting, and vocalization. The task was performed during the light phase of the circadian cycle.

Grip test

The grip test was used to determine the muscle strength of the limbs of the mice. The task was performed during the light phase of the circadian cycle. The mice were tested on a grid (22×28.5 cm^2) with a gap width of 1 cm. During the 5 min of testing, animal was hanging on the inverted grid with its fore-limbs and hind-limbs (illumination level 130 lux). Mice that were able to hold on for 5 min were considered to have passed the test. In between test sessions, the grid was cleaned with 70% ethanol.

Sucrose preference test

Animals were solitarily housed for fifteen days in cages with free access to two water bottles. The first 4 days were used as an adaptation period. Furthermore, the natural preference of the mice to one of the bottles was assessed. From day 5, every second day, one of the water bottles was substituted with a bottle containing sucrose solution of an increasing concentration. On the remaining days, mice had access to

water as a washout period. As a consequence, mice obtained access to a sucrose solution of the following concentrations: 0.5% (day 5), 1% (day 7), 2% (day 9), 4% (day 11), 8% (day 13), 16% (day 15). The sucrose solution was always given in the least preferred of the two bottles. The sucrose preference was calculated as the amount of sucrose solution consumed relative to the total amount of liquid consumed that day (sucrose and water together).

Statistical analysis

One-way ANOVA, with genotype as a main factor and a Bonferroni post hoc test, was used to compare the RWA of the WT, HE and KO mice. Levene's test of equality of error variances showed that the assumption of the one-way ANOVA was met. Fisher exact test was used to compare the outcome of the categorical parameters of the SHIRPA test battery between genotypes. The independent samples Kruskal-Wallis test was used to compare the three genotypes in terms of the performance on the rotarod, as well as for the locomotor activity in the open arena. All analyses were performed with SPSS 20.0 (IBM). Data are presented as a mean ± standard error of the mean. For significance thresholds, a *P*-value of 0.05 was used.

Results

Basic characteristics

The WT, HE and KO mice did not differ in terms of: 1) baseline body weight ($\chi^2(2)=0.493$, $P<0.781$; Figure 4.1a); 2) baseline food intake ($\chi^2(2)=0.336$, $P<0.845$; Figure 4.1b); 3) brown adipose tissue ($\chi^2(2)=2.690$, $P<0.260$; data not shown); 4) white adipose tissue ($\chi^2(2)=1.628$, $P<0.443$; data not shown); nor 5) total adipose tissue ($\chi^2(2)=2.600$, $P<0.273$; data not shown).

Running wheel activity

There was a gene-dose effect of *Nfatc2* gene deletion on the daily running wheel activity levels in mice adapted to the running wheel (Figure 4.1c; average of days 5-7). One-way ANOVA showed a significant difference between the genotypes in terms of the RWA ($F(2, 41)=9.44$, $P<0.001$). Bonferroni post hoc test showed that both, the HE (n=14, $P<0.05$) and the KO (n= 15, $P<0.001$) mice, run significantly less than the WT (n=15) controls (Figure 4.1d).

Specificity of the RWA phenotype

We assessed whether the observed difference in the levels of RWA may be explained by sensorimotor or motor coordination differences between the WT, HE and KO

mice. In order to do so, we used the accelerating rotarod, the SHIRPA test battery, and the grip strength test.

Rotarod performance. The KO (n=13), HE (n=13) and WT (n=9) mice were tested for their performance on the accelerating rotarod. The independent sample Kruskal-Wallis test also showed that there was no difference between the genotypes in terms of the latency to fall of the accelerating rotarod ($\chi^2(2)=1.955$, $P<0.376$), nor in terms of the maximal revolutions of the rotarod per minute ($\chi^2(2)=1.950$, $P<0.377$) (Figure 4.1 e-f).

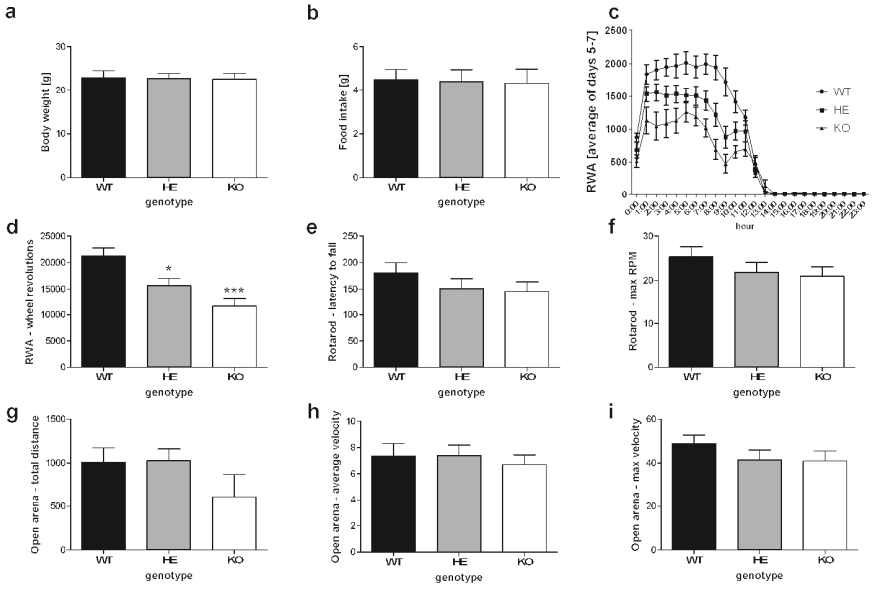

Figure 4.1. Baseline body weight (a), food intake (b), running wheel activity (RWA) tests (c-d), performance on the accelerating rotarod (e-f), and in the open arena of the SHIRPA test battery (g-i) per genotype (WT = wild type, HE = heterozygous, KO = knockout). Panel a) average baseline body weight in grams. b) Average baseline food intake in grams. c) 24-hrs RWA rhythms of the WT, HE and KO mice as an average of the last three days of the RWA test; data show the RWA over 24 hours. d) Average running wheel revolutions for the days 5-7 of running wheel activity. The HE and KO mice run significantly less than the WT controls. b) Latency to fall of the accelerating rotarod. c) Maximal revolutions per minute (RPM) reached on the accelerating rotarod. d) Total distance moved in the open arena over 2 minutes of testing. e) Average velocity of locomotor activity in the open arena over 2 minutes of testing. f) Maximum velocity of locomotor activity in the open arena over 2 minutes of testing. All data are presented as the mean ± SEM; * $P<0.05$, *** $P<0.001$ in comparison to the WT mice.

SHIRPA test battery. The KO (n=13), HE (n=13) and WT (n=9) mice did not differ for any of the categorical parameters tested in the SHIRPA test (data not shown as all the mice reached maximal scores on these parameters). The independent sample Kruskal-Wallis test also showed that there was no difference between the genotypes for the total locomotor activity in the novel arena ($\chi^2(2)=1.117$, $P<0.572$), maximal velocity in the novel arena ($\chi^2(2)=2.387$, $P<0.303$), nor for the average velocity in the novel arena ($\chi^2(2)=2.964$, $P<0.227$) (Figure 4.1 g-i).

Grip test. Almost all mice managed to stay under the grid for the total duration of the test. Therefore, there was no difference between the KO (n=6), HE (n=5) and WT (n=4) in the grip strength test (data not shown). Three mice (of different genotypes) fell off the grid before the end of the test. This happened, however, while they tried to clime on the other side of the grid and not due to muscle strength problems.

Sucrose preference test

We assessed whether the RWA difference between the genotypes may be attributed to the differences in reward sensitivity. In order to do so, we tested the KO (n=8), HE (n=8) and WT (n=8) mice in a sucrose preference test with increasing percentages of sucrose over time. The independent sample Kruskal-Wallis test showed that the mice of different genotypes did not differ at any time point in terms of the sucrose preference (Figure 4.2 a). Furthermore, WT, HE and KO mice did not differ in terms of baseline water intake that was corrected by individual body weight levels (Figure 4.2 b, $\chi^2(2)=2.240$, $P<0.326$).

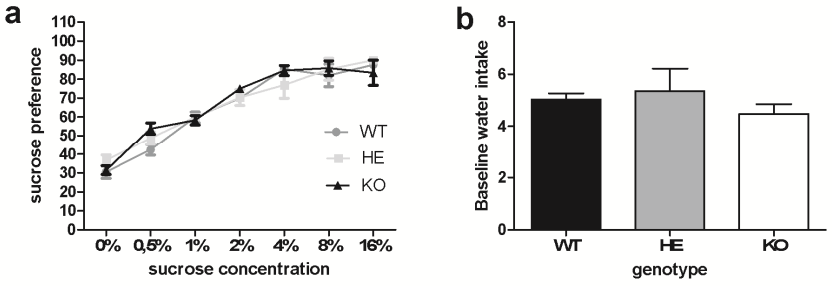

Figure 4.2. Baseline sucrose preference (a) and water intake (b) per genotype (WT = wild type, HE = heterozygous, KO = knockout). No significant differences were found between the genotypes. All data are presented as the mean ± SEM.

Discussion

The present study showed that *Nfatc2* deficiency in mice has a gene-dose effect on the voluntary running wheel activity levels. The effect of *Nfatc2* gene deficiency on RWA levels may potentially result from the involvement of *Nfatc2* in the regulation of the physical capability to express RWA or the motivation to engage in PA. Interestingly, no genotype effects were observed for locomotor activity levels in a novel open arena, SHIRPA test parameters (e.g. neurological reflexes), rotarod performance for sensorimotor coordination, muscle strength, and reward sensitivity. In addition, no effects were observed in metabolic parameters, such as food intake and the size of the adipose tissue.

The current data suggest that the Nfatc2 deficiency has a specific effect on the levels of RWA. Taking under consideration the lack of differences between the WT and KO mice in SHIRPA test battery as well as rotarod test, the reduction in RWA levels in the current data set cannot be explained by the sensory system or sensorimotor coordination deficits.

Thus far, several *Nfatc2* KO mice have been generated using different KO techniques (Heyer et al., 1997; Hodge et al., 1996; Xanthoudakis et al., 1996). In one of the *Nfatc2* KO lines (Hodge et al., 1996), it has been shown that *Nfatc2* deficiency causes a lean phenotype, deficits in fat tissue accumulation, and significantly reduction in leptin levels (Yang et al., 2006). The influence of *Nfatc2* on plasma leptin levels may be direct as the leptin promoter contains *Nfatc2* binding sites (Liefers et al., 2005). Changes in adipogenesis and consequently in leptin levels could significantly influence energy balance of an organism. It is known that leptin signaling in the sympathetic and the central nervous system (Polito et al., 2000) regulates energy balance by increasing physical activity and thermogenesis (Hwa et al., 1997; Scarpace et al., 1997; van Dijk 2001; van Elburg et al., 2007). However, these specific *Nfatc2* KO mice were also characterized by decreased body growth and locomotor activity – two phenotypes which were not observed in KO mice used in the current experiments (Xanthoudakis et al., 1996). Furthermore, we detected no differences in the size of the adipose tissue between *Nfatc2* WT, HE and KO mice. These observations point to the fact that the phenotypic consequences of the gene KO are not fully comparable between the different KO lines and may reflect the effect of genetic background differences.

It is well established that RWA possesses rewarding properties (Belke and Garland, Jr. 2007; Brene et al., 2007; Garland, Jr. et al., 2011b; Kagan and Berkum 1954; Lett et

al., 2000; Novak et al., 2012; Sherwin 1998). The rewarding value of RWA may be explained evolutionarily when one considers the importance of high motivation to remain physically active in order to obtain food and find mating partners, despite pain, effort and potential danger (Garland, Jr. et al., 2011b). Changes in the sensitivity for natural reward could cause a decrease in the motivation to run on the running wheel, and consequently result in a decrease in the RWA. However, we could show that, in case of the currently used *Nfatc2* KO line, there was no reduction in reward sensitivity (as measured by the sucrose preference test) in comparison to the WT littermates.

The role of *Nfatc2* transcription factor in muscle tissue development has been demonstrated (Calabria et al., 2009; Schulz and Yutzey 2004). It is likely that *Nfatc2* regulates the growth of multinucleated muscle cells during skeletal muscle development (Abbott et al., 1998; Horsley et al., 2001). The myoblast fusion in *Nfatc2* KO mice (Hodge et al., 1996) was shown to be defective, which results in diminished cross-sectional fiber area as well as delayed muscle regeneration (Horsley et al., 2001). Furthermore, the proportion of the slow- and fast-twitch fibers is altered as the consequence of the *Nfatc2* KO (Horsley et al., 2001). Finally, the grip strength of a double *Nfatc2* and *Nfatc4* KO line was decreased in comparison to the WT control (Arron et al., 2006). No similar reports are available for the KO line used in the current study (Xanthoudakis et al., 1996). Furthermore, using the SHIRPA test battery as well as the grip test we could show that the reduction in RWA levels are not caused by muscle strength reduction in *Nfatc2* deficient mice. As mentioned earlier, consequences of a KO may be only partly comparable between different KO lines. Therefore, further experiments are needed to characterize muscle growth and muscle regeneration in the currently used *Nfatc2* KO line. As different fiber types are important for long and short-term engagement in physical activity, it is possible that the observed RWA differences between the WT and KO mice result from altered physical exercise capacity. This hypothesis gains further support in studies conducted by Courtney and Massett (2012). Using an association mapping in 34 inbred mouse strains tested on a treadmill they could show that 32.6% of exercise capacity variance can be explained a single nucleotide polymorphism in an intron of *Nfatc2* gene (with an T allele causing a significant reduction in exercise time).

In summary, the data showed that the *Nfatc2* deficiency causes a specific and gene-dose dependent reduction in the RWA in mice. On the basis of the current data, this decrease in RWA cannot be accounted for by disturbances in the sensory or motor systems, nor by the sensory-motor coordination. Furthermore, the natural reward

sensitivity did not differ between the mice of different genotypes suggesting that the observed differences are not caused by altered rewarding properties of RWA. Further experiments are needed to elucidate the mechanisms by which *Nfatc2* contributes to exercise capacity.

Hippocampal *Ptpn1* Expression Affects Food Anticipation

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Abstract

The ability for an organism to predict the timing of an upcoming meal is essential for survival and is dependent on nutrient status and on proper synchronization to environmental stimuli related to meal arrival. Using chromosome substitution strains, we report that a locus on Chr2 is linked to food anticipatory activity (FAA) in mice exposed to a daily scheduled limited food access paradigm. Since the hippocampus has been implicated in memory formation and learning processes relevant to associative processes, we performed gene expression microarray analysis on hippocampi of mice expressing high and low FAA. This revealed differential expression of *Ptpn1*, a gene located in the FAA locus on mouse Chr known to affect memory and learning processes and implicated in central regulation of energy homeostasis. To study the functional involvement of hippocampal *Ptpn1* expression in FAA, we show that reducing hippocampal *Ptpn1* expression by RNA interference attenuates FAA. Collectively, we provide strong evidence for a novel role of hippocampal *Ptpn1* function in food anticipation.

Introduction

The ability to seek and find food is crucial for survival. Therefore, feeding behavior and accompanied behaviors are regulated tightly by central and peripheral systems (Schwartz et al., 2000; Woods and Seeley 2000). As part of this evolutionary conserved process, the consummatory phase of feeding behavior is preceded by the appetitive phase that reflects the motivational drive to fulfill the physiological need to eat (Berridge and Robinson 1998; Kas et al., 2004). For example, insects, fish and mammalian species, when provided limited access to food, show locomotor activity increase in the hours immediately preceding daily scheduled feeding time (Mistlberger 1994). The intensity of this so-called food anticipatory activity (FAA) is directly related to the levels of food intake during scheduled feeding (Kas et al., 2004) and has been suggested to reflect the natural motivation to search for food (Mistlberger 1994). It has been shown that the expression of daily timed anticipatory behavior is independent from the master light-entrainable oscillator located in the suprachiasmatic nuclei (Mistlberger 1994), but involves the hippocampus and cortical areas of the brain (Wakamatsu et al., 2001).

Recent studies have shown that mouse inbred strains express different levels of FAA when exposed to a daily scheduled limited food access paradigm (Gelegen et al.,

2007; Gelegen et al., 2008), indicating genetic background differences underlying the anticipation to food. Chromosome substitution (CS) strains of mice have been used successfully to facilitate the mapping of genetic factors that control trait differences (quantitative trait loci (QTLs)) in the progenitor strains of the CS panel (Nadeau et al., 2000). In these strains, a single chromosome of the host background strain (C57BL/6J) is substituted by the corresponding chromosome of the donor strain (A/J). Phenotypic differences between a CS strain and the genetic background strain (C57BL/6J) indicate that at least one genetic locus on the particular chromosome contributes to the observed (behavioral) phenotype. Using an F_2 population of the Chr2 CS strain (CSS2), we identified a QTL contributing to FAA during daily scheduled limit food access. Furthermore, we tested whether a candidate gene in this locus, in particular protein tyrosine phosphatase, nonreceptor type 1 (*Ptpn1*), contributes to FAA in C57BL/6J mice. In summary, we present genetic, molecular and behavioral data showing that hippocampal *Ptpn1* plays an important role in the manifestation of anticipatory behavior towards food under food-restricted conditions.

Methods

Animals and housing

Initial breeding pairs for CS strains, their progenitors (C57BL/6J and A/J mice) and additional strains were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). F_1 hybrids were generated by crossing CS-strain 2 (CSS2) females and males to C57BL/6J males and females, respectively. An F_2 population (CSS2 F_2, n=111, females) was generated by intercrossing the F_1 hybrid males and females. In addition, C57BL/6J and A/J females were bred in the animal facility of the Brain Center Rudolf Magnus (Utrecht, The Netherlands) and were 3-4 months old at the start of the experiment. Following weaning, at 3-4 weeks, female mice were socially housed in cages (n=2-5 mice per cage) in a room maintained on a 12/12 light-dark cycle (lights on at 2 a.m.), with an ambient temperature of 22 ± 2 °C. Animals were given ad libitum food and water, unless stated otherwise. All described procedures were approved by the ethical committee on the use and care of animals of the University Medical Center Utrecht and Utrecht University, The Netherlands. For ethical reasons, it was decided that mice were to be removed from the experiment when mice lost more than 20% of their initial baseline body weight; 83 F2 mice were available for the QTL analysis.

Daily scheduled limited food access paradigm

After an adaptation phase of 7 days in cages where individually housed mice had *ad libitum* access to food, water and running wheel, mice were exposed to a daily limited food access schedule (restriction phase) for 4 consecutive days. During this period, food with an energy intake of 3.31 kilocalorie per gram was available only in the beginning of the dark phase for 2h. Baseline measures for body weight and food intake were generated on the basis of the average value during the last three days of the adaptation phase (measured just before the dark period). Baseline running wheel activity (wheel revolutions) levels were determined as the average running wheel activity of the last two days of the adaptation phase. The following parameters were assessed daily; body weight, running wheel activity, food intake and water intake. Body weight was measured prior to the dark phase, just before daily food access. Based on a review by Mistlberger (1994), food anticipatory activity (FAA) was defined as the running wheel activity 4h prior food access on days on scheduled feeding (Mistlberger 1994). As running wheel activity increases to a greater extent in C57BL/6J mice as compared to A/J mice, we analyzed the ratio of food anticipation calculated as the percentage FAA of total running wheel activity. At the end of day 3 of the restriction phase, when FAA has clearly developed, mice were sacrificed by decapitation. Brains and fat tissues were immediately dissected under RNase free conditions. All tissues were stored at -80°C until further processing.

DNA samples, genetic marker analysis and map construction

Genomic DNA was isolated from spleen and/or tail from F1-hybrids, F2-intercross mice and CSS2, A/J and C57BL/6J mice, using a phenol/chlorophorm/iso-amylalcohol protocol (Laird et al., 1991). A total of 23 DNA markers, dispersed throughout mouse chromosome 2, was used to map a region on chromosome 2 associated with FAA in the F2 population (see Table 5.1). Genomic DNA was amplified by polymerase chain reaction (PCR) with primer sets for microsatellite markers and analyzed by agarose gel electrophoresis with 4% agarose in 1 × TAE buffer. The bands visualized by ethidium bromide staining were analyzed for length polymorphisms with reference to the polymorphisms of each marker between C57BL/6J and A/J mice. In addition single nucleotide polymorphisms SNPs were selected using the Celera-database based on the presence of allelic differences between the mouse strains A/J and C57BL/6J: rs27524348, rs27498297, rs27434812, rs28277299, rs13476894, rs27292002, rs27289000, rs27619825, rs27289254. For the selected SNPs, Taqman Assay by Design was ordered (Applied Biosciences, Foster City, CA, USA). SNP analysis was performed as described in Kas et al. (2009c). Segregation ratio of the genotypes of individual markers was checked by means of the Chi-squared goodness-of-fit-test.

None of the markers showed ($P < 0.05$) segregation distortion. Cox et al. (2009) have constructed a revised genetic map of the mouse genome and demonstrated that utilization of the revised map improves QTL mapping. Therefore, marker positions were taken from this map by using the 'mouse map converter' (http://cgd.jax.org/mousemapconverter/).

Quantitative Trait Loci (QTL) analysis

For all days of measurements, the trait 'food-anticipatory activity' corrected for individual total activity level (%FAA)' was normally distributed. The QTL location was determined by using the scanone function in the R/qtl package and using cross as an additive and interactive covariate (Broman et al., 2003). Because the traits was normally distributed the interval mapping module was used. Results were expressed as LOD scores. LOD score threshold (5% significance) was determined using 10,000 random permutations; an association was assumed significant when the LOD score was ≥ 2.58.

Microarray expression analysis

Gene expression profiling was performed on hippocampi dissected from female CSS2 F_2 mice selected from the 5% extremes of their FAA. Eight mice were selected from the CSS2 F_2 population, 4 mice from each of the extremes for FAA; mice that demonstrated highest FAA (FAA group) and those that did not anticipate to food (non-FAA group). The selected groups differed only in their FAA and were matched for daily wheel running activity, food intake during restriction and body weight loss after restriction.

RNA was extracted from the hippocampus of each of these mice using the guanidium thiocyanate method (Chomczynski and Sacchi 1987) with TRIzol reagent (Invitrogen Life Technologies, UK). Double-stranded cDNA was synthesized from 4 μg of total RNA by reverse transcription using T7-Oligo(dT) promotor primer and then biotin-labeled cRNA was made from the cDNA template by in vitro transcription (One-Cycle Target Labeling kit, Affymetrix, Santa Clara, USA). cRNA was fragmented and hybridized to the Mouse 430A 2.0 Gene Expression Array (Affymetrix, Santa Clara, USA). The arrays were subsequently washed, stained with phycoerythrin streptavidin and scanned according to standard Affymetrix protocols. The Affymetrix 430A 2.0 microarrays consist of over 22,690 probesets representing approximately 14,000 well-characterized mouse genes, designed from the UniGene database (Build 107, June 2002) and draft assembly of the mouse genome from the Whitehead Institute for Genome Research (MGSC, April 2002).

The raw intensity data (cell files) were normalized and the probe set data modeled to produce a single expression value using three different methods: 1) Model-Based Expression Indexes (MBEI) model with Perfect Match – Mismatch (PM-MM), 2) Perfect Match-only (PM-only) fits using the dChip software (Li and Wong 2001) and invariant-set normalization, and 3) Robust Multichip Average (RMA) model using RMA express with quantile normalization (Irizarry et al., 2003). Student's t-test was used to compare the extreme groups with the statistical package R. FDR control using Q=0.05 was used to correct the *P*-value for multiple testing.

Design of the short-hairpin RNAs

To identify possible functional siRNA/shRNA sequences against *Ptpn1* gene of the mouse, we used bioinformatic tools on the website of Biopredsi and Invitrogen. Two sets of two oligonucleotides (Stratagene, Amsterdam, The Netherlands) with SapI and XbaI overhangs (shRNA1 and shRNA2) were ordered. Additionally, a set of scrambled shRNA oligonucleotides was ordered as a control (control-shRNA). The sequence of the shRNA oligos were; shRNA1: TTT**GCGGCTATTTACCAGGACA TT***CTTCCTGTCA***AATGTCCTGGTAAATAGCCGC**TTTTT, for shRNA2: TTT**GGA GATGGTCTTTGATGA***CTTCCTGTCA***TCATCAAAGACCATCTCC**TTTTT, for control-shRNA: TTT**GCATTCGTTTCAACCATAATT***CTTCCTGTCA***AATTATGG TTGAAACGAATGC**TTTTT (**target sequence**, *loop sequence*). Oligonucleotides were annealed and ligated into SapI- and XbaI-digested pAAV-shbase. In this vector, the shRNA are driven by a mouse U6 promoter containing a terminator sequence after the shRNA sequence. In addition, this plasmid also expresses GFP under a CMV promoter hybridized to a β-actin intron. The GFP gene is followed by an independent terminator sequence. The *Ptpn1* mouse cDNA was cloned into p3xflag-renilla (p3xflag-renilla was a kind gift from M. Vooijs), and this resulted in *Ptpn1*-renilla fusion plasmid. pcDNA4/TO-luc (luciferase) was a kind gift from M. van der Wetering. All constructs were verified by sequencing.

Luciferase assay

HEK293T cells in 24-well plates were transfected using polyethylenimine (PEI; Polysciences, Amsterdam, The Netherlands). Per well, 5 ng pcDNA4/TO-luc, 250 ng *Ptpn1*-renilla, and 812 ng pAAV-shRNA were transfected (molar ratio *Ptpn1*-renilla:pAAV-shRNA was 1:4). Three days after transfection, the cells were lysed in passive lysis buffer (Promega) according to the manufacturer's protocol. Samples were assayed with a dual luciferase kit (Promega) and measured using a Victor 96-well plate reader (PerkinElmer, Groningen, The Netherlands). All values were normalized to luciferase (to correct for transfection efficiencies). Subsequently, the

different pAAV-shRNA's were normalized to pAAV-shbase. Results were also compared for each pAAV-shRNA against *Ptpn1* to the results of pAAV-shRNA vector containing control-shRNA. Virus was produced for the shRNA (shRNA-1) with the best knock-down efficiency in comparison to the control vector (Figure 5.1).

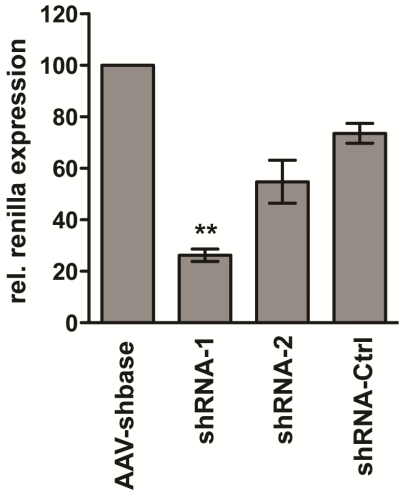

Figure 5.1. In vitro *Ptpn1* knockdown by shRNA constructs. Renilla-luciferase assay was conducted to test the effectiveness of Ptpn1 gene knockdown by both shRNA constructs. One-way ANOVA showed significant differences between the different shRNAs ($F(3,4)= 42.29$, $P<0.01$). Dunnett's post-hoc analysis revealed a significant decrease in gene knockdown for shRNA1 ($P<0.01$) as compared to the control shRNA.

Virus production and purification

Human embryonic kidney (HEK) 293T cells were maintained at 37°C with 5% CO_2 in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and non-essential amino acids. HEK293T cells were cultured in fifteen 150 × 150 mm cell culture dishes. Two hours before transfection, 10% FCS-DMEM was replaced with 2% FCS-DMEM. All transfections were performed with polyethylenimine (PEI) as described before (Reed et al., 2006). pAAV-shbase was co-transfected with the helper plasmid pDP1 (Grimm et al., 2003) (Plasmid factory, Bielefeld, Germany) in a molar ratio of 1:1. The following day, the transfection mix was substituted by the 2% FCS-DMEM. The production and purification was essentially performed as described before (Zolotukhin et al., 2002). Sixty hours post-transfected cells were collected, pelleted and resuspended in an ice-cold lysis solution (150mM NaCl, 50 mM Tris, pH 8.4) and stored at -20°C until further use.

To lyse the cells, two additional freeze-thaw cycles were performed between dry-ice-ethanol (100%) and a 37°C water bath. Subsequently, the cells were incubated for 30 minutes with 50 U/ml Benzonase (Sigma, the Netherlands) at 37°C and centrifuged. After centrifugation, the supernatant was loaded onto an iodixanol gradient (60%, 40%, 25%, 15%, supernatant (Optiprep, Lucron bioproducts, Belgium)) in quickseal tubes (Beckman Coulter, The Netherlands). After ultracentrifugation (75 min, 70.000 rpm at 18°C) in Ti70 rotor (Beckman Coulter, the Netherlands), the 40% layer was extracted and used for anion exchange chromatography with 5 ml HiTrapQ columns (GE Healthcare, The Netherlands). A gradient with buffer was created to elute the virus from the column and 2 ml fractions were collected and stored at 4°C. These fractions were then screened by PCR and the positive fractions were pooled and transferred to a Centricon Plus-20 Biomax-100 concentrator column (Millipore, The Netherlands) to concentrate the virus and exchange the buffer to PBS. The purified virus was then stored in aliquots at -80°C. Before use, AAV1 vector titers (genome copies/ml) were determined by real-time quantitative PCR in a LightCycler (Roche) using primers detecting BGHpolyA (forward primer BGHpolyA: 5'-CCTCGACTGTGCCTTCTAG; reverse primer BGHpolyA: 5'- CCCCAGAATAGAAT GACACCTA. The titer obtained for AAV1 virus containing *Ptpn1*shRNA1 was 7.8×10^{12} genomic copies/ml.

Ptpn1 knockdown in hippocampus

C57BL/6J female mice (12 wks old, n=16) were randomly assigned to either the control (*Ctrl*^{hippo}) or the *Ptpn1* knockdown treatment group (*Ptpn1*^{hippo}). Animals were anaesthetized with isoflurane (Pharmachemie BV, The Netherlands) and analgesia was provided pre-operatively by subcutaneous administration of 5 mg/kg carprofen (Rimadyl®, Pfizer Animal Health, The Netherlands). Hamilton syringe needles were bilaterally targetted at the hippocampus. In each hemisphere, a total of purified virus (1×10^9 genomic copies/µl) containing either a shRNA targeting the *Ptpn1* mRNA (*Ptpn1*^{hippo}, n=8) or control shRNA (*Ctrl*^{hippo}, n=8) was delivered over a period of 5 minutes (0.2 µl/min, total volume 1 µl), after which the needles were kept in place for 5 minutes before slow removal. Hippocampus coordinates were 2.0 mm posterior from bregma, 1.8 mm lateral from midline, and 2.0 mm ventral below the surface of the brain; toothbar set was used to obtain flat skull position.

Verification gene knockdown and virus injection sites *in situ* hybridization

Frozen brains collected from the animals at the end of the experiment were sliced under RNAase-free conditions using a cryostat (Leica, Rijswijk, the Netherlands) and thaw-mounted onto RNAase-free SuperFrost Plus slides (Menzel-Gläser,

Braunschweig, Germany). Coronal 16 mm thick cryosections series were obtained for the area comprised between -1 and -3 mm posteriorly from bregma, as determined by morphological analysis based on Paxinos and Franklin's mouse brain atlas (Paxinos and Franklin 2001), representative of the hippocampal region in which spread of the viral vectors was predicted to occur. Slides were stored at -80ºC until further processing.

Injection sites and viral spread were localized by in situ hybridization (ISH) for GFP followed by a light methyl green counterstaining, which allowed histological analysis without interfering with microscope detection of the GFP probe's signal. ISH was performed with a digoxigenin (DIG)-labeled antisense GFP RNA probe, generated using as a template a 748bp GFP sequence fragment. The sequence was excised from lentilinker vector with BamHI and SalI restriction enzymes, and subsequently inserted into a pBlueScript SK+ plasmid (Stratagene, La Jolla, CA, USA). The pBluescript vector containing GFP cDNA was used for a PCR reaction with T3 (forward), T7 (reverse) primers and Taq polymerase. The PCR product was then purified and served as a template for subsequent in vitro transcription with a RNA labeling mix and T7 RNA polymerase.

ISH were performed according to standard procedure. In short, slides were fixated for 10 min in fresh 4% paraformaldehyde in phosphate-buffered saline and acetylated for 10 min in a solution containing 245 ml H2O, 3.3 ml triethanolamine, 438 µl HCl (37%), and 625 µl acetic anhydride. Sections were washed with PBS and prehybridized for 2 h in a prehybridization solution (50% deionized formamide, 5× SSC, 5× Denhardt's solution, 250 µg/ml baker's yeast, and 500 µg/ml sonificated salmon sperm DNA). Hybridization was performed overnight at 68 °C with 400 ng/ml DIG-labeled probe added to 150 µl hybridization solution each slide, covered with nescofilm. After following washing steps and preincubation, the sections were incubated overnight at 4 °C with alkaline phosphatase-conjungated mouse anti-DIG Fab fragment (Roche), 1:5000 diluted in buffer 1 with 1% heat inactivated fetal calf serum. Sections were washed and subsequently incubated in the dark over night with a solution containing NBT/BCIP solution (Roche). The color reaction was stopped by adding 10 mm Tris/HCL, 5 mm EDTA, pH 8.0. Methyl green counterstaining was subsequently performed by immersion of the slides in 1% methyl green for 2 minutes, followed by a brief wash in demi-water, dehydration with quick passages in 96–100–100% ethanol and 2 x 3 minutes clearing washes in xylene. Slides were afterwards embedded in Entellan (Merck) and allowed to dry in air overnight.

Verification of potential neuronal damage.

In situ hybridization of miRNA-124. The expression of miRNA-124 was detected in the infected hippocampal neurons to check for signs of neuronal damage. ISH was performed using miRCURY LNA™ Detection probe (Exigon, product no. 88066-15), 3'- and 5'- double digoxygenin (DIG)-labeled, against miR124. The ISH was conducted according to the protocol described before (Silahtaroglu et al., 2003) with small modifications. In short, slides were fixated for 10 min in fresh 4% paraformaldehyde in phosphate-buffered saline and acetylated for 10 min in a solution containing 245 ml H2O, 3.3 ml triethanolamine, 438 µl HCl (37%), and 625 µl acetic anhydride. Sections were treated with ProteaseK (15 ul of 5 mg/ml per 75 ml 1x PBS) at room temperature for 5 min. Slice were prehybridized at room temperature using hybridization buffer (10ml formamide (50%), 5ml 20xSSC (5x), 2ml 50xDenhardt's (5x), 250 ul 20mg/ml yeast RNA (200 mg/ml), 1000 ul 10mg/ml salmon sperm DNA (500 mg/ml), 0.4 g Roche blocking reagents and 1.75 ml MQ) while the hybridization mix was prepared. Hybridization was performed for 2 hours at 55 °C with hybridization mix (4 ul of 250nM LNA DIG-labeled probe stock solution per 200ul hybridization mix; final concentration 5nM). Each slide was covered with nescofilm. After following washing steps and preincubation, the sections were incubated overnight at 4 °C with alkaline phosphatase-conjungated mouse anti-DIG Fab fragment (Roche), 1:2500 diluted in blocking solution. Sections were washed and subsequently incubated in the dark for 3 hours with a solution containing NBT/BCIP (Roche) solution (200 ul NBT/BCIP solution, 240 ul 2.4mg/ml levamisol, 25 ul of 20% Tween and 9.5 ml (0.1M Tris pH 9.5/0.1M NaCl/50 mM MgCl2)). Double labeled immunohistochemistry was performed after hybridization.

Double labeled immunohistochemistry of NeuN. Neurons in the brain sections stained for miRNA-124 were marked by the use of double labelled immunohistochemistry for NeuN. Brain sections were blocked using 1% FCS in PBS/0.2% TritonX100 for 1 hour at RT. Next, mouse anti-NeuN (1:200, Millipore) diluted in blocking solution was applied to the sections for overnight incubation at 4°C. After washing in PBS, sections were incubated in secondary antibody goat-anti-mouse Alexa 594 (1:600, Invitrogen, USA) in blocking solution. Images were taken using Axioscope A1 fluorescence microscope (Carl Zeiss).

Verification of the gene knockdown by radioactive *in situ* hybridization**.**

Expression of *Ptpn1* in the hippocampus of the treated animals was detected and quantified by radioactive *in situ* hybridization with a ^{33}P-labeled antisense RNA probe against a *Ptpn1* sequence fragment, obtained via PCR amplification using

specific primers (forward primer: 5'-GCACTTCTGGGAGATGGTGT-3'; reverse primer: 5'-GTAAGAGGCAGGTGTCAGCC-3'), followed by auto-radioactivity analysis. In brief, defrosted brain sections were fixed in 4% PFA in PBS for 10 minutes, washed in PBS, then pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 minutes, washed again in PBS, and dehydrated in graded ethanol (30–50–60–70–80–96–100%) followed by 100% chloroform and 100% ethanol. The sections were dried in air, and subsequently hybridized overnight at 72ºC with the *Ptpn1* RNA probe ($1x10^6$ cpm) in a hybridization mix containing 50% deionized formamide, 2x SSC, 10% dextrane sulphate, 1x Denhardt's solution, 5 mM EDTA, 10 mM phosphate buffer (pH 8.0) and 500 mg/ml of baker's yeast tRNA (150 ml total mix volume per slide), after probe denaturation by heating at 80ºC for 5 minutes. After hybridization, the slides were rinsed shortly in 5x SSC at 72ºC, transferred for 2 hours in 0.2x SCC at 72ºC and let cool down on air; they were then washed briefly in 0.2x SSC at room temeperature and dehydrated in graded ethanol (30–50–70–96–100%) diluted in 0.3Mammonium acetate (5 minutes per step). Afterwards, the sections were dried in air and exposed to Bio-Max MR X-ray film (Kodak, Rochester, NY, USA) for 20 days. The films were subsequently developed and expression of *Ptpn1* in the hippocampus was semi-quantitatively analyzed by converting film absorbance values detected in the hippocampal CA1/CA2, CA3 and dentate gyrus areas to radioactivity concentrations, using a standard calibration curve. All autoradioactivity analyses were performed using ImageJ (US National Institutes of Health, Bethesda, MD, USA), in brain sections obtained from regions where viral vector-directed expression of GFP was observed. Measurements were also taken from stratum radiatum–stratum lacunosum-moleculare subfields to represent hemisphere-specific background radioactivity measures. Therefore, related samples were used to correct radioactivity values detected in the hippocampal regions of choice. Radioactivity data was subsequently averaged per region across hemispheres and slices for each animal.

Statistical analysis

The data were expressed as means with standard error of the mean (SEM) unless otherwise specified. One sample Kolmogorov-Smirnov test was used to check the Gaussian distribution and Levene's test was used for the homogeneity of variances. Two-sided *P* values of <0.05 were considered statistically significant. For comparison of percentage food anticipatory activity of the chromosome substitution strains with a single control background strain, significance levels were corrected using Dunnett's approach (Belknap 2003). Statistical analysis was carried out using SPSS version 21.0 for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism version 5.0.

Results

Chromosomal mapping of food anticipatory activity

In order to identify mouse chromosome(s) harboring genetic pathways underlying anticipation to food, twenty CS strains and their two progenitor strains (C57BL/6J and A/J) were exposed to a daily scheduled limited food access paradigm (see also Figure 5.2). Consistent with our previous results, C57BL/6J mice anticipate towards the upcoming food access by significantly increasing their running wheel activity prior to food access, whereas A/J mice do not increase running wheel activity when exposed to the restricted feeding schedule (Figure 5.3, panels a and b). When analyzing the activity pattern of all CS strains, only CS strain 2 showed a decreased food anticipatory activity as compared to both A/J and C57BL/6J mice. The more detailed activity pattern of the CSS2 mice reveals that CSS2 mice do not develop FAA to the daily scheduled feeding paradigm. CSS2 strain exhibits dark phase activity levels similar to anticipating C57BL/6J mice, indicating that the reduced FAA is not caused by general motor activity deficits (Figure 5.3c). The food intake and body weight loss during the restricted feeding schedule are depicted in Figure 5.4. Taken together, we show that mouse chromosome 2 is highly involved in the expression of food anticipation as measured by running wheel activity.

Identification of QTL on mouse chromosome 2 for food anticipation

To identify QTLs underlying FAA, a CSS2 F_2 population was generated and all mice (n=111) were exposed to the daily scheduled limit food access. As depicted in Figure 5.5a, percentage FAA (%FAA = total FAA as percentage of total RWA) in C57BL/6J and CSS2 mice was significantly higher as compared to A/J mice (respectively, P=0.002 and P=0.02). In the CSS2 F_2 population, the distribution of %FAA could be defined by three separate groups expressing less than 5% FAA, between 5-10% FAA and higher than 10% FAA similar to the expression of food anticipation seen in A/J mice, CSS2 mice and C57BL/6J mice respectively (see Figure 5.5b).

To map the QTL on chromosome 2, each individual mouse was genotyped for 23 polymorphic markers (see Table 5.1). Interval mapping showed a significant QTL for percentage FAA (%FAA) with a maximum and significant LOD score of 5.98 that was located between 164.6 and 170.4 Mb (-1LOD support interval) (see Figure 5.5c) (Lynch and Walsh 1998). This 5.8 Mb QTL interval contained approximately 74 protein coding genes and accounted for 28.2.% of the variance in the FAA levels in the F_2 population.

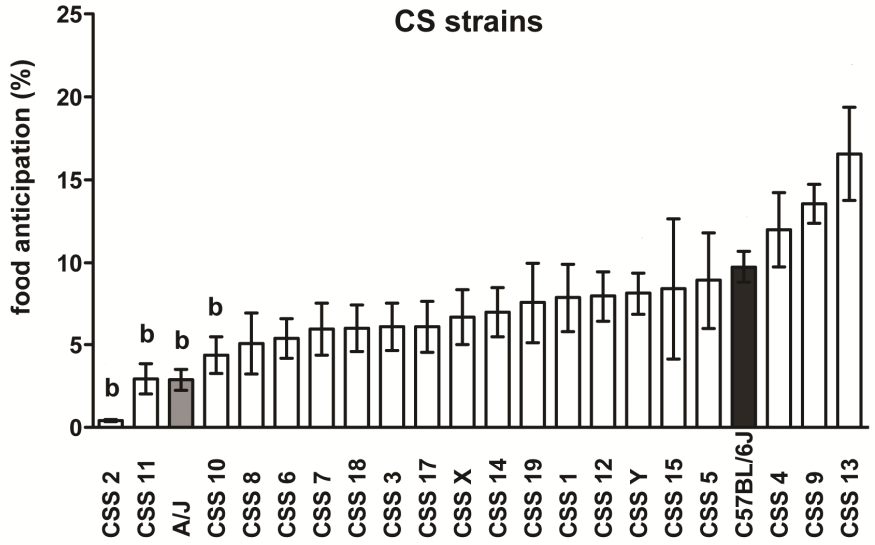

Figure 5.2. Food anticipatory activity (FAA) in all CS and their progenitor strains C57BL/6J (black) and A/J (grey). FAA was calculated as the percentage of total running wheel activity in individual animals exposed to daily schedule limited food access. Significant differences in FAA of the individual CS strains compared to the progenitor strain C57BL/6J are indicated by 'b'.

Gene expression analysis reveals possible hippocampal *Ptpn1* involvement

To identify hippocampal candidate genes within the genetic locus on mouse chromosome 2 we investigated mRNA gene expression levels in order to identify differentially expressed genes between mice with low and high FAA. We carried out a microarray analysis on mRNA from hippocampi of CSS2 F_2 mice selected on the basis of extreme values of their FAA. No statistically significant results were observed at the FDR level but when probesets were filtered according to a *P* value, a sharp increase was observed after P=0.01 suggesting that the most significant changes in expression occurred at a level of P<0.01 (Table 5.2). Using all three probeset summary models, we found 96 probesets with a *P* value <0.01, which represented the most robust changes in hippocampal gene expression observed between the two extreme groups of mice. Functional annotation of the most significant differences in gene expression was applied and comparison with the literature was made using Chilibot (http://www.chilibot.net/; (Chen and Sharp 2004)) and Webgestalt (http://bioinfo.vanderbilt.edu/webgestalt/; (Zhang et al., 2005)) to investigate whether there were any genes that may be potential candidates mediating the FAA.

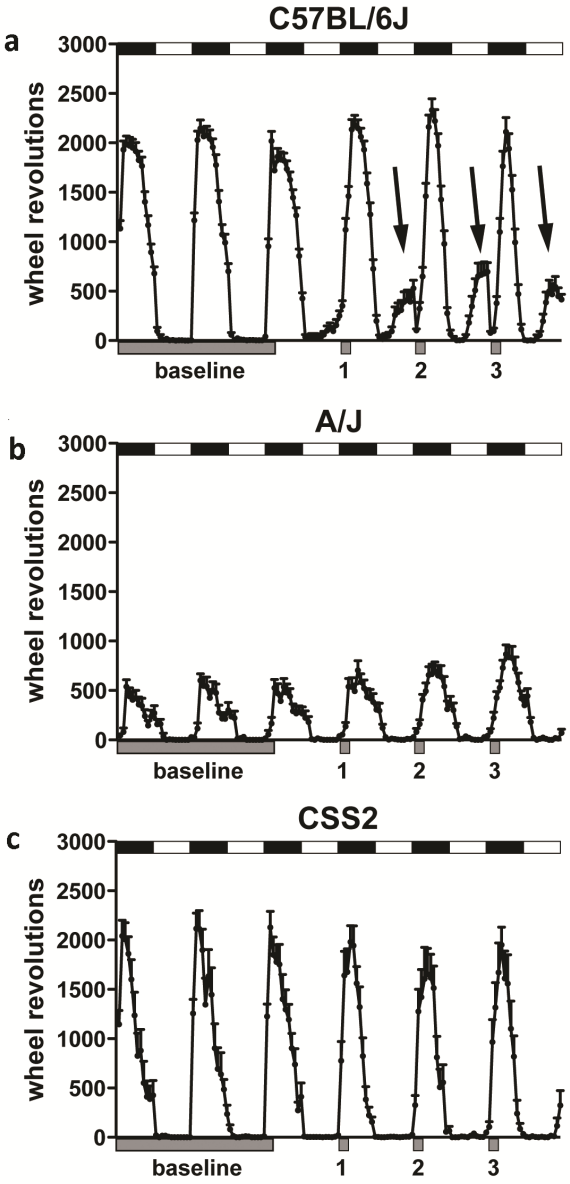

Figure 5.3. Hourly running wheel pattern of the progenitor strains C57BL/6J mice (panel a) and (panel b) A/J mice. Filled bars indicate the dark phase, open bars the light phase. Grey bars on the x-axis indicate the time of food access. After analysis of the hourly running wheel pattern of all three CS strains, it becomes clear that CS strain 2 lacks food anticipation (panel c).

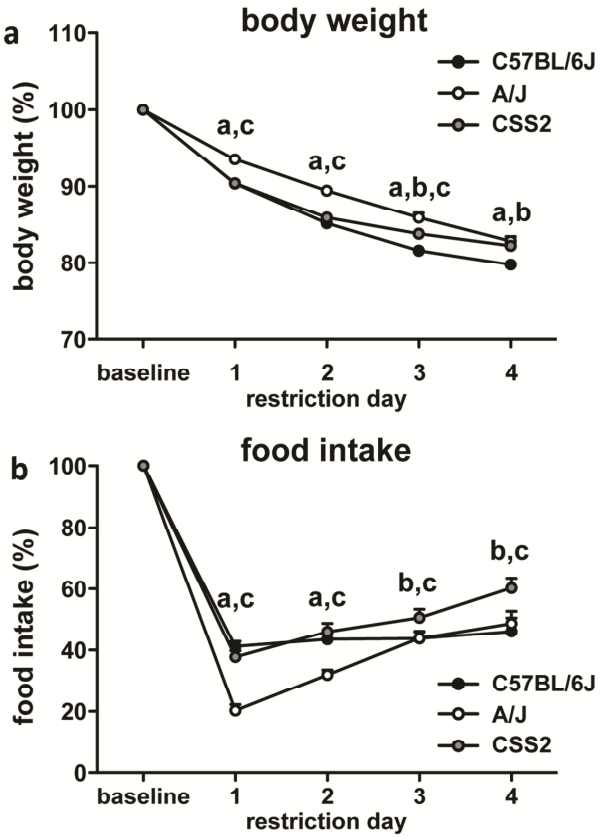

Figure 5.4. Percentage body weight loss (panel a) and percentage food intake (panel b) in C57BL/6J, A/J and CSS2 mice exposed to the daily scheduled limited food access paradigm. Significant differences between C57BL/6J and A/J mice are indicated by 'a', while 'b' indicates significant differences between C57BL/6J and CSS2 mice, and 'c' indicates significant differences between A/J and CSS2 mice. Statistical significance was set at $P<0.05$.

The most interesting candidate gene displaying a significant difference in gene expression was protein tyrosine phosphatase, non-receptor type 1 (*Ptpn1*), for which two probe sets showed a *P*-value lower than 0.01 (Table 5.2). Not only is *Ptpn1* within the QTL for FAA on chromosome 2 but there is also a known polymorphism between the progenitor strains of the CSS panel (A/G SNP in intron 3; rs27292002). Brain specific *Ptpn1* gene knockout mice exhibit reduced weight and adiposity, and increased activity and energy expenditure (Klaman et al., 2000). Moreover, several studies have reported a link between *Ptpn1* and leptin (Bence et al., 2006; Zabolotny et al., 2002) and it has been suggested that it is one of the key negative regulators of both leptin and insulin signalling (Dadke and Chernoff 2003; Goldstein 2001).

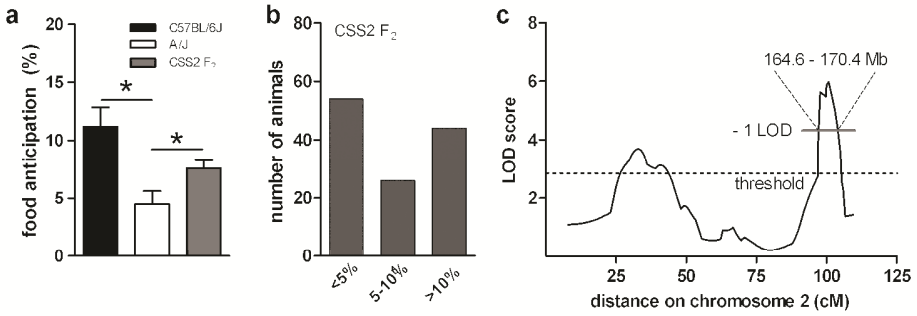

Figure 5.5. QTL mapping on mouse chromosome 2. (a) Percentage food anticipatory activity (%FAA) in C57BL/6J (black), A/J (white), and CSS2 F_2 mice (grey). Statistical significance was set at $P<0.05$. (b) Distribution of the percentage FAA in CSS2 F_2 mice (n=83). (c) Identification of a QTL for %FAA (LOD = 5.98), with 1-LOD support interval indicated by the grey line. Significance threshold is indicated with the dotted line at 2.85.

Ptpn1 knockdown in hippocampus downregulates food anticipatory activity

To investigate the functional relationship between *Ptpn1* gene expression levels in the hippocampus and FAA, a local shRNA-directed knockdown approach was used. In order to determine whether shRNA-directed knockdown of *Ptpn1* expression in the hippocampus was successfully obtained in mice subsequently exposed to the daily scheduled limited food access, *Ptpn1* mRNA expression levels were determined separately for CA1/CA2, CA3 and dentate gyrus hippocampal regions and compared between control and *Ptpn1* knockdown groups (Figure 5.6a,b). AAV shRNA injections in the hippocampus led to a significant reduction of *Ptpn1* mRNA levels in the dentate gyrus, while *Ptpn1* mRNA expression levels in the CA1/CA2 and CA3 regions of the hippocampus remained unaffected (Figure 5.6c). This *Ptpn1* gene knockdown led to behavioural changes as mice injected with virus containing shRNA targeting the *Ptpn1* (*Ptpn1*^{hippo}) had reduced FAA as compared to controls (Contr^{hippo}), while overall activity levels were unaffected between the two experimental groups (Figure 5.6e,f). Moreover, we performed a correlation analysis in order to determine whether the variability in FAA observed during the restricted feeding schedule could be explained by differences in *Ptpn1* mRNA expression levels in the dentate gyrus. Indeed, as plotted in Figure 5.6d, the *Ptpn1* mRNA expression levels in the dentate gyrus were significant positively correlated with the magnitude of FAA (Pearson's r=0.642, P=0.045).

Verification of potential neuronal damage

ISH against miRNA-124 and IHC against NeuN analysis showed that all AAV transfected neurons in the DG and CA fields (marked by NeuN) expressed miRNA-124 (Figure 5.7)). There was also no reduction in the number of NeuN positive cells due to the virus injection. This indicates that the observed behavioural differences was gene knock-down specific.

Table 5.1. DNA markers (fourteen microsatellites and nine SNPs) used for QTL analysis. Marker positions were taken from the 'mouse map converter' (http://cgd.jax.org/mousemapconverter/).

marker	microsatellite	Position (Mb)
1	D2Mit117	8.66
2	D2Mit417	26.46
3	D2Mit370	40.65
4	D2Mit458	50.71
5	D2Mit156	56.93
6	D2Mit380	69.46
7	D2Mit94	80.02
8	D2Mit66	84.66
9	D2Mit206	106.75
10	rs27524348	109.56
11	rs27498297	117.00
12	rs27434812	127.34
13	D2Mit525	131.51
14	D2Mit493	153.80
15	D2Mit51	163.89
16	rs28277299	164.68
17	rs13476894	165.88
18	rs27292002	167.79
19	rs27289000	168.40
20	rs27619825	169.90
21	rs27289254	171.76
22	D2Mit113	173.18
23	D2Mit148	178.543

Table 5.2. List of all candidate genes identified by microarray analysis

Probe set	Gene Symbol	Gene Name	*P*-value	Chr
1417065_at	*Egr1*	early growth response 1	1,43E-05	18
1449977_at	*Egr4*	early growth response 4	1,49E-05	6
1422502_at	*Parp1*	poly (ADP-ribose) polymerase family, member 1	5,05E-05	1
1448119_at	*Bpgm*	2,3-bisphosphoglycerate mutase	1,62E-04	6
1422930_at	*Icam4*	intercellular adhesion molecule 4, Landsteiner-Wiener blood group	1,69E-04	9
1427076_at	*Mpeg1 /// LOC671359*	macrophage expressed gene 1 /// similar to macrophage expressed gene 1	1,95E-04	19
1454078_a_at	*Gal3st1*	galactose-3-O-sulfotransferase 1	2,01E-04	11
1448945_at	*Pllp*	plasma membrane proteolipid	2,27E-04	8
1438670_at	*Ptpn1*	protein tyrosine phosphatase, non-receptor type 1	2,53E-04	2
1448249_at	*Gpd1*	glycerol-3-phosphate dehydrogenase 1 (soluble)	3,74E-04	15
1415834_at	*Dusp6*	dual specificity phosphatase 6	4,34E-04	10
1448606_at	*Edg2*	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	4,87E-04	4
1448830_at	*Dusp1*	dual specificity phosphatase 1	4,94E-04	17
1435321_at	*3732412D22Rik*	RIKEN cDNA 3732412D22 gene	5,45E-04	5
1418025_at	*Bhlhb2*	basic helix-loop-helix domain containing, class B2	5,52E-04	6
1454711_at	*Trio*	triple functional domain (PTPRF interacting)	5,66E-04	15
1449623_at	*Txnrd3*	thioredoxin reductase 3	5,79E-04	6
1423160_at	*Spred1*	sprouty protein with EVH-1 domain 1, related sequence	5,87E-04	2
1424568_at	*Tspan2*	tetraspanin 2	6,15E-04	3
1419816_s_at	*Errfi1*	ERBB receptor feedback inhibitor 1	6,87E-04	4
1450875_at	*Gpr37*	G protein-coupled receptor 37	6,94E-04	6
1451469_at	*D530005L17Rik*	RIKEN cDNA D530005L17 gene	7,93E-04	4
1448956_at	*Stard10*	START domain containing 10	8,47E-04	7
1452277_at	*Arsg*	arylsulfatase G	8,87E-04	11
1416432_at	*Pfkfb3*	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	9,03E-04	2
1418472_at	*Aspa*	aspartoacylase (aminoacylase) 2	9,34E-04	11
1437527_x_at	*Mcl1*	myeloid cell leukemia sequence 1	9,52E-04	3
1454712_at	*Mcart1*	mitochondrial carrier triple repeat 1	1,07E-03	4
1415899_at	*Junb*	Jun-B oncogene	1,15E-03	8
1424567_at	*Tspan2*	tetraspanin 2	1,36E-03	3
1425382_a_at	*Aqp4*	aquaporin 4	1,40E-03	18
1455581_x_at	*9530028C05*	hypothetical protein 9530028C05	1,42E-03	6
1418674_at	*Osmr*	oncostatin M receptor	1,45E-03	15
1417575_at	*Otub2*	OTU domain, ubiquitin aldehyde binding 2	1,46E-03	12
1448149_at	*Ctnna1*	catenin (cadherin associated protein), alpha 1	1,48E-03	18
1417625_s_at	*Cmkor1*	chemokine orphan receptor 1	1,78E-03	1
1428421_a_at	*2700085E05Rik*	RIKEN cDNA 2700085E05 gene	2,01E-03	11
1448229_s_at	*Ccnd2*	cyclin D2	2,09E-03	6
1450020_at	*Cx3cr1*	chemokine (C-X3-C) receptor 1	2,10E-03	9
1449694_s_at	*Commd5*	COMM domain containing 5	2,19E-03	15
1449099_at	*Lrba*	LPS-responsive beige-like anchor	2,40E-03	
1418932_at	*Nfil3*	nuclear factor, interleukin 3, regulated	2,41E-03	13
1451961_a_at	*Mbp*	myelin basic protein	2,45E-03	18
1419103_a_at	*Abhd6*	abhydrolase domain containing 6	2,72E-03	14
1451130_at	*2010315L10Rik*	RIKEN cDNA 2010315L10 gene	3,18E-03	8
1438167_x_at	*Flcn*	Folliculin	3,53E-03	11
1418255_s_at	*Srf*	serum response factor	3,53E-03	17
1417068_a_at	*Ptpn1*	protein tyrosine phosphatase, non-receptor type 1	3,59E-03	2
1438705_at	*AI465270*	expressed sequence AI465270	3,62E-03	
1420405_at	*Slco1a4*	solute carrier organic anion transporter family, member 1a4	3,80E-03	6

1415799_at	*Wbp11*	WW domain binding protein 11	4,16E-03	6
1431724_a_at	*P2ry12*	purinergic receptor P2Y, G-protein coupled 12	4,27E-03	3
1449682_s_at	*Tubb2b*	tubulin, beta 2b	4,50E-03	13
1424905_a_at	*Slc39a11*	solute carrier family 39 (metal ion transporter), member 11	4,58E-03	11
1416200_at	*9230117N10Rik*	RIKEN cDNA 9230117N10 gene	4,64E-03	19
1423100_at	*Fos*	FBJ osteosarcoma oncogene	4,64E-03	12
1456424_s_at	*Pltp*	phospholipid transfer protein	4,74E-03	2
1423073_at	*Cmpk*	cytidylate kinase	4,79E-03	4
1422557_s_at	*Mt1*	metallothionein 1	4,92E-03	8
1434403_at	*Spred2*	sprouty-related, EVH1 domain containing 2	5,52E-03	11
1418937_at	*Dio2*	deiodinase, iodothyronine, type II	5,64E-03	12
1422206_at	*B3galt1*	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1	6,12E-03	2
1450958_at	*Tm4sf1*	transmembrane 4 superfamily member 1	6,58E-03	3
1417626_at	*Pde4dip*	phosphodiesterase 4D interacting protein (myomegalin)	6,65E-03	3
1416129_at	*Errfi1*	ERBB receptor feedback inhibitor 1	6,67E-03	4
1429884_at	*Srgap2*	SLIT-ROBO Rho GTPase activating protein 2	6,71E-03	1
1423619_at	*Rasd1*	RAS, dexamethasone-induced 1	6,77E-03	11
1449799_s_at	*Pkp2*	plakophilin 2	7,04E-03	16
1418311_at	*Fn3k*	fructosamine 3 kinase	7,25E-03	11
1450019_at	*Cx3cr1*	chemokine (C-X3-C) receptor 1	7,27E-03	9
1455007_s_at	*Gpt2*	glutamic pyruvate transaminase (alanine aminotransferase)2	7,36E-03	8
1451538_at	*Sox9*	SRY-box containing gene 9	7,46E-03	11
1452160_at	*Tiparp*	TCDD-inducible poly(ADP-ribose) polymerase	7,58E-03	3
1460180_at	*Hexb*	hexosaminidase B	7,66E-03	13
1417273_at	*Pdk4*	pyruvate dehydrogenase kinase, isoenzyme 4	7,81E-03	6
1420871_at	*Gucy1b3*	guanylate cyclase 1, soluble, beta 3	7,93E-03	3
1425859_a_at	*Psmd4*	proteasome (prosome, macropain) 26S subunit, non-ATPase, 4	9,06E-03	3
1450389_s_at	*Pip5k1a*	phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha	9,28E-03	19
1418884_x_at	*Tuba1*	tubulin, alpha 1	9,36E-03	15
1422605_at	*Ppp1r1a*	protein phosphatase 1, regulatory (inhibitor) subunit 1A	9,37E-03	15
1425231_a_at	*Zfp46*	zinc finger protein 46	9,45E-03	4
1423750_a_at	*Sf1*	splicing factor 1	1,10E-02	19
1418204_s_at	*Aif1*	allograft inflammatory factor 1	1,14E-02	17
1424737_at	*Thrsp*	thyroid hormone responsive SPOT14 homolog (Rattus)	1,19E-02	7
1418166_at	*Il12rb1*	interleukin 12 receptor, beta 1	1,22E-02	8
1428311_at	*Ccdc6*	coiled-coil domain containing 6	1,28E-02	10
1428097_at	*2510009E07Rik*	RIKEN cDNA 2510009E07 gene	1,31E-02	16
1424331_at	*Rab40c*	Rab40c, member RAS oncogene family	1,35E-02	17
1417042_at	*Slc37a4*	solute carrier family 37 (glycerol-6-phosphate transporter), member 4	1,38E-02	9
1416125_at	*Fkbp5*	FK506 binding protein 5	1,40E-02	17
1416318_at	*Serpinb1a*	serine (or cysteine) peptidase inhibitor, clade B, member 1a	1,42E-02	13
1425560_a_at	*S100a16*	S100 calcium binding protein A16	1,44E-02	3
1424427_at	*Tada1l*	transcriptional adaptor 1 (HFI1 homolog, yeast) like	1,44E-02	1
1417168_a_at	*Usp2*	ubiquitin specific peptidase 2	1,47E-02	9
1426081_a_at	*Dio2*	deiodinase, iodothyronine, type II	1,49E-02	12
1448620_at	*Fcgr3*	Fc receptor, IgG, low affinity III	1,49E-02	1

Chr = chromosome

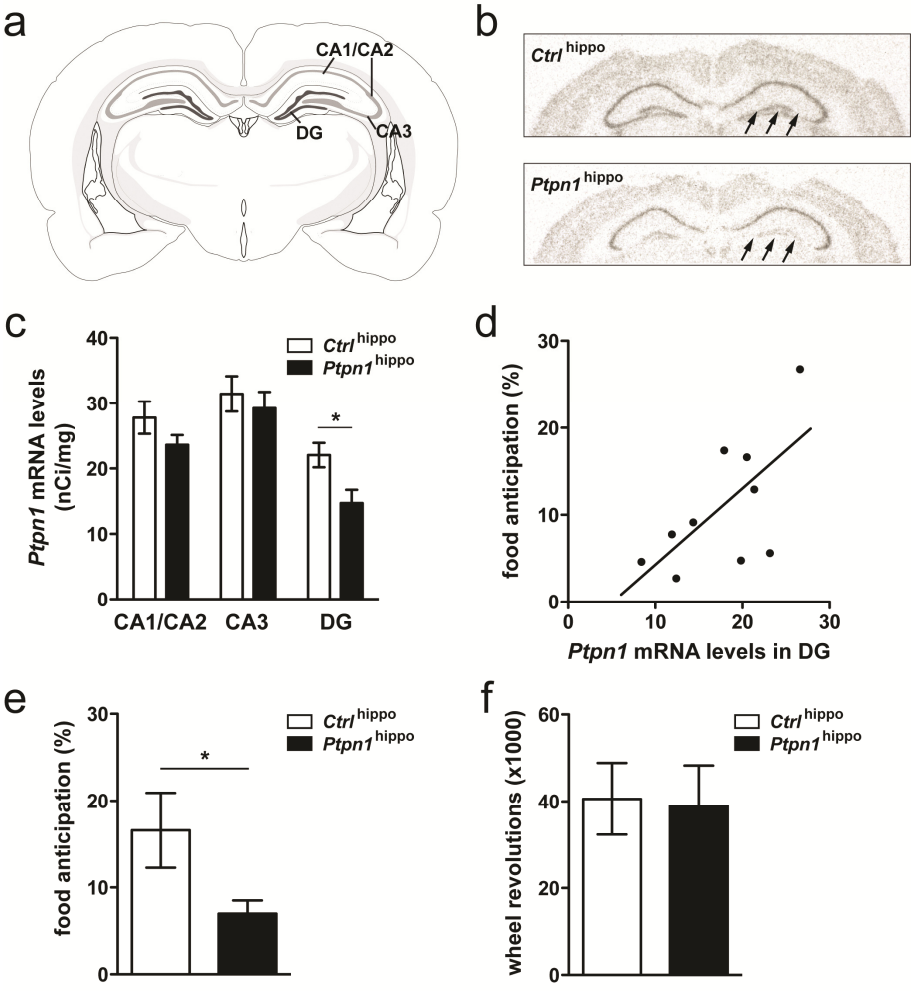

Figure 5.6. *Ptpn1* gene knockdown in the hippocampus attenuates food anticipation. (a-b) Expression of *Ptpn1* mRNA levels in the CA1/CA2, CA3, and dentate gyrus region of the hippocampus revealed by radio-active in situ hybridization in C57BL/6J mice injected in the hippocampus with the AAV1 virus containing shRNA against *Ptpn1* mRNA (*Ptpn1*^{hippo}) compared to mice injected with a control-shRNA(*Ctrl*^{hippo}). (c) Quantitative analysis of *Ptpn1* mRNA levels in the different hippocampal regions in *Ptpn1*^{hippo} mice as compared to *Ctrl*^{hippo} mice. Statistical significance was set at $P<0.05$. (d) Bivariate correlation analysis between *Ptpn1* mRNA expression levels within the dentate gyrus and percentage food anticipatory activity (%FAA) in individual mice. (e-f) Percentage food anticipatory activity (%FAA) and total running wheel activity in *Ptpn1*^{hippo} and *Ctrl*^{hippo} mice. Statistical significance was set at $P<0.05$.

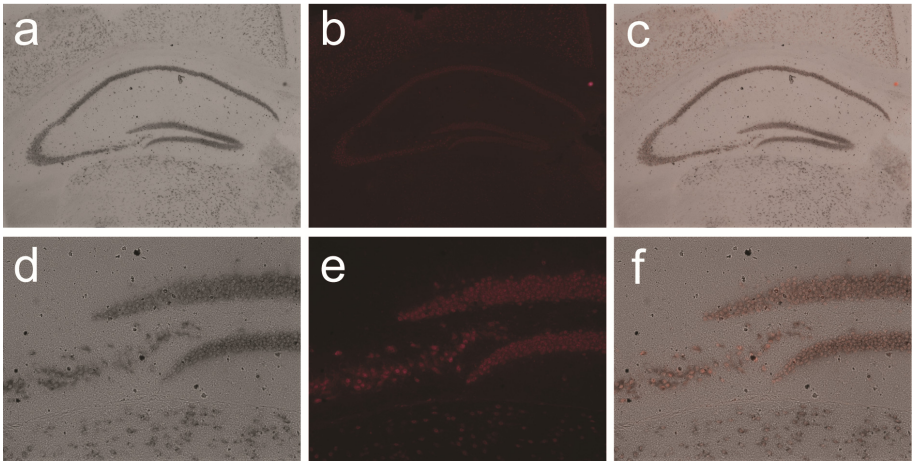

Figure 5.7. Investigation for neuronal damage by AAV shRNA injection. (a,d) *In situ* hybridization against miRNA-124 and (b.e) immunostaining for NeuN were performed to show that the infected hippocampal neurons were still viable and expressed neuronal markers. (c,f) Analysis of the NeuN staining showed that all AAV transfected hippocampal neurons in the dentate gyrus and CA regions also expressed miRNA-124. In addition, there was no visible reduction in the amount of NeuN positive neurons due to injection, transfection or shRNA expression.

Discussion

In the present study we show that *Ptpn1* gene expression levels in the hippocampus are functionally related to food anticipatory activity (FAA) in response to daily scheduled limited food access, an evolutionary conserved phenotype observed in a wide variety of species, including mammal species. This finding was based on complementary molecular, genetic, and behavioural phenotyping approaches. First, in a behavioural phenotype-driven genetic screen, a narrow genetic QTL region on mouse chromosome 2 was mapped for FAA in mice exposed to a daily scheduled limited access paradigm. Second, using microarray-based mRNA gene expression analysis, we identified differential expressed mRNA levels of *Ptpn1* in hippocampal tissue of low and high FAA expressing F_2 individuals generated for the genetic fine mapping of the locus. Of these differentially expressed genes, *Ptpn1* mRNA was significantly decreased in low FAA expressing F_2 mice when compared to high FAA expressing F_2 mice. These results indicate that *Ptpn1* gene regulation in the hippocampus may be related to FAA. To provide proof for this, we performed hippocampal shRNA-directed gene knockdown technology and showed that

targeted reduction of *Ptpn1* in the hippocampus is functionally related to FAA in mice. Vector directed downregulation of *Ptpn1* in the hippocampus resulted in a significant reduction in FAA and individual quantified levels of *Ptpn1* gene expression in this brain region, especially in the dentate gyrus, were significantly correlated with the individual levels of FAA.

Several observations lead us to conclude that the effect of *Ptpn1* on FAA is not related to other possible confounding factors that could influence the levels of FAA expressed. First, total running wheel levels in CSS2 mice as well as C57BL/6J mice in which we induced hippocampal *Ptpn1* reduction (through shRNA interference) were similar to control C57BL/6J mice. These findings indicate that general motor activity functioning is not impaired as a function of *Ptpn1* gene expression levels. Furthermore, metabolic parameters, such as food intake and body weight, were not affected both in CSS2 mice as well as in C57BL/6J mice following targeted *Ptpn1* gene expression in the hippocampus. Previous studies have shown that the levels of FAA are strongly related to the levels of food intake during a daily scheduled limited food access paradigm (Kas et al., 2004). However, the present findings indicate that the FAA levels cannot be affected by the consummatory phase nor by the levels of body weight as they did not differ between the low and high FAA expressing animals. Finally, shRNA technology may induce tissue damage that could indirectly affect the levels of FAA. By additional miRNA-124 in situ hybridisation and NeuN immunohistochemistry experiments we have shown that the applied shRNA interference technology did not induce alteration in miRNA pathways, tissue damage or neuronal apoptosis. Therefore, we could exclude nonspecific side effects of viral vector injections. Based on these findings, we conclude that *Ptpn1* gene expression levels in the hippocampus are specifically related to the anticipation to food during daily scheduled limited food access.

Various brain regions have been previously implicated in the regulation of FAA. The hippocampus is, based on a relationship with FAA, an important neuronal circuitry in the regulation of temporal learning and associated processes (Poulin and Timofeeva 2008). In addition, proper synchronisation with environmental time cues that predict meal availability is required for adequate FAA expression. Previous studies have shown that the so-called entrainment to daily scheduled food availability is independent from the suprachiasmatic nucleus of the hypothalamus, the master clock critical for the generation of circadian rhythms and the synchronisation to the 24 hrs light/dark cycle (Mistlberger and Marchant 1999; Mistlberger 1994). Nevertheless, other secondary (peripheral) clocks have been

identified in order to synchronize local processes to the rhythm of the SCN master clock. Interestingly, genes that are important for the master clock function, such as Period, are expressed in the dentate gyrus of the hippocampus. Furthermore, Period gene expression levels in the dentate gyrus shows robust circadian patterns which are modified by daytime restricted feeding (Angeles-Castellanos et al., 2007; Lamont et al., 2005; Verwey et al., 2007), and the development of FAA (Wakamatsu et al., 2001). In the present study, hippocampal *Ptpn1* downregulation following shRNA interference was specifically associated with the levels of *Ptpn1* in the dentate gyrus, opening the intriguing possibility that the dentate gyrus may have secondary synchronizing properties in relation to daily scheduled non-photic environmental cues, such as food availability.

Ptpn1 is a known negative regulator of leptin receptor signalling (Johnson et al., 2002). Leptin is a hormone that signals satiety to the brain after being released from adipocytes, its peripheral source (Jequier 2002; Schwartz et al., 2000). During events of limited food access, plasma leptin levels will decrease and will subsequently provide a signal to the brain for adequate behavioural responses during those moments of reduced food availability. For example, recent studies have shown that general behavioural hyperactivity during daily scheduled limited food access can be lowered by leptin infusion (Hillebrand et al., 2005), indicating that endogenous leptin levels are related to behavioural hyperactivity during times of limited food availability. In this way, endogenous reduced leptin levels may be the signal to the dentate gyrus of the hippocampus to promote FAA for adequate preparation to an upcoming meal (Carneiro and Araujo 2009). As Ptpn1 is a negative regulator of leptin signalling, levels of *Ptpn1* may directly modulate leptin actions in this brain region. Consequently, *Ptpn1* gene down-regulation during limited food access may mimic signals of satiety. For instance, low Ptpn1 expression levels may result in high levels of leptin signalling even in situations of hunger, when leptin release is relatively low.

It is of note that several other interesting candidates were identified in the list of genes with the most significant differential expression (see Table 5.2). These included the immediate early genes (IEGs) and early growth response 1 (*Egr1*) and 4 (*Egr4*). IEGs are genes which expression is induced rapidly and are used as markers in tissues that are particularly active during a response to a stimulus (Li et al., 2005). *Egr1* is induced following thyroid hormone T(3) increased food intake (Kong et al., 2004) and in response to leptin administration (Bjorbaek et al., 2001). Dual specificity phosphatase 1 (*Dusp1*), one of the MAPK signalling cascade proteins, was decreased

in the non-FAA mice. Interestingly, *Dusp1* knockout mice are resistant to diet induced obesity due to enhanced energy expenditure (Wu et al., 2006). There was a decrease in non-FAA mice in expression of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*Pfkfb3*), a gene also located on chromosome 2. Interestingly, expression of *Pfkfb3* has been shown to be decreased during starvation in both starved lean and obese Zucker rats (Perez et al., 1998). The results presented in the current paper support the involvement of *Ptpn1* in the regulation of FAA; however, they do not exclude a role of other candidate genes present in the candidate QTL region.

Together, this study provides functional evidence for *Ptpn1* as a newly identified regulator of FAA. Targeted down regulation of this gene in the hippocampus, especially in the dentate gyrus, showed that this brain region is important for the synchronisation to non-photic environmental cues. As a downstream regulator of satiety hormone signalling sensitivity, we propose that *Ptpn1* plays a central role in the preparation and synchronisation to daily scheduled meals, such as in times of limited food access.

The Expression of Excessive Exercise Co-segregates with the Risk of Developing an Eating Disorder in Women

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Abstract

Excessive exercise (EE) is an important symptom of eating disorders (ED) and is a likely risk factor for developing ED, however, no population-based studies have been performed on the relationship between EE and obtaining ED diagnosis. The aim of this study was to examine the co-occurrence of EE and ED diagnosis in a general population of women. Data for 778 females (age min=30, max = 55) from the Saint Thomas Twin Registry, London were used. Phenotypes analyzed included self-reported time spent on physical activity per week, ED diagnosis, Eating Disorder Inventory results (EDI-III), age, BMI and kinship (twin pair). Generalized Estimating Equation analysis showed that only EE (>5 hours of exercise per week) and Bulimia Subscale of EDI-III were significantly associated with obtaining ED diagnosis throughout the life. These data revealed that the odds of ever being diagnosed with an ED are more than 2.5 times higher for excessive exercisers compared to individuals with lower activity levels. These data support the notion that EE may be an important risk factor for developing an ED in women.

Introduction

Anorexia nervosa (AN) and bulimia nervosa (BN) are mental disorders which are most prevalent among young females (between 15 and 25 years of age) (Lucas et al., 1999) and are characterized by the highest mortality rate among psychiatric disorders (10-15%) (Hoek 2006). The high mortality rate results from extreme emaciation and subsequent neuroendocrine adaptations (Haas et al., 2005). Body weight loss leading to emaciation is facilitated in individuals with an eating disorders (ED) who are also hyperactive (especially those who express high levels of physical exercise). The sub-group of ED sufferers with high levels of physical activity possesses a specific psychological profile (Shroff et al., 2006) and uses different ways for body weight control when compared to other ED patients (Brewerton et al., 1995; Davis and Kaptein 2006; Shroff et al., 2006; Solenberger 2001). Furthermore, occurrence of EE has a negative consequence for the course of ED (Casper and Jabine 1996; Shroff et al., 2006; Solenberger 2001; Strober et al., 1997). Taken together, EE has high clinical importance. It has been proposed that EE may also be present pre-morbidly in this group of ED patients (Kron et al., 1978), however, it has not been established whether hyperactivity is a risk factor for the development of ED using a population based study.

Hyperactivity in ED may manifest itself in various forms ranging from maintaining active posture (e.g. rather standing than sitting) to fidgeting and sporting (Alberti et al., 2013). Among these different forms of hyperactivity, high levels of physical activity (sporting, exercising) are relatively the easiest to operationalize and measure. Nevertheless, one can find various definitions of hyperactivity in research regarding high activity levels in ED (excessive exercise – EE, compulsive exercise, heightened activity, hyperactivity etc.). Furthermore, different cut-off points are used to split a population of interest into a high and a low active group (Hebebrand et al., 2003). The split may be based, for example, on a population median score (Solenberger 2001) or an arbitrary amount of hours spent on exercise per day or week (Davis 1995).

There is no doubt that some individuals suffering from ED may be described as hyperactive. The paradoxical restlessness and different forms of hyperactivity despite the extreme emaciation were reported already in the first descriptions of ED (Gull 1997; Hebebrand et al., 2003; Pearce 2004). There is, however, no agreement regarding the percentage of subjects with ED that can be classified as hyperactive (estimates range from 1/3 to 2/3; (Hebebrand et al., 2003)). Furthermore, it is not reported how many of the hyperactive ED sufferers exercise excessively and how many are hyperactive in a different manner. There is also no consensus whether the ED population has elevated physical activity levels in comparison to control subjects. Some researchers showed a clear difference in activity levels between ED and control groups (Blinder et al., 1970; Bratland-Sanda et al., 2010; Davis et al., 2005) while others could not confirm this difference (Davis et al., 2005; Hechler et al., 2008).

Concomitantly, the above mentioned observations show the importance of EE for the course of ED, however, are not equivalent to the statement that EE is a risk factor for the occurrence of ED. Indeed, it is not determined in how many of individuals with ED EE precedes the onset of ED, although self-reported retrospective studies showed that in some cases EE precedes the occurrence of dieting and body weight loss (Davis et al., 1994; Davis 1997; Kron et al., 1978). Interestingly, the elevated level of physical activity correlates with levels of food restriction (Holtkamp 2004), and 75% of AN patients report that their EE increased during the most strict dieting (Davis et al., 1994). This may support the theory that EE during ED is an evolutionarily conserved mechanism which is activated in response to severe body weight loss and food limitation (Bratland-Sanda et al., 2010; Epling and Pierce 1992; Guisinger 2003) and not a pre-morbid characteristic. Furthermore, it is not determined how high proportion of healthy adolescent girls and young women can be classified as EE.

Only comparison of a number of people classified as EE between the healthy and ED population (matched for age etc.) may suggest that EE is a risk factor for the development of ED.

There is a substantial body of research regarding high levels of physical activity and other forms of hyperactivity in subjects suffering from ED. The majority of studies that have focused on the relationship between the ED and EE are case-control studies. The case-control design is, however, not suitable to determine if an examined variable is a risk factors for a disorder of interest. Therefore, we decided to examine the relation between the expression of EE and the risk of obtaining an ED diagnosis in a sample of women representing the general population. In order to achieve this goal, we used data on EE and ED of adult females from a large general population-based observational study.

Methods

Participants

In the current study, we investigated data obtained from the Saint Thomas Twin Registry (TwinsUK). TwinsUK is a population-based sample of Caucasian twin pairs from Great Britain which was described in detail elsewhere (Spector and MacGregor 2002; Spector and Williams 2006). We initially obtained data (age, ED diagnosis, physical activity, EDI-III) of 3288 females enrolled to TwinsUK between 1997 and 2007 and that had non-missing data for at least one variable requested. Table 6.2 shows the exact group counts for each of the variables. For the final analysis, we included 778 participants who were in the desired age group (30–55 years old in 2007, mean age 44.16, SD=7.49) and for whom all the required data (on age, physical activity, ED diagnosis and EDI-III) was available. Zygosity was established by standardized questionnaire, which was confirmed by DNA fingerprinting. The study was approved by the St Thomas' Hospital research ethics committee.

Please note that we used the data from TwinsUK because this particular population-based registry contains the data required for our analysis (ED diagnosis and physical activity data). The fact that TwinsUK is a twin register was irrelevant to our study design. We corrected for the possible confounding effect of the kinship in our general estimating equation analysis.

Procedure

Data were obtained from the TwinsUK in an electronic data file. The data were gathered by TwinsUK during visits of participants at the Department of Twin Research & Genetic Epidemiology, King's College London, UK or by submitting self-reported questionnaires sent to participants to their home. The dates at which the data from particular questionnaires were obtained were as follows: Q10 – 2005 (physical activity), Q11A – 2004-2008 (ED diagnosis), Q13 – 2006 (ED diagnosis), Q16 – 2008 (EDI-III), Q17D – 2007-2010 (physical activity).

Age

We conducted the analysis in an age group between 30 and 55 years of age in order to include a maximum number of participants with a known ED status. The decision was based on the fact that a majority of women obtains a diagnosis of ED between the age of 15 and 25 (Lucas et al., 1999). The data analyzed were obtained in a cross sectional study design in which participants were asked if they had obtained ED diagnosis ever in life (before the TwinsUK assessment). Inclusion of participants younger than 30 years of age could lead us to falsely classify numerous participants as not diagnosed with an ED (participants who developed ED after the assessment by TwinsUK). We also applied a maximum cutoff for age for participants included in the final generalized estimating equations (GEE) model. It resulted from the fact that there was an increase in prevalence of ED since the 1970s which most probably followed an improvement in an ED diagnosis. Thus participants who in the 1970s were older than in their twenties might be individuals with an undiagnosed episode of ED. Age of participants in the year 2008 was used as an inclusion criterion for the analysis.

ED diagnosis

The assessment regarding participants' possible diagnosis of ED was conducted by the TwinsUK between 2004 and 2008. The diagnosis was established using self-reported questionnaires (so called Q11A and Q13 questionnaires). We included a given participant into an ED group if this person admitted (in the Q11A or Q13 questionnaire) to obtaining an ED diagnosis. The questions included in the TwinsUK assessment were as follows: 1) Questionnaire Q11: *Have you ever been told by a doctor or other health professional that you had an eating disorder (anorexia or bulimia nervosa)*; 2) Questionnaire Q13: *Have you ever had anorexia*; 3) Questionnaire Q13: *Have you ever had bulimia*. In order to unify the answers obtained in both questionnaires, the answers of two questions from Q13 questionnaire were combined to deliver information if a participant has ever been diagnosed with an ED (without specifying

the type of ED). Data of both questionnaires (Q11 and Q13) were available for a sub-population of the TwinsUK. Thus, the data from both questionnaires were combined as follows: answer from Q11 questionnaire was used primarily. In case of missing data, the answers from Q13 questionnaire were used.

As mentioned above, the possible ED diagnosis was established on the basis of self-reported questionnaires and was not confirmed by an independent observer or medical records. We strived to check the accuracy of the ED classification. In order to do so, we took into account the scores from EDI-III (for the description of EDI-III, please see the following methods sub-section). We could not use the score of EDI-III to confirm the ED diagnosis for every TwinsUK participant for three reasons. Firstly, limited number of participants answered the EDI-III questionnaire. Secondly, there is no established threshold, for EDI-III scale, that can be used to diagnose an individual with an ED. Thirdly, many of the participants, who admitted to ever being diagnosed with an ED, might not suffer for ED any more at the assessment by TwinsUK. Two arguments speak for this conclusion: 1) the diagnosed and not-diagnosed groups did not differ in terms of their BMI (Mann-Whitney U test, $p=0.069$); 2) The average age in the TwinsUK population included in the current analysis was 44.16 and did not differ between diagnosed and not-diagnosed groups (Mann-Whitney U test, $p=0.077$). Therefore, we could not use the scores of EDI-III to validate the diagnosis of each of the participants. However, we used the scores of EDI-III to confirm that the group classified as ever diagnosed with an ED had higher average scores of ED pathology measured with EDI-III than the control group (nonED group). Mann-Whitney U test showed that people ever diagnosed with an ED had significantly higher scores of EDI Drive Towards Thinness, Body Dissatisfaction and Bulimia Subscale in comparison with the group that was never diagnosed with ED (Table 6.1).

Additional argument for reliability of a self-reported ED diagnosis comes from the lifetime prevalence of ED in TwinsUK population. A subpopulation of TwinsUK sample (those who filled in Q13 questionnaire) stated whether they have been diagnosed with either AN or BN (n=1541 and 1543, respectively). Percentages of individuals who admitted that they had been diagnosed with AN (1.9%) or BN (1.2%) were comparable to the numbers reported previously (Hudson et al., 2007). The lifetime prevalence was higher for participants included in the GEE analysis (AN: 2.6%, BN: 1.8%).

Table 6.1. Differences in results of EDI-III subscales between the participants from control (nonED) and eating disorders (ED) group. EDI DTT – drive toward thinness scale of Eating Disorder Inventory; EDI BD – body dissatisfaction scale of Eating Disorder Inventory; EDI BS – bulimia scale of Eating Disorder Inventory.

	nonED				**ED**				**Mann-Whitney U test**		
	n	Mean (SD)	Min	Max	n	Mean (SD)	Min	Max	U	Z	*P*<
EDI DTT	1230	17.40 (6.98)	7	42	95	22.87 (8.75)	7	41	79224	5.80	.001
EDI BD	1208	33.53 (11.74)	10	60	94	38.49 (12.76)	13	60	69998	3.76	.001
EDI BS	1242	12.40 (4.75)	8	48	101	16.71 (8.10)	8	48	83814	5.66	.001

EDI-III

Part of the Eating Disorder Inventory, so called EDI-III, was handed in to participants of TwinsUK study in 2006. EDI-III consists of 25 items grouped into three subscales: Drive Towards Thinness (DTT), Body Dissatisfaction (BD) and Bulimia Subscale (BS). The scores obtained on those 3 scales may be used by clinicians to screen for individuals at risk of developing an ED. EDI-III is a very commonly used tool with a high reliability and validity to assess ED symptomatology, with higher scores of EDI-III subscales indicating more profound pathology (Bratland-Sanda et al., 2010; Clausen et al., 2011; Davis et al., 1990; Wildes et al., 2010). Internal consistency of EDI-III scales in our sample was good for all the scales (Cronbach's alpha=0.84, 0.90, 0.87 for DTT, BD and BS respectively). In the current analysis, sum of scores for each subscale was calculated separately and used to validate the difference between the ED and nonED groups.

Activity measures

Physical activity of participants from TwinsUK study was assessed on the basis of self-reported questionnaires (so called Q10 and Q17D). The questionnaire Q10 included the following questions: 1) *Currently, how many minutes per week do you spend in weight bearing activity? e.g. aerobics, running, dance, football, basketball, racquet sports etc. (do not include walking or gardening)*; 2) *Currently, how many minutes per week do you spend in non-weight bearing activity? e.g. swimming, cycling, yoga, aqua aerobics etc.* The questionnaire Q17D included the following questions: 1) *During the last week, how many hours did you spend on each of the following physical activities? / Physical exercise such as swimming, jogging, aerobics, football, tennis, gym workout, power walking etc.*; 2) *During the last week, how many hours did you spend on each of the following physical activities? / Cycling, including to work and during leisure time*; 3) *During the last week, how many hours did you spend on each of the following physical activities? / Gentler exercise,*

Yoga and Pilates. Data of questionnaire Q10 were used preferentially as they were available for 3233 participants (in comparison to only 2683 in case of Q17D questionnaire). If data from questionnaire Q10 for a given participant were not available, the score from questionnaire Q17D was used. Finally, the amount of sport was categorized as EE if a participant spent five or more hours per week to exercise as proposed by Davis (1995).

Estimate of heritability of physical activity

Heritability was established in the current sample as an attempt to test reliability of the physical activity assessment method (self-reported questionnaires sent to participant's home address). For that purpose, we compared heritability in the TwinsUK sample with previously established heritability for physical activity in the UK population.

Statistical analysis

Data were analyzed using SPSS19 software (Statistical Package For Social Sciences, Windows package version 19. IBM Company, 2010). GEE model with kinship (belonging to the same twin pair) as a subject effect was used to calculate the risk of obtaining an ED diagnosis throughout the life as a function of EE, age, BMI and EDI scales. For heritability estimation, the concordance of the phenotype was calculated for monozygotic (MZ) and dizygotic (DZ) twin pairs using Pearson correlation. The heritability (h^2) was calculated according to equation h^2=[concordance(MZ) – concordance(DZ)] * 2. Mann-Whitney U-test was used to compare ED participants, included in the GEE analysis, who belonged to the EE and nonEE (non-excessive exercise) groups.

Results

Characterization of the sample

The descriptive statistics of all variables used in the analysis are shown in Table 6.2. We aimed at assessing if there was a sampling bias for participants included in the final GEE analysis (the inclusion criteria being the availability of the data for the variables used in the analysis). Therefore, we compared the participants who were included with those who were not included in the final analysis. There were no differences for age, BMI, EDI DTT, EDI BD and EDI BS between total cases included and not included in the final GEE analysis (Table 6.2).

Additionally, we investigated the ED prevalence (Table 6.3) and level of heritability for physical activity. Almost 9% of participants who filled in ED related questionnaires reported that they have ever been diagnosed with an ED. This percentage was slightly lower (6.9%) for the participants included in the GEE analysis. In the sample 1438 cases belonged to the monozygotic twin pairs, whereas 1726 belonged to the dizygotic twin pairs. For the remaining 124 individuals, no data on zygosity was present, however, it was known whether the data from their sibling was present in the TwinsUK dataset. The heritability of physical exercise in the TwinsUK sample was estimated to be 62%, which stays in agreement with the literature (Stubbe et al., 2006).

Table 6.2. Descriptive statistics for: (1) all the participants whose data were obtained from the TwinsUK; (2) participants included in the generalized estimating equations (GEE) analysis; (3) participants not included in the GEE analysis. EDI DTT – drive toward thinness scale of Eating Disorder Inventory; EDI BD – body dissatisfaction scale of Eating Disorder Inventory; EDI BS – bulimia scale of Eating Disorder Inventory; PA – physical activity; GEE – general estimating equation analysis.

	TwinsUK sample		**included in GEE**		**not included in GEE**	
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
Age	3288	46.16 (7.48)	778	46.29 (6.60)	2510	43.51 (7.61)
EDI DTT	1300	18.31 (7.35)	778	17.95 (7.43)	522	18.85 (7.21)
EDI BD	1283	34.96 (11.91)	778	34.64 (11.79)	505	35.47 (12.08)
EDI BS	1316	13.06 (5.44)	778	12.89 (5.18)	538	13.31 (5.79)
PA [hr/week]	1894	1.34 (1.86)	778	1.42 (1.92)	1116	1.28 (1.81)
BMI	1942	26.33 (5.33)	778	26.22 (5.33)	1164	26.40 (5.34)

Lower BMI or age of participants classified into EE group could confound conclusions about association between EE and risk of developing ED. To avoid this, we tested if there is a difference in age, BMI or EDI scales between participants belonging to EE and nonEE groups. Mann-Whitney U test analysis showed that there were no statistically significant differences between the EE and nonEE groups for: age (P<0.810), BMI (P<0.222), EDI DTT (P<0.115), BD (P<0.767) and BS (P<0.588).

Table 6.3. The frequencies of the occurrence of ED diagnosis and participation in excessive exercise in the TwinsUK sample and in cases included in the general estimating equation (GEE) analysis.

	TwinsUK		Included in GEE	
	n	%	n	%
ED diagnosis	2434		778	
- no	2242	92.1	724	93.1
- yes	192	7.9	54	6.9
Excessive exercise	1894		778	
- no (nonEE)	1804	95.25	735	94.5
- yes (EE)	90	4.75	43	5.53

Life-long probability of obtaining the ED diagnosis

The probability of obtaining an ED diagnosis throughout life was assessed depending on the EDI scales and the individuals' participation in EE. BMI and age were taken into account as potential confounders. The kinship-effect variable was used to adjust for the baseline similarities within the families. As a consequence 778 cases that had the data for all the variables used in GEE were included in the analysis. Those cases belonged to 539 twin pairs. Table 6.4 summarizes the results from the multivariate GEE model. The results revealed a significant positive association of participation in EE (p=0.03) and the score on the EDI BS (p=0.01) with the probability of obtaining the ED diagnosis. The odds ratio for EE participation was 2.64, while the odds ratio for the BS EDI was 1.09. The remaining scales of the EDI questionnaire, BMI, and age did not show a significant effect.

We investigated the possibility that the obtained GEE result is an artifact caused by co-segregation of variables used in the model. We conducted a Kruskal-Wallis k-samples analysis to compare 4 groups created by EE and ED cross-tabulation (nonED-nonEE, nonED-EE, ED-nonEE, ED-EE). There were no significant differences between the groups for age ($\chi^2(3)=3.748$, $P<0.291$) and BMI ($\chi^2(3)=5.687$, $P<0.129$) however there were significant differences between the 4 groups for EDI DTT ($\chi^2(3)=24.997$, $P<0.001$), BD ($\chi^2(3)=9.437$, $P<0.025$) and BS ($\chi^2(3)=17.904$, $P<0.001$). Post hoc analysis using Mann-Whitney U test showed that there were no differences between the EE and nonEE groups within the ED and nonED subpopulations (data not shown). There were significant differences for EDI-III scales between the ED and nonED populations within the nonEE (EDI DTT, $P<0.001$; BD, $P<0.010$; BS, $P<0.0.002$) and EE groups (EDI DTT, $P<0.017$; BS, $P<0.007$; but not BD, $P<0.068$). These data indicated that age, BMI and EDI-III scales do not co-segregate with EE and that EDI

BS contributes, independently from EE, to the chance of developing ED throughout the life.

Table 6.4. Result of multivariate general estimating equation (GEE) analysis. EDI DTT – drive toward thinness scale of Eating Disorder Inventory; EDI BD – body dissatisfaction scale of Eating Disorder Inventory; EDI BS – bulimia scale of Eating Disorder Inventory.

	B	SE	*P* value	Exp(B) [95% CI]
(Intercept)	-1.76	1.64	0.28	0.17 [0.01 – 4.29]
Excessive exercise	0.97	0.46	0.03	2.64 [1.08 – 6.50]
Age	-0.03	0.03	0.21	0.97 [0.92 – 1.02]
BMI	-0.08	0.05	0.15	0.93 [0.83 – 1.03]
EDI BD	0.01	0.02	0.58	1.01 [0.97 – 1.06]
EDI DTT	0.04	0.03	0.12	1.05 [0.99 – 1.11]
EDI BS	0.08	0.03	0.01	1.09 [1.02 – 1.16]

Discussion

The present study revealed that participation in EE co-occurs in the general population with an increase in a life-long probability of developing an ED. The analysis, with the use of the TwinsUK sample, showed that the odds of ever being diagnosed with an ED are 2.64 times higher for people who engage in EE (exercising more than 5 hours per week) than for people with lower activity levels. In addition, the odds of obtaining ED diagnosis are also higher for participants with higher scores on the Bulimia Scale (BS) of EDI-III (1.09) suggesting that the chance of obtaining an ED diagnosis increases significantly with every point obtained on the BS scale. The reported influences of EE and BS are independent of the participant age and current BMI.

The association between EE and ED risk found in this study cannot be explained by selective sampling of participants from TwinsUK population to the final GEE model because the population used for the GEE analysis did not differ from general TwinsUK population for age, BMI, PA and EDI-III scales. The percentage of participants with EE expression was slightly higher in the set of cases included in the GEE than in the general TwinsUK population. However, in both groups the percentage of excessive exercisers was comparably higher for the ED group (total TwinsUK sample: 10.40%, GEE analysis: 17.39%) than the control group (total TwinsUK sample: 4.02%, GEE analysis: 5.08%).

The current study suggests a co-occurrence of EE with ED. From a physiological point of view, EE can be seen as a direct risk factor for developing ED as it facilitates body weight loss. It is known that specific sports are associated with an increased risk for developing an ED (e.g., ballet and athletics) (Johnson et al., 1999; Sundgotborgen 1994; Taub and Blinde 1992; Wichstrom 1995). In contrast, sport participation may also protect against ED development by, for example, increasing self-esteem or decreasing body dissatisfaction (Smolak et al., 2000). Furthermore, various environmental factors, not related to any pathology, may contribute to increased physical activity levels, e.g. parents' and friends' participation in physical exercise (Springer et al., 2006; Wagner et al., 2004). Thus, EE is not pathological per se and becomes a risk factor for the development of ED in combination with other characteristics. For example, in previous studies it was postulated that the motivational aspect of exercise determines if EE contributes to the development of ED (Bratland-Sanda et al., 2010) (Mond et al., 2008; Mond et al., 2004; Mond et al., 2006). These studies showed that the motivational aspects, such as 'exercising to improve body shape' and 'experiencing guilt if exercise was omitted' were associated with ED pathology. The aim of the current study was to assess whether knowledge of EE levels of an individual may also convey information about increased risk of developing ED.

There are various discrepancies between the reports on high physical activity in ED regarding the percentage of patients affected (Brewerton et al., 1995; Davis 1997; Hebebrand et al., 2003), premorbid versus evoked by disease character of hyperactivity (Davis et al., 1994; Davis 1997; Kron et al., 1978) and differences between ED and control groups (Blinder et al., 1970; Bratland-Sanda et al., 2010; Davis 1995; Davis et al., 2005; Hechler et al., 2008). These discrepancies may result from: 1) different operationalizations of high activity levels, 2) heterogeneity of the ED population and differences between the studied populations, 3) between population differences in severity of ED, and 4) between population differences in physical activity levels of control groups (dependent on many factors, including age and cultural factors). In order to eliminate some of these confounders, we used data from a cross-sectional general population study with data on the life long prevalence of ED diagnosis and current EE. The group of participants was representative for the occurrence of both phenotypes. Up to now there were few population based studies on the relation between EE and ED pathology. Two of them, conducted in adolescents addressed the question of relation between levels of physical activity and ED related symptoms such as Eating Disorder Inventory scores (Bratland-Sanda and Sundgot-Borgen 2011; Wichstrom 1995) but not with ED diagnosis. As a consequence

of the relative young age of the participants, a proportion of the participants may develop EE or ED phenotypes later in life. The remaining papers reported associations of EE with ED related behaviors (Mond et al., 2008; Mond et al., 2004; Mond et al., 2006). However, the definition of EE was different than the one used in current study namely, EE was defined on the basis of motivation for exercise and feeling of guilt when an exercise session was omitted. The researchers did not find an association between the EE and the Eating Disorder Examination Questionnaire (EDE-Q). The discrepancies between those and our findings may result from the fact that the EDE-Q is a self-report questionnaire, which estimates ED pathology in the past 28 days. Current study showed a strong association of EE and ED diagnosis in a life-long perspective.

In order to make a general conclusion from the current study, we assessed if the sample was representative for the general population. We compared the prevalence of ED and the heritability of physical activity between the sample and previously reported data. The amount of females ever being diagnosed with the ED was higher (8%) than previously reported (Attia 2010; Klein and Walsh 2004) for the total sample. This may reflect the fact that participants who suffered from ED were more prone to fill in ED related questionnaires than those who never suffered from ED. However, in the final sample of 778 cases taken for the GEE analysis the percentage of participants ever diagnosed with ED was 6.9% and that number is comparable with previous reports (Smink et al., 2012). Based on the TwinsUK physical activity data set, physical exercise heritability was estimated as 62% which stays in accordance with heritability data published previously (Stubbe et al., 2006).

We could also show that people who indicated that they were ever diagnosed with ED have higher scores on the EDI-III scales. This finding further confirms the justification of the ED categorization. The short version of EDI-III containing three scales (DTT, BD, BS) is widely used by clinicians to screen for individuals at risk for developing an ED (Bratland-Sanda et al., 2010; Clausen et al., 2011; Davis et al., 1990; Wildes et al., 2010). We added the three EDI scales to the final GEE analysis in order to examine the relative importance of association between EE and ED. Those three scales were not related to the commitment to exercise in the study by Pini et al. (2007) suggesting that the commitment to exercise and EDI scales have a rather additive effect to the development of ED. Interestingly, only the score of BS was significantly associated with the probability of developing an ED throughout the life. There are no data on the consistency of EDI-III measured phenotype with age. However, our

result may suggest that bulimic behaviors and attitudes, but not body image and shape dissatisfaction, may increase lifelong risk of developing and ED.

There are also some limitations with respect to the current study. One of them is the lack of differentiation for the subtypes of the ED diagnosis. However, taking under consideration the high rate of cross-over between the subtypes of AN as well as between AN and BN (Eddy et al., 2008; Peat et al., 2009) and the occurrence of EE in AN as well as in BN (Brewerton et al., 1995; Davis 1997), it does not seem necessary to use split diagnoses in case of the current research hypothesis. Furthermore, ED diagnosis was assessed on the basis of self-reported questionnaire and not confirmed by objective observer or medical record. Additionally, the assessment was conducted in participants of 30 years old or older. Some of the participants might have chosen not to reveal their past psychiatric illness history. Nevertheless, the significantly higher scores of EDI-III for the ED group from TwinsUK together with the lifetime prevalence comparable with prevalence reported previously (Hudson et al., 2007) suggest that the self-reported ED diagnosis was agreeably reliable in the perspective of the total sample.

Finally, the present study is a cross-sectional study on the association between ED diagnosis and specifically defined EE which was derived from self-reported physical activity measurement. We cannot be sure if the proposed model could be extended to data obtained using objective activity measurements or other EE criteria. The data suggest that EE could be an important risk factor for developing ED; however, the cross-sectional study design does not allow us to make any conclusion about the time course of occurrence of both phenotypes and the direction of their causal relation. A longitudinal study starting in children and continuing to late adulthood are needed to test this hypothesis. Nevertheless, current study suggests that EE is a risk factor for developing ED pathology in the general population of adult women.

Longitudinal Changes in the Physical Activity of Adolescents with Anorexia Nervosa and their Influence on Body Composition and Leptin Serum Levels After Recovery

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Abstract

Objective: Patients with anorexia nervosa (AN) are often observed to have high levels of physical activity, which do not necessarily diminish after a successful therapy. Previous studies have shown that body fat tissue recovery in these patients is associated with a disproportional restoration of the adipocyte hormone, leptin. Therefore, we wondered whether the individual variation in physical activity in AN patients prior to treatment may be related to body fat percentage and plasma leptin level outcome. **Method:** Body fat percentage, leptin serum, and physical activity levels (accelerometer) were measured in adolescents with AN (n=37, age 13 to 17.5 years) at initial assessment, at the end of study participation (median 12 months), and at one-year follow-up. **Results:** Accelerometer data were used to split the patients in two groups: those with low (n=26) and those with high levels of physical activity (HLPA, n=11). These groups did not differ in terms of age, IQ, presence of menses, BMI and season of admission. The HLPA group was characterized by a longer total duration of illness. Physical activity levels during therapy decreased for the group with initially HLPA and increased for the group with low levels of physical activity (to comparable levels). Physical activity remained stable after one year. The increase in body fat percentage and leptin levels were dependent on the recovery status; however, recovered patients with initially HLPA had significantly higher fat mass during the follow-up. **Discussion:** HLPA, an important modulator of AN progression in adolescents, can be successfully diminished by therapeutic intervention. Among recovered patients, those with initially HLPA had higher fat mass levels than those with low levels of physical activity. This finding suggests that HLPA are an important modulator of the body composition recovery mechanism.

Introduction

Hyperactivity is one of the core symptoms of anorexia nervosa (AN)(Gull 1997; Pearce 2004). It was reported that about 30-80% of individuals with AN can be characterized as hyperactive (Hebebrand et al., 2003). Hyperactivity may be differentiated in AN patients in three ways: 1) excessive exercise (i.e. exercising at least 6 hours per week (Davis et al., 1994) or an exercise that, when postponed, evokes negative emotional states (Hubbard et al., 1998; Mond et al., 2006)); 2) high commitment to exercise despite its adverse consequences for ones health or social contacts (Davis et al., 1993); or, 3) restlessness which may express itself in constantly active posture, fidgeting or inability to sit still (Alberti et al., 2013; Beumont et al.,

1994). Various studies have shown that hyperactive AN individuals differ from those who are not characterized by hyperactivity (Casper and Jabine 1996; Shroff et al., 2006; Strober et al., 1997). Hyperactive individuals with AN are at higher risk for relapse (Casper and Jabine 1996; Strober et al., 1997) and are usually diagnosed at a younger age (Shroff et al., 2006). It is suggested that AN sufferers exercise in order to deal with negative affections (Bratland-Sanda et al., 2010). This may be reflected in studies that found positive (Holtkamp 2004) as well as a negative (Carrera et al., 2012) correlations between levels of activity and anxiety measurements. Hyperactivity can manifest itself in various forms, such as restlessness and fidgeting or engagement in intensive physical exercise. Intensive physical exercise, though not pathological per se, facilitates body weight loss in the acute phase of the AN and hampers the efforts to restore body weight (Shroff et al., 2006). Just as high physical activity levels in AN patients may be a premorbid factor of the disease, the elevated physical activity levels assessed during an eating disorder (ED) may just reflect their premorbid high physical activity levels (Davis et al., 1994; Kron et al., 1978). However, some argue that hyperactivity is a consequence of extreme food intake limitation (Epling and Pierce 1992; Holtkamp et al., 2004). Discrepancies were also observed in clinical evidence (Bratland-Sanda et al., 2010; Kron et al., 1978). Because of these discrepancies, it is unclear whether one should expect a decrease in physical activity during treatment. To our knowledge, the issue of changes in physical activity levels during treatment and their consequences of these changes in recovery were never assessed in adolescents with ED. One exception is a case study reporting a slight but not complete decrease in hyperactivity in one adolescent ED patient after treatment (Inoko et al., 2005).

Body fat percentage (fat%) drops severely in individuals with AN due to weight preoccupation, restricted feeding, and/or exercise regime. Patients' interest is devoted to restricting fat intake with relatively maintained protein and vitamin intake (Hebebrand et al., 2007). As a consequence, percent body fat is even lower in persons with ED than in underweight females without ED matched for BMI (Kopp et al., 1997; Tolle et al., 2003; von Prittwitz et al., 1997). Therapeutic interventions aim, among other goals, to restore body weight and body composition; however, various research groups have shown abnormalities in fat tissue restoration in AN patients after regaining body weight. Namely, fat tissue tends to restore faster in abdominal regions than at the extremities (Grinspoon et al., 2001; Iketani et al., 1999; Mayer et al., 2005; Mayer et al., 2009; Mayo-Smith et al., 1989; Orphanidou et al., 1997). This effect is no longer observed one year after recovery (Mayer et al., 2009).

Changes in body fat percentage at the acute phase of the disease (Hebebrand et al., 2007) and during recovery (Mathiak et al., 1999) are reflected in the plasma levels of leptin, a hormone secreted by fat tissue. Leptin plays pleiotropic roles in energy assessment and expenditure. When animals are food deprived, and as a consequence develop hyperactivity, leptin injections diminish their activity level (Hillebrand et al., 2005; Verhagen et al., 2009; Verhagen et al., 2011). As a consequence, leptin levels were hypothesized to be inversely related to the heightened activity levels in ED. It has indeed been shown that high physical activity (Holtkamp et al., 2003b) and subjective motor restlessness (Exner et al., 2000) correlate with low plasma leptin levels. Weight restoration in individuals suffering from AN is followed by an increase in leptin, sometimes above the normal range (Hebebrand et al., 1997; Holtkamp et al., 2003a; Wabitsch et al., 2001); however, hyperleptinemia is not always detected (Djurovic et al., 2004; Popovic et al., 2004). If hyperleptinemia occurs, leptin levels are said to drop back to a normal range within weeks or months (Hebebrand et al., 1997). The abrupt increase in leptin to levels exceeding those normally observed in age and BMI matched controls may hamper body weight restoration and was associated with heightened risk of relapse (Holtkamp 2004). Hyperleptinemia can partly be a consequence of sudden increase in body fat percentage in weight-restored AN patients. Thus, body fat percentage changes can modulate changes in various ED symptoms, including hyperactivity and excessive exercise. Other hormonal changes were also observed in acutely ill individuals with AN, such as elevated levels of ghrelin (Soriano-Guillen et al., 2004; Tolle et al., 2003), a hormone secreted by the stomach in response to fasting (Inui 2001). Body weight increase in recovered individuals with AN is accompanied by elevated ghrelin levels (Otto et al., 2001; Tolle et al., 2003).

In the present study, we measured the physical activity of adolescent ED patients by using an accelerometer. The measurements were performed longitudinally: at the initial assessment, at the end of study (maximum of 12 months after the initial assessment) and at one-year follow-up. First, we tested the hypothesis that high levels of physical activity decrease in adolescent ED patients as a result of treatment. We also evaluated if this effect is still present at one-year follow-up. Second, we assessed whether high levels of physical activity at the acute phase of the illness (the initial assessment) would negatively influence the recovery rate. Finally, as body composition and leptin levels at recovery have an influence on relapse, we investigated if high levels of physical activity in AN patients influence the body composition and leptin levels restoration at recovery and at one-year follow-up.

Methods

Participants

Female adolescent patients (n=51) were referred to the treatment center (by e.g., general practitioners or mental health professionals) because of an expected diagnosis of AN. Patients were between 13 and 17.5 years old and were still living with their parents. Six patients who did not fulfill the weight criterion, but whose weight was clearly below that which is expected from their own growth curves were diagnosed as having Eating Disorders Not Otherwise Specified and were also included in the study. Comorbidity was not common in our sample. The majority of patients (63%) were diagnosed with restrictive type of AN and the remaining with purging type of AN. The type of AN was not related to the physical activity level of the AN patients (Fisher exact test: $P<0.44$).

Study information included a letter sent to the patients' home address followed by an interview with the subject, the parents, and a researcher during the first visit to the treatment center. Upon confirmation of the eating disorder diagnosis and after obtaining informed consent, a diagnostic assessment followed. All 51 patients were enrolled in a treatment procedure. Out of these, 14 patients were not included in the analysis due to missing data for objectively measured physical activity (PA) (in three cases, the Actiwatch malfunctioned; two patients did not wear the Actiwatch; and nine patients showed long periods of inactivity, which indicated Actiwatch misuse). Therefore, the final sample included in the current study consisted of 37 patients (23 inpatients and 14 outpatients). The sample was used in a previous study by Carrera and colleagues (Carrera et al., 2012); their study assessed the influence of ambient temperature on the expression of excessive physical activity in AN patients.

Ethics statement

All participants provided a written informed consent. In case of minor participants, the written consent was also obtained from the next of kin. All procedures were approved by the Medical Ethics Committee of the University Medical Center Utrecht.

Treatment Center

The study took place between January 2006 and May 2009 at the Rintveld Center for Eating Disorders in Zeist (Altrecht Mental Health Institute). All treatments were covered by the Dutch health insurance and were without additional costs for patients and their families. Rintveld is a specialist center for eating disorders and is one of the two top clinical centers in The Netherlands. This center offers multidisciplinary in- and outpatient treatment for adolescent (<18 years) and adult patients. In addition, if

necessary, medical treatment may be provided by the nearby Meander Hospital in Amersfoort. The staff of the adolescent treatment program consists of psychiatrists and other physicians, dietitians, family therapists, psychomotor therapists (Probst et al., 2010), creative therapists, psychologists, and nurses. An integrated approach that aimed at recovery of weight, eating pattern, and body attitude, as well as normalizing family relations and developmental and social skills, was offered to the patients in a system-oriented stepped and matched care model. Additional treatments were assigned to patients according to their needs and were adjusted during treatment as appropriate. Clinical staff aimed at limiting patients' hyperactivity when possible. Patients attended school at home or at the center whenever possible. Weight gain was targeted at 0.5–1.0 kg/week in accordance with clinical guidelines.

Time frame of the study

All patients obtained treatment for the full year or until they recovered or dropped out of the treatment. Additionally, patients took part in a longitudinal study with assessment repeated every second month for a maximum of one year. Thus, measurements were taken at initial assessment and at 2, 4, 6, 8, 10 and 12 months after the initial assessment. As most patients stayed in the treatment and participated in the study for the whole year, the median duration of the study was 12 months (minimum 2 months, maximum 12 months). Fifteen patients dropped out of the study sooner than 12 months (i.e., patients who withdrew between initial assessment and the 12th month, terminated treatment against advice, or terminated treatment with the agreement of their therapist after at least partial remission) (for overview, see Figure 7.1). The last assessment taken for each of the patients was considered 'the end of study' for that patient. Measurements were taken also one year after the end of the study (follow-up). There was no difference in the duration of study participation between patients classified as having high levels of physical activity and those who were classified as having low levels of physical activity (Mann-Whitney test: U=129.00, P<0.66). Additionally, we calculated the duration of illness prior to the initial assessment (duration of illness) as a difference between age of AN onset and the date of admission to the clinic.

Data collection

At initial assessment. Demographic information collected during the first visit included the results of a physical examination, psychiatric interview, and an assessment of ED and comorbid psychopathology. A trained psychologist assessed the presence of AN characteristics based on DSM-IV criteria using the eating-

disorder examination (EDE). Furthermore, we used standardized BMI (sdBMI) smaller than 1.0 (comparable with BMI 19 in adults) as the weight criterion for AN. Additionally, the patients were examined using the Eating Disorder Inventory-2 (EDI-2) (Garner 1991); the Morgan and Russell Outcome Assessment Schedule (MROAS); the Comprehensive Psychopathological Rating Scale (CPRS-S-A); and the Information, Vocabulary, Block Design, and Picture Completion tasks of the Wechsler Intelligence Scale for Children-3 (WISC3). Finally, physical activity, fat%, as well as serum leptin and ghrelin levels, were measured.

At the end of study and at follow-up. Data on sdBMI, presence of menses, EDI-2, MROAS, CPRS-S-A, physical activity, fat%, serum leptin, and ghrelin levels were collected at the end of study and at follow-up.

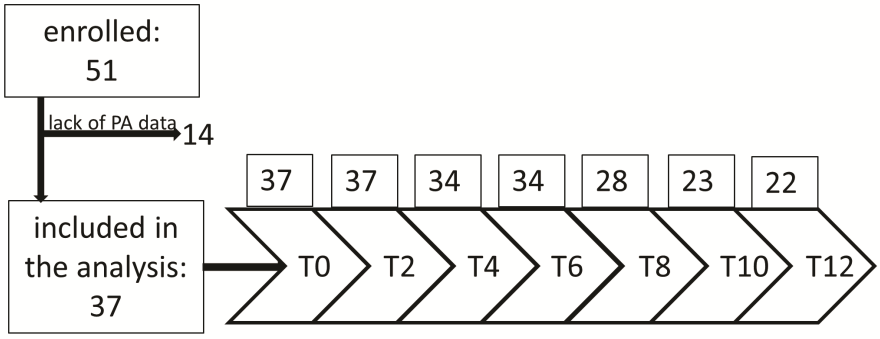

Figure 7.1. Timeframe of the study. The diagram shows the number of patients in the study at each data measurement point between enrolment, initial assessment (T0), and maximal duration of treatment (T12).

Recovery definition

For study purposes, we used MROAS to split the patient population into the recovered (MROAS score ≥ 9) and the non-recovered group (MROAS score < 9). Patients were classified as recovered or non-recovered at the end of study and at follow-up study independently. The MROAS is often used in AN research and provides a quantitative measure (scores 0–12) of current outcome state divided over five subscales: nutritional state, menstrual function, mental state, psychosexual adjustment, and socio-economic status. Higher MROAS scores indicate better outcome states. The composite MROAS score can also be used to evaluate patient's outcome state in three categories: Good (composite score ≥9), Intermediate (≥4 and ≤8), and Poor (<4) (Lee et al., 2003).

Standardized BMI (sdBMI)

We used BMI transformed into sdBMI scores on the basis of the standard growth curves for the Dutch population (Growth Analyser 3.5, Dutch Growth Foundation, Rotterdam, NL). The use of the sdBMI values instead of BMI values was motivated by the fact that this study involved adolescent patients (13.5 – 17 years of age) and because we analyzed the longitudinal data of each patient (BMI at intake and at the end of study). Average BMI changes significantly during puberty. It may reach values considered to be low according to standard cut-off points. However, these values may still be within a healthy range when the standard growth curves for the age and gender are considered. Adolescent patients should gain weight at a level that is considered appropriate for their age. The amount of body weight loss during illness is potentiated by the fact that they drift away from their normal growth curve. Therefore, sdBMI, in comparison with BMI, is considered a more precise description of the body growth of adolescents.

Objective assessment of physical activity

Physical activity (PA) was measured using an accelerometer (Actiwatch model AW 4; Cambridge Neurotechnology, Cambridge, United Kingdom). The Actiwatch was strapped to the patient's right ankle and was worn for three consecutive weekdays, from 9 pm on the first day to 9 pm on the fourth day, except while swimming and showering. The epoch length (sampling time) for the Actiwatch was set to 1 minute. Night activity (23:00–07:00) and sequences of >10 min of consecutive zero counts were excluded from the recordings. This procedure was similar to that recently used in the field of eating disorders (Bratland-Sanda et al., 2010). Thereafter, the data were summarized as counts per day and patients were excluded from analyses if more than 30% of the day was not available for two of the three days. Activity data from days 1 to 3 were averaged to determine daily physical activity for each patient. Data analysis was undertaken to determine the periods of time (%) at varying intensity levels of physical activity. The ranges (in counts per minute) for the activity intensities were <200 for sedentary activity (SA), 200 to <1800 for light activity (LA), and ≥1800 for moderate-to-vigorous physical activity (MVPA), as validated by Puyau et al. (Puyau et al., 2002) for the Actiwatch device worn on the lower right leg. Patients were subdivided into groups characterized by high levels of physical activity and low levels of physical activity (HLPA and LLPA groups, respectively) on the basis of their participation in moderate or vigorous physical activity measured by the accelerometer. Accelerometer calibration studies showed that MVPA readouts correspond to high levels of physical activity such as participation in sports (Puyau et al., 2002). Thus, total time spent on MVPA was used to split the population into

the two groups by using a cutoff of 2.5 hours of MVPA readout per three days of measurements, which corresponds to the definition used in previous publications (Bratland-Sanda et al., 2010).

Physiological parameters

Body weight and fat% were measured using a TANITA Body Composition Analyzer TBF-300 (Tanita Corporation, Tokyo, Japan). Blood samples were obtained by venipuncture for hormonal analysis. Plasma samples were stored at −80°C prior to determination. Leptin was measured using a sensitive Radio-Immuno-Assay (RIA) (Sensitive Human Leptin RIA Kit, Linco, St. Charles, Missouri, U.S.A.), intra-assay CV of 5.63%, and inter-assay CV of 5.66%. Ghrelin was measured using a sensitive Radio-Immuno-Assay (RIA) (Human (total) Ghrelin RIA Kit, Linco, St. Charles, Missouri, U.S.A.), intra-assay CV of 6.4%, and inter-assay CV of 16.3%. Relative hyperleptinemia was defined as leptin levels above the 95 percentile for a given BMI for girls 5.8-18.99 years old based on previously published observations (Hebebrand et al., 1997).

Data analysis

Data of all patients with valid data for at least the first two months of the study were analyzed. Missing data were imputed according to the last-observation-carried-forward method. According to this method, the last observed data point after initial assessment may be used for all subsequent missing data points until the end of the study for a given patient. Student's t-test was used to compare the LLPA and the HLPA groups as well as the recovered and the non-recovered groups. Effect size for the t-test results was calculated using Pearson's r. Repeated measures ANOVA (with Greenhouse-Geisser correction if necessary; in that case ε value is reported) was used to analyze physical activity data at initial assessment, at the end of study and at follow-up. Effect size for repeated measures ANOVA was calculated using partial Eta^2 ($\eta^2_{partial}$). Fisher exact test was used for the in- and outpatient analysis. Chi-square and Kaplan-Meier analyses were used to evaluate recovery and recovery rate differences between the LLPA and the HLPA group. Cramer's V coefficient was used as an estimate of the effect size for the chi-square test. Two-way ANOVA, with physical activity group classification and recovery status as main factors, was used to compare the characteristics of the recovered and the non-recovered LLPA and HLPA patients. In all cases, Levene's test of equality of error variances showed that the assumption of the two-way ANOVA was met. Spearman's rho correlation analysis was used to examine associations between fat%, plasma leptin, and plasma ghrelin levels with psychological variables measured with EDI-2 and CPRS-S-A. All analyses

were performed with SPSS 20.0 (IBM) and data are presented as mean ± standard error of the mean. For significance thresholds, a *P*-value of 0.05 was used throughout, unless the *P*-value was corrected for multiple comparisons as stated at the results section.

Results

Population characteristics

All the participants (n=37, all those who had physical activity data at initial assessment) were females, aged 13 to 17.5 years at initial assessment (mean=15.15, SD=1.21), with average IQ of 104.8 (SD=13.11), and BMI between 12.6 and 18.4 (mean=15.66, SD=1.38). Out of those patients, 30% were classified as belonging to the HLPA group at initial assessment based on the objective accelerometer data (LLPA n=26, HLPA n=11).

Table 7.1. Characteristics of the sample at the end of study. Characteristics of the nonRec (non-recovered) and the Rec (recovered) groups at the end of study. EDI-2 = Eating Disorder Inventory, Interpers. distrust = interpersonal distrust; interoceptive aw.= interoceptive awareness, CPRS-S-A = Comprehensive Psychopathological Rating Scale, e.s. = effect size. Student's t-test, significant results are in bold.

	nonRec (n=18) Mean (SD)	**Rec (n=19)** Mean (SD)	**t(df)**	***P*<**	**e.s.**
BMI	18.656 (0.424)	19.311 (0.345)	-1.205 (35)	0.24	0.200
sdBMI	-1.033 (0.260)	-0.479 (0.189)	-1.73 (31.4)	0.09	0.295
CPRS-S-A					
anxiety	6.563 (4.60)	5.250 (3.553)	0.937 (32)	0.36	0.163
obsessions	8.781 (5.709)	4.056 (3.753)	2.81 (25.4)	**0.01**	0.487
depression	8.562 (6.129)	4.722 (4.226)	2.147 (32)	**0.04**	0.355
EDI-2					
drive for thinness	33.28 (7.61)	27.89 (10.48)	1.780 (35)	0.08	0.288
body dissatisfaction	41.94 (10.50)	36.47 (12.47)	1.439 (35)	0.16	0.236
bulimia scale	13.11 (3.71)	11.53 (4.85)	1.112 (35)	0.27	0.185
ineffectiveness	37.06 (9.69)	33.89 (8.35)	1.065 (35)	0.29	0.177
perfectionism	19.61 (5.71)	18.11 (4.98)	0.856 (35)	0.40	0.143
interpers. distrust	22.89 (6.22)	21.05 (5.17)	0.979 (35)	0.33	0.163
interoceptive aw.	29.78 (8.34)	30.16 (7.53)	-0.146 (35)	0.86	0.025
maturity fears	27.11 (7.50)	22.79 (5.25)	2.041 (35)	**0.05**	0.326
ascetism	28.50 (7.72)	25.53 (6.30)	1.287 (35)	0.21	0.213
impulse regulation	27.33 (5.81)	25.16 (6.34)	1.086 (35)	0.29	0.181
social insecurity	30.33 (6.91)	25.95 (5.98)	2.068 (35)	**0.05**	0.330

Recovery

At the end of study, 51.4 % of patients met the criteria for recovery according to MROAS (19 recovered (Rec), 18 non-recovered (nonRec) patients). The Rec group gained more body weight than the nonRec group; however, the two groups did not differ significantly in terms of BMI at the end of study. At this time point, the Rec and nonRec group differed significantly in terms of the Obsession and Depression scales of CPRS-S-A, as well as for Maturity Fears and Social Insecurity scales of EDI-2 (Table 7.1). These differences were observed despite the fact that at initial assessment, there were no differences between the Rec group and the nonRec group for CPRS-S-A or EDI-2 (data not shown). Between the end of study and the follow-up, six additional patients recovered. However, six other patients relapsed. It is of note that the relapse rate in our sample (16%) was comparable to the one reported in the literature (Strober et al., 1997). At one-year follow-up, the nonRec group had a significantly lower BMI and sdBMI than the Rec group (Table 7.2). Furthermore, the nonRec group had higher scores than the Rec group for CPRS-S-A (all scales) as well as for EDI-2 (Ineffectiveness, Interpersonal Distrust, Interoceptive Awareness, Maturity Fears, and Social Insecurity) (see Table 7.2).

Characterization of LLPA and HLPA groups

We compared the LLPA and HLPA patients at initial assessment to test whether other characteristics co-occurred in our sample of patients with high levels of physical activity. No differences between the LLPA and the HLPA groups were observed for age, IQ ($n_{(nonEE)}$=23, $n_{(EE)}$=11), BMI, presence of menses (n=31 no menses, n=1 regular menses, n=4 premenarche, n=1 use of anticonceptive pill), EDI-2 total score, EDI-2 subscales, and CPRS-S-A subscales (Table 7.3). Furthermore, outpatients were not more likely to be included in the HLPA group than inpatients (LLPA inpatients (n=15), LLPA outpatients (n=11), HLPA inpatients (n=8), HLPA outpatients (3); Fisher's exact test, P<0.48). However, the LLPA and the HLPA patients differed in terms of duration of illness prior to initial assessment (Figure 7.2). Student's t-test showed that the HLPA group had significantly longer duration of illness before admission to Rintveld Clinic than the LLPA group (t(35)=-2.472, P<0.02, r=0.99) with a large effect size (Figure 7.2a). This data indicate that the HLPA group had an earlier age of onset of the illness.

Decrease in physical activity during treatment

In order to test the hypothesis that physical activity decreases during treatment, we compared the time course of total physical activity between LLPA and HLPA groups

Table 7.2. Characteristics of the sample at follow-up. Characteristics of the nonRec (non-recovered) and the Rec (recovered) groups at follow-up. EDI-2 = Eating Disorder Inventory, Interpers. distrust = interpersonal distrust; interoceptive aw.= interoceptive awareness, CPRS-S-A = Comprehensive Psychopathological Rating Scale, e.s. = effect size. Student's t-test, significant results are in bold.

	nonRec (n=18)	**Rec (n=19)**			
	Mean (SD)	**Mean (SD)**	**t(df)**	***P* <**	**e.s.**
BMI	17.95 (1.95)	19.71 (1.72)	-2.918 (35)	**.006**	0.442
sdBMI	-1.66 (1.35)	-0.47 (0.71)	-3.374 (35)	**.002**	0.495
CPRS-S-A					
anxiety	8.50 (5.26)	4.36 (3.58)	2.711 (32)	**0.01**	0.432
obsessions	9.47 (5.43)	4.47 (3.82)	3.129 (32)	**0.01**	0.484
depression	9.59 (5.38)	4.44 (4.36)	3.077 (32)	**0.01**	0.478
EDI-2					
drive for thinness	31.78 (6.49)	28.11 (12.17)	1.15 (27.7)	0.26	0.213
body dissatisfaction	39.78 (8.39)	39.32 (12.50)	0.13 (31.6)	0.89	0.023
bulimia scale	14.61 (5.82)	11.58 (3.67)	1.906 (35)	0.07	0.307
ineffectiveness	38.56 (9.61)	31.47 (10.00)	2.194 (35)	**0.04**	0.348
perfectionism	19.39 (4.63)	16.42 (4.86)	1.901 (35)	0.07	0.306
interpers. distrust	24.33 (6.70)	19.58 (5.32)	3.397 (35)	**0.02**	0.498
interoceptive aw.	31.72 (7.34)	26.47 (7.35)	2.172 (35)	**0.04**	0.345
maturity fears	27.11 (6.86)	22.89 (5.04)	2.139 (35)	**0.04**	0.340
ascetism	27.11 (7.38)	23.11 (7.09)	1.684 (35)	0.10	0.274
impulse regulation	28.17 (6.53)	24.11 (7.04)	1.817 (35)	0.08	0.294
social insecurity	31.33 (6.08)	23.74 (5.38)	4.030 (35)	**0.01**	0.563

at initial assessment, at the end of study, and at one-year follow-up. We conducted repeated measures ANOVA to assess changes over time of the physical activity levels of the patients. The physical activity levels were measured using an accelerometer and the patients were grouped on the basis of their physical activity (PA) levels. For daily physical activity levels, both the PA classification and the interaction between time and PA classification were significant, thus indicating that PA levels differed between the LLPA and the HLPA groups in a time-dependent manner. The Student's t-test, which was conducted as a post hoc test (corrected *P*-value: $P=0.0167$), showed that the HLPA group had a significantly higher total daily physical activity than the LLPA group at initial assessment ($t(38.37)=-9.137$, $P<0.001$, $r=0.99$) (Figure 7.2b and Table 7.4). This difference disappeared during treatment due to the fact that the HLPA group decreased in their total activity while the LLPA group on average slightly increased in their activity.

Table 7.3. Basic characteristics of the sample split by physical activity levels. Student's t-test showed no differences between the LLPA (low levels of physical activity) and the HLPA (high levels of physical activity) groups at initial assessment. EDI-2 = Eating Disorder Inventory, Interpers. distrust = interpersonal distrust; interoceptive aw.= interoceptive awareness, CPRS-S-A = Comprehensive Psychopathological Rating Scale. All effect sizes (e.s.) are small or negligible.

	LLPA (n=26)	**HLPA (n=11)**			
	Mean (SD)	**Mean (SD)**	**t(df)**	***P*<**	**e.s.**
Age	15.262 (1.353)	15.345 (1.010)	-0.184 (35)	0.86	0.031
IQ	103.783 (12.446)	106.755 (13.124)	-0.640 (32)	0.53	0.112
BMI	16.188 (1.408)	15.582 (1.654)	1.138 (35)	0.26	0.189
sdBMI	-2.185 (1.026)	-2.827 (1.755)	1.398 (35)	0.17	0.229
CPRS-S-A					
anxiety	9.340 (4.377)	11.556 (4.572)	-1.287 (32)	0.21	0.222
obsessions	9.280 (4.880)	12.833 (5.006)	-1.861 (32)	0.07	0.313
depression	9.827 (13.222)	13.222 (5.197)	-1.657 (33)	0.11	0.277
EDI-2					
drive for thinness	34.115 (8.155)	35.500 (5.418)	-0.515 (35)	0.61	0.087
body dissatisfaction	42.269 (10.267)	42.818 (10.255)	-0.149 (35)	0.88	0.025
bulimia scale	12.962 (5.466)	15.182 (6.493)	-1.068 (35)	0.29	0.178
ineffectiveness	38.31 (8.57)	42.77 (8.29)	-1.462 (35)	0.15	0.240
perfectionism	19.04 (5.80)	20.27 (5.12)	-0.611 (35)	0.55	0.103
interpers. distrust	22.50 (5.94)	26.09 (5.75)	-1.696 (35)	0.10	0.276
interoceptive aw.	34.19 (7.80)	34.82 (6.95)	-0.230 (35)	0.82	0.039
maturity fears	26.54 (5.94)	30.41 (7.79)	-1.650 (35)	0.11	0.269
ascetism	27.92 (5.95)	30.10 (6.59)	-0.955 (34)	0.35	0.162
impulse regulation	27.43 (7.55)	29.40 (6.87)	-0.720 (34)	0.48	0.123
social insecurity	28.23 (4.94)	31.00 (6.73)	-1.360 (34)	0.18	0.227

Influence of HLPA on recovery rate

We aimed at assessing if HLPA detected at initial assessment diminished the recovery rate of adolescent AN patients. There was no difference in recovery rate for both the LLPA and the HLPA groups ($\chi^2(1)=1.408$, $P<0.24$, Cramer's V=0.195, for sample sizes see Figure 7.3a). Kaplan-Meier survival analysis with PA classification as a factor showed that there was no effect of PA classification on the recovery rate (mean=10.72, 95% CI 9.57-11.87; mean=10.57, 95% CI 8.87-12.28, for the LLPA and the HLPA groups respectively). Thus, our data suggested that HLPA at initial assessment had no negative influence on recovery rate of the adolescent AN patients.

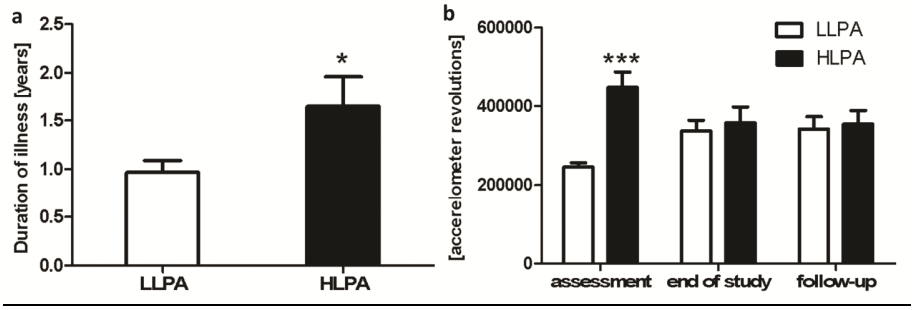

Figure 7.2. Characterization of LLPA and HLPA patients. a) Duration of illness, defined as time between obtaining the first diagnosis and initial assessment at Rintveld clinic. Duration of illness is significantly higher for the HLPA (high levels of physical activity) group than for the LLPA (low levels of physical activity) group. Mean±SEM, Student's t-test, * P<.05. b) Physical activity of the LLPA and the HLPA groups at initial assessment, at the end of study, and at follow-up. Data are expressed as mean ± SEM; Repeated measures ANOVA, Student's t-test as a post hoc test: *** P<.001 in comparison to the LLPA group at the same time point.

Changes in PA levels in recovered and non-recovered patients

We tested the hypothesis that HLPA patients who recovered had lower physical activity levels at initial assessment than those who did not recover. We also assessed if changes in levels of PA were more profound for the Rec than for the nonRec patients from the HLPA and the LLPA groups. In order to do so, we split the population into four groups on the basis of PA classification and of recovery according to MROAS. As a consequence four groups were compared: LLPA-nonRec, LLPA-Rec, HLPA-nonRec, and HLPA-Rec. We conducted two-way ANOVA, with PA classification and recovery status as factors, to assess daily physical activity levels at assessment, at the end of study, and at follow-up (Figure 7.3a, Table 7.5).

At the initial assessment, there was a significant effect of PA classification, recovery status, and interaction on daily physical activity levels. Post hoc analysis (Student's t-test, corrected P-value: P=0.025) showed significant difference between Rec and nonRec patients in the HLPA group with a large effect size ($t(9)=-2.989$, P<0.02, r=0.71). HLPA patients, who recovered at the end of study, had significantly higher physical activity levels at initial assessment than those patients who did not recover. At the end of the study and at follow-up, the effect of PA classification, recovery status, and interaction between the two variables were not significant.

Influence of HLPA on restoration of fat% and on plasma leptin levels

We investigated the influence of HLPA at initial assessment on the restoration of fat% and on plasma leptin and ghrelin levels after treatment (at the end of study and

at one-year follow-up). For that purpose, we split the population into four groups as explained in the analysis above. We conducted a two-way ANOVA analysis, with PA classification and recovery status as factors, to characterize fat% at initial assessment, at the end of study and at follow-up. Due to the availability of a small number of blood samples for patients in the HLPA-Rec group at initial assessment, we only analyzed leptin and ghrelin levels at the end of study and at follow-up.

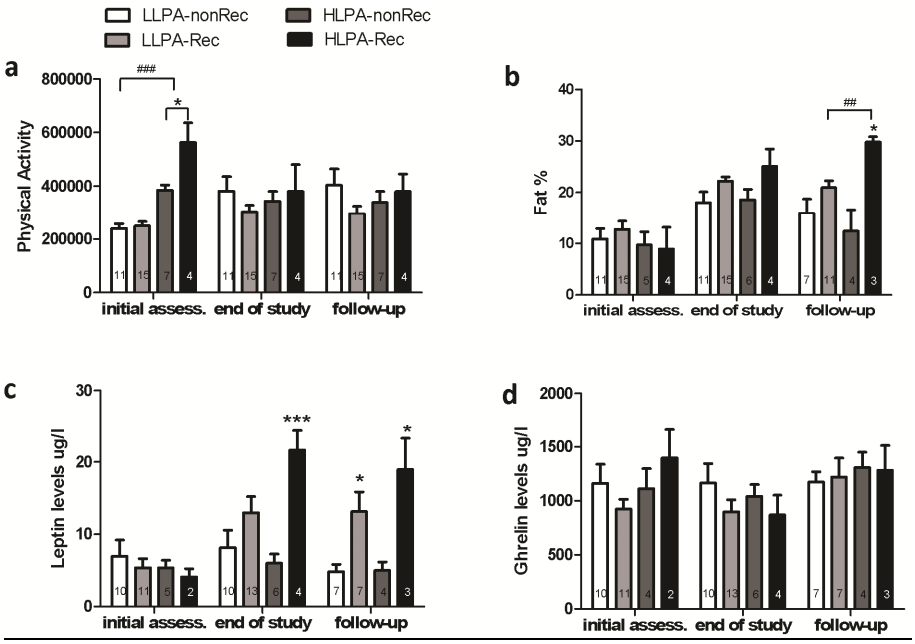

Figure 7.3. Differences between recovered and non-recovered LLPA and HLPA patients. Daily physical activity scores (a), fat% (b), leptin serum levels (c), and ghrelin serum levels (d) plotted for recovered and non-recovered LLPA and HLPA groups at 3 time points (initial assess. = initial assessment). Data are expressed as mean ± SEM; Two-way ANOVA, Student's t-test as a post hoc test: * P<.05, *** P<.001 between the recovered (Rec) and non-recovered (nonRec) patients in the same group (LLPA or HLPA) and at the same time point; ## P<.01, ### P<.001 between the LLPA and the HLPA groups at the same time point. Numbers within bars indicate sample size per group.

Results for fat% are shown in Figure 7.3b and Table 7.5. At initial assessment, there were no differences in terms of fat% between the recovered and the non-recovered HLPA and LLPA groups. At the end of study, Rec patients had higher fat% than nonRec patients and there was no significant effect of PA classification or interaction on body fat%. At follow-up, recovered patients had higher fat% than non-recovered patients and this effect depended on PA classification as the interaction effect was significant. Post hoc analysis (Student's t-test; corrected P-value: #=0.025) showed

significant difference between the LLPA and the HLPA recovered patients with a large effect size (t(12)=-3.309, *P*<0.007, r=0.99). There was also a significant difference, with a large effect size, between recovered and non-recovered HLPA patients (t(5)=-3.424, *P*<0.02, r=0.69); however, there was no difference between recovered and non-recovered LLPA patients (t(16)=-1.828, *P*<0.09, r=0.42).

Results for leptin levels are shown in Figure 7.3c and Table 7.5. Two-way ANOVA showed that at the end of study, the recovery status had a significant large effect on plasma leptin levels; however the PA classification or interaction had no effect. Post hoc analysis (Student's t-test; corrected *P*-value: *P*=0.025) showed significant difference between recovered and non-recovered HLPA patients for leptin levels with a large effect size (t(8)=-5.886, *P*<0.001, r=0.90). Also at the follow-up, there was a significant effect of recovery status on leptin levels but not significant effects of PA classification or interaction. The effect sizes for PA classification and interaction were large, suggesting that a larger sample size might be required to obtain significant effect. Post hoc analysis (Student's t-test; corrected *P*-value: *P*=0.025) showed significant differences (with large effect sizes) between recovered and non-recovered patients in the LLPA group (t(12)=-2.859, *P*<0.02, r=0.64) and the HLPA group (t(5)=-3.605, *P*<0.02, r=0.85). Results for ghrelin levels are shown in Figure 7.3d and Table 7.5. There were no significant effects for ghrelin at the end of study and at follow-up.

Associations of psychological measurements with observed biological parameters

We assessed how the biological measures relate to the psychological measures at one-year follow-up in our sample. We used Spearman's rho correlation analysis to examine associations between fat%, plasma leptin, and plasma ghrelin levels with psychological variables measured with EDI-2 and CPRS-S-A (Table 7.6). Analysis showed that fat% at follow-up correlated significantly and negatively with all the subscales of CPRS-S-A and with four EDI-2 subscales (Ineffectiveness, Interpersonal Distrust, Maturity Fears and Social Insecurity). As fat% and leptin correlated significantly (Spearman's rho = 0.569, *P*<0.01), it is not surprising that plasma leptin levels at follow-up correlated significantly and negatively with all the subscales of CPRS-S-A and with six EDI-2 subscales (Ineffectiveness, Interpersonal Distrust, Interoceptive Awareness, Maturity Fears, Impulse Regulation, and Social Insecurity). Finally, ghrelin plasma levels correlated significantly and positively with two EDI-2 subscales (Body Dissatisfaction and Interpersonal Distrust). Taking under consideration the number of correlations, 5% of the significant associations are false positives (about 3 of the 42 correlations). Therefore, majority of the correlations obtained should be considered valid.

Discussion

We conducted a longitudinal study in adolescent anorexia nervosa patients to assess the effects of treatment on high physical activity levels as well as to investigate the consequences of high levels of physical activity on physiological recovery. We showed that patients who had high levels of physical activity at initial assessment were characterized by earlier age of onset of the illness. During the treatment, physical activity levels of AN patients normalized (decreased for the HLPA and increased for the LLPA groups) at the end of treatment and remained stable at one-year follow-up. High levels of physical activity at initial assessment did not influence recovery rate in this sample. However, patients who engaged in high levels of physical activity had profound increases in fat% and leptin levels at recovery. The changes in body composition and leptin levels observed at recovery were still maintained at one-year follow-up.

The data showed the normalization of physical activity levels of the adolescent AN patients during treatment, and this effect was maintained one year later. Patients who were initially highly active diminished their physical activity, while patients who were characterized by low activity increased their physical activity. The increase in the PA of patients from the LLPA group may be potentially explained by the emaciation of patients at initial assessment, which might have resulted in decreased physical activity levels. As patients from the LLPA group partly restored their body fat%, their physical activity levels increased. As far as the decrease of physical activity of patients in the HLPA group is concerned, there are two possible explanations for this finding. First, this effect may be considered a positive verification of the hypothesis that extreme food limitation and body weight loss may be evolutionarily conserved triggers for hyperactivity observed in AN patients (Epling and Pierce 1992; Holtkamp 2004). Second, the decrease in activity at the end of study might have been caused by the efforts of the clinical staff to decrease patients' hyperactivity. The effect of reduction of PA was present also at follow-up. This may suggest that members of the patients' social surroundings learned about the negative effect of hyperactivity on the health status of AN patients. As a result, they may put more pressure on patients to stay less active. Finally, we cannot fully exclude the possibility that the observed normalization of physical activity seen in both groups is merely an effect of regression toward the mean. The latter would take place if the PA scores of both the LLPA and the HLPA groups at initial assessment were extreme in comparison to the average scores of the adolescent AN population.

Table 7.4. Physical activity changes during treatment. Results of repeated measures ANOVA on changes of PA (physical activity) during treatment. Significant *P*-values are marked in bold. *ε=0.764.

	PA classification			Time			Interaction		
	F (df)	*P* <	η^2partial	F (df)	*P* <	η^2partial	F (df)	*P* <	η^2partial
PA	4.392 (1,35)	**0.05**	0.111	0.002 (1.53,53.47)*	1.00	<0.001	11.758 (1.53)	**0.001**	0.273

Table 7.5. Results of the two-way ANOVA on differences between the recovered and the non-recovered LLPA and HLPA AN patients. As = initial assessment, EoS = the end of study, F-up = follow-up. Significant *P*-values are marked in bold. *ε=0.764.

	PA classification			Recovery status			Interaction		
	F (df)	*P* <	η^2partial	F (df)	*P* <	η^2partial	F (df)	*P* <	η^2partial
PA									
As	74.764 (1,33)	**0.001**	0.69	12.432 (1,33)	**0.002**	0.27	9.972(1,33)	**0.004**	0.23
EoS	0.146 (1,33)	0.71	0.004	0.163 (1,33)	0.69	0.005	1.241 (1,33)	0.27	0.04
F-up	0.032 (1,33)	0.86	0.001	0.382 (1,33)	0.54	0.006	1.922 (1,33)	0.18	0.03
Fat%									
As	0.949 (1,31)	0.34	0.03	0.040 (1,31)	0.84	0.001	0.272 (1,31)	0.61	0.01
EoS	0.705 (1,32)	0.41	0.02	7.005 (1,32)	**0.05**	0.18	0.328 (1,32)	0.57	0.01
F-up	0.968 (1,21)	0.34	0.07	17.602 (1,21)	**0.001**	0.23	5.404 (1,21)	**0.03**	0.11
Leptin									
EoS	1.432 (1,29)	0.24	0.05	14.295 (1,29)	**0.001**	0.33	4.001 (1,29)	0.06	0.12
F-up	1.450 (1,17)	0.25	0.17	20.087 (1,17)	**0.001**	0.32	1.297 (1,17)	0.27	0.32
Ghrelin									
EoS	0.199 (1,29)	0.66	0.007	1.679 (1,29)	0.206	0.06	0.078 (1,29)	0.78	0.003
F-up	0.334 (1,17)	0.57	0.02	0.004 (1,17)	0.95	0.03	0.043 (1,17)	0.84	0.06

However, this is not the case. In a new study that is currently being conducted at the Rintveld clinic, a new group of adolescent AN patients has comparable PA scores to the population used in the current study. Also, the percentage of patients classified as HLPA is identical between the two populations.

Contrary to what is expected, our data show that HLPA patients who recovered at the end of study were characterized by even higher physical activity levels at initial assessment than the non-recovered HLPA patients. The data may support previous findings that high levels of physical activity do not necessarily result in poorer outcomes after treatment. It was previously shown that, in AN patients, high physical activity levels are partly driven by body weight preoccupation (Davis 1995). Furthermore, the relation between high exercise levels and disordered eating is partially mediated by reasons to exercise (Thome and Espelage 2007). These variables were, however, not assessed in the current study.

Previously, it was reported (Shroff et al., 2006) that AN patients who were highly physically active were younger at the first clinical interview. Our findings also showed that the age of onset of AN was earlier in the HLPA group than in the LLPA group. We propose that high levels of physical activity may mask the progress and severity of AN for patients and their social surroundings. This is in agreement with previous hypotheses that one of the functions of high physical activity in individuals with AN is the denial of the negative outcomes of severe dieting (Kron et al., 1978).

The recovery rate during one-year follow-up was comparable to those previously reported in the literature (Carter et al., 2004). As was predicted, recovery was associated with an increase in body fat% and leptin levels. In our sample, the fat% increase was more profound in a group of patients who were classified to the HLPA group at the acute phase of the illness. The effect on body fat% was maintained and even strengthened at one-year follow-up. Thus, we suggest that previous history of HLPA changes fat pad restoration in response to re-feeding. We also observed relative hyperleptinemia (assessed dependent on the BMI for age and gender matched controls) during recovery, consistent with previous reports (Hebebrand et al., 1997; Holtkamp et al., 2003a). However, contrary to a previous report (Hebebrand et al., 1997), elevated leptin levels did not normalize at one-year follow-up. Although, 1) leptin levels at the end of study and at follow-up mimicked the group differences observed in body fat% at these time points; and, 2) though relative hyperleptinemia (assessed dependent on the BMI for age and gender matched controls) was observed in all of the HLPA-Rec patients and only in half of the LLPA-Rec patients, the results were not statistically significant. This is most probably due to the small HLPA-Rec

group size and high variability in leptin levels. Finally, both fat% and leptin plasma levels in the current study correlated negatively at follow-up with psychological measurements such as anxiety, obsessions, depression (as measured with CPRS-S-A), and various subscales of EDI-2. These findings suggest that the higher fat% and leptin levels are at one-year recovery, the better the psychological outcome as measured by CPRS-S-A and EDI-2 (lower scores of psychopathology measured by these questionnaires).

Although plasma leptin levels correlate with body fat%, leptin levels are likely to respond to short-term changes due to various stimuli such as food intake. As a consequence, slow changes in fat% are more informative of patients' physical state than the dynamic leptin measurements. Nevertheless, plasma leptin levels may convey important information. First, in the current study, at follow-up, leptin was a much better predictor of psychological outcome than fat% measurement. Second, leptin levels may inform us about the propensity of a person to develop hyperactivity. Gelegen et al. (2007) showed that a mouse strain that is sensitive to the activity-based anorexia model has higher baseline plasma leptin levels in comparison to a strain that is more resistant to activity-based anorexia. Both strains are characterized by comparable plasma leptin levels as a consequence of restricted feeding. Thus the susceptible mouse strain (that becomes hyperactive during food restriction) is characterized by a stronger decline of leptin between baseline and experimentally evoked food restriction. This could lead to the hypothesis that the stronger the decline of leptin due to food restriction, the higher the sensitivity is to restricted caloric assessment and perhaps to the hyperactivity that is evoked by it. Additional research is needed to further test this hypothesis.

We did not observe any effects of recovery on ghrelin levels. The high within-group variance of ghrelin levels suggests that factors, other than the ones included in the current study, may have influenced ghrelin levels.

The main limitation of the study is the drop-out of patients between initial assessment, end of study, and follow-up. As a consequence, some of the groups have a small headcount. Therefore, it is not justified to generalize the findings on the influence of HLPA on recovery of fat% and leptin levels to all AN patients. Another limitation of the study is the lack of a control group, which makes it impossible to state whether the HLPA group is characterized with higher physical activity levels than healthy controls or only relative to the LLPA group. Further studies are necessary to replicate our findings.

Despite the limitations, the current study contains several findings of clinical relevance. On the one hand, HLPA may have a negative influence on the course of AN. For example, our data suggest that HLPA may mask the development of AN from patients and their social surroundings. This fact may cause a delay in searching for professional help and may prolong the time of illness without appropriate treatment. On the other hand, one should distinguish possibly different influences of HLPA on health status of AN patients during disease onset, maintenance, and recovery from AN. First, in our sample, HLPA did not have negative consequences on the recovery rate of adolescent AN patients. This observation surely results from the fact that the treatment was successful in diminishing the levels of PA in our sample. HLPA was, however, associated with higher body fat percentage and higher leptin levels in recovered patients (at the end of study and at follow-up) in comparison to the LLPA group. These observations may have negative as well as positive influences on long-term remission. It has been previously shown that high leptin levels may counteract therapeutic efforts by increasing PA and decreasing food intake (Farooqi et al., 2002; Heymsfield et al., 1999; Hwa et al., 1997; Scarpace et al., 1997; van Dijk 2001; van Elburg et al., 2007). However, in the current study, we did not observe an increase in PA at one-year follow-up in the group of patients with high plasma leptin levels. Furthermore, the correlation analysis has shown that the higher the fat% and leptin levels, the better the psychological outcome as measured using CPRS-S-A and EDI-2 questionnaires. This may suggest that although HLPA contribute to the development of AN and may counteract treatment effort, it may also have a positive side. Namely, HLPA patients who recover and manage to maintain low PA levels may have better treatment outcomes than LLPA patients. It is possible that previous HLPA result in higher fat% levels after refeeding, which may in turn result in higher leptin plasma levels in comparison to the LLPA group. This may have an unexpected positive influence on the psychological status of AN patients as leptin is known to diminish hypothalamic-pituitary-adrenal axis response to stress (Malendowicz et al., 2007). This hypothesis requires, however, further research as well as replication of current findings in a larger sample.

In summary, the data show that AN patients who are characterized by HLPA at the acute phase of the illness are characterized by longer duration of illness prior to admission to specialized treatment. High activity levels in adolescent AN patients do not hamper per se the progress in treatment. However, body composition recovery differs between the recovered LLPA and the recovered HLPA groups. Namely, the increase in fat% is higher for the recovered HLPA than for the recovered LLPA patients, even at one-year follow-up. Furthermore, these findings suggest that HLPA

at initial assessment have long lasting consequences for body composition and consequently for levels of psychopathology at recovery.

High Leptin Levels in Recovered AN Patients Reflect Individual Variation in Body Weight Loss During Illness Progression

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Abstract

Objective: The plasma leptin levels in recovered anorexia nervosa (AN) patients often exceed the normal values of age- and body weigh-matched controls and are higher than predicted on the basis of the amount of the body fat (its source). As these high leptin levels may constitute a risk factor for a relapse, we were interested in examining a potential source of this phenomenon. The semi-starvation of AN patients results in series of neuroendocrine changes, including a strong reduction of plasma leptin levels. As the magnitude of leptin level reduction during starvation may depend on the individual premorbid body weight, we hypothesized that leptin levels in recovered patients depend on the amount of body weight loss during the development of the illness. **Method:** Female AN participants (n=44, mean age=15.18) were included in this longitudinal study. Participants' body fat percentage, plasma leptin levels and normalized BMI were measured at initial assessment and the end of study participation (median 12 months, mean 9.55 months). **Results:** Plasma leptin levels at the end of the study strongly correlated with the amount of body weigh loss between the premorbid state and the acute phase of the illness, in the recovered group (r_s= 0.839, P<0.002), but not in the non-recovered group (r_s= - 0.371, P<0.325). **Conclusion:** The plasma leptin levels in freshly recovered adolescent anorexia nervosa patients reflect the magnitude of body weight loss during the progression of the illness.

Introduction

Anorexia nervosa (AN) is a psychiatric illness with the highest mortality rate among psychiatric disorders (Hoek 2006). Therapies are effective in restoring body weight, however, many patients relapse (Herpertz-Dahlmann et al., 2001) especially during the first year after discharge from the clinic (Carter et al., 2012). Better understanding of biological consequences of starvation and body weight restoration will contribute to the improvement of treatment programs.

Leptin plays a key role in the regulation of energy intake and expenditure. In individuals with a normal body weight, high leptin levels cause a reduction in food intake (Farooqi et al., 2002; Heymsfield et al., 1999; van Dijk 2001). Moreover, they signal high body fat percentage and lead to degradation of fat stores (Farooqi et al., 2002; Heymsfield et al., 1999; van Dijk 2001). Additionally, in individuals with normal body weight, high leptin levels lead to increased energy expenditure through

physical activity and thermogenesis (Hwa et al., 1997; Scarpace et al., 1997; van Dijk 2001; van Elburg et al., 2007). However, the plasma leptin levels as well as the role of leptin change during the course of the AN (Hebebrand et al., 2007; Prentice et al., 2002).

At the acute phase of the illness, plasma levels of this adipocyte hormone are severely reduced in comparison to controls even if corrected for body fat percentage (Haas et al., 2005; Hebebrand et al., 2007; Polito et al., 2000). The reduction of plasma leptin levels evoked by semi-starvation in AN patients results in a series of neuroendocrine changes (Ahima et al., 1996; Eckert et al., 1998; Haas et al., 2005; Hebebrand et al., 2007; Polito et al., 2000) as well as a drop in baseline metabolic rate (Polito et al., 2000). These changes can be seen as an adaptation to the 'chronically low energy intake' and may prevent further weight loss (Ahima et al., 1996; Haas et al., 2005).

The recovery of body weight (and adipose tissue) leads to increase in the plasma leptin to the levels which are higher than predicted on the basis of leptin levels of age and body weight matched controls (Eckert et al., 1998; Hebebrand et al., 1997; Hebebrand et al., 2007; Mantzoros et al., 1997; Wabitsch et al., 2001). This effect may be observed for months after body weight gain (Eckert et al., 1998; Hebebrand et al., 1997; Mantzoros et al., 1997; Wabitsch et al., 2001). Taking under consideration the role of leptin in the regulation of energy intake and expenditure, these high plasma leptin levels are one of possible triggers of a relapse in AN patients (Hebebrand et al., 1997; Holtkamp et al., 2003a; Holtkamp 2004; Mantzoros et al., 1997). Despite the clinical relevance of this phenomenon, the etiology of the observed high plasma leptin levels is not definitely established. It was hypothesized that the speed and extent of body weight recovery may explain the elevation of serum leptin in recovered AN patients (Hebebrand et al., 2007). However, the correlation between leptin levels and body fat% decreases during body weight restoration (Eckert et al., 1998; Hebebrand et al., 1997), suggesting that leptin levels are partially independent of fat% during refeeding (Hebebrand et al., 2007). Thus, the increase in plasma leptin levels after recovery cannot be exclusively explained by the increase in fat% which accompanies an increase in body weight (Eckert et al., 1998).

The severity of starvation determines the extent of neuroendocrine abnormalities observed in AN patients (Eckert et al., 1998) and has an effect on the speed and extent of the physiological and psychological recovery (Hebebrand et al., 2007). The severity of the disorder has previously been indicated by the degree of leptin levels decrease in the acute phase of AN (Hebebrand et al., 2007). Finally, Havel and

colleagues (1996) have shown that the decrease of leptin, due to a body weight drop, is greater in individuals with higher premorbid body weight. Therefore, we hypothesized that plasma leptin levels of recovered AN patients depend on the magnitude of body weight loss during the progression of the illness. We tested this hypothesis in a longitudinal study of adolescent AN patients.

Methods

Patients

Female adolescent patients (n=51) were referred to the treatment center (by e.g., general practitioners or mental health professionals) because of an expected diagnosis of AN. All patients were between 13 and 17.5 year old and were still living with their parents. Six patients who did not fulfill the weight criterion, but whose weight was clearly below expected from their own growth curves were diagnosed as Eating Disorders Not Otherwise Specified and were also included in the study. These patients did not differ from the rest of the study population at the assessment (when considering normalized BMI, leptin, fat%, Morgan and Russell Outcome Assessment Schedule (MROAS), Eating-Disorder Examination (EDE) and amenorhea) nor at the end of study (when considering normalized BMI, leptin, fat% and MROAS).

Study information included a letter sent to the patients' home address followed by an interview with the subject, as well as with the parents and a researcher during the first visit to the treatment center. Upon confirmation of the eating disorder diagnosis and after obtaining informed consent, a diagnostic assessment followed. The final sample consisted of 44 patients for whom we had the data of the MROAS at the end of study and therefore their recovery status could be established for the current analysis. Finally, out of these 44 patients, only 22 provided data on their pre-morbid body weight and only 19 had also data for plasma leptin levels. Therefore, only 19 patients (10 recovered and 9 non-recovered) were included in the correlation analysis of plasma leptin levels with change in normalized BMI due to the illness. All participants provided a written informed consent. In case of minor participants, the written consent was also obtained from the next of kin. All procedures were approved by the Medical Ethics Committee of University Medical Center Utrecht, NL.

Time frame of the study

All patients obtained treatment for the full year or until they recovered or dropped out of the treatment. Additionally, patients took part in a longitudinal study with assessment repeated every second month for a maximum duration of one year. Thus, measurements were taken at initial assessment and then 2, 4, 6, 8, 10 and 12 months afterwards. Eighteen patients dropped out of the study (i.e., patients who resigned between initial assessment and the end of study, terminated treatment against advice, or terminated treatment with the agreement of their therapist after at least partial remission) or recovered sooner than at one year (Figure 8.1). Missing data were imputed according to the last-observation-carried-forward method. According to this method, the last observed data point after initial assessment was used for all subsequent missing data points until the end of the study for a given patient. There was no difference between recovered and non recovered groups in the number of patients who left the study before 12 months (Figure 8.1). As most patients stayed in treatment and participated in the study for 12 months (n=26, recovered group: n=13, non-recovered group n=13), median duration of the study was 12 months (minimum 2 months, maximum 12 months; mean duration: 1) for the whole population= 9.55 months; 2) for recovered group = 9.64; 3) for non-recovered group = 9.45). The last assessment taken for each of the patients individually was considered 'the end of study' for that patient in the current analysis.

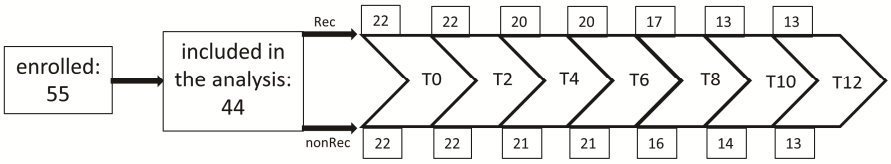

Figure 8.1. Schematic representation of the number of patients enrolled in the study, included in the analysis and remaining at different time points of the study; from intake (T0) to the last month (T12). Rec = recovered, nonRec = non-recovered

Treatment Center

The study took place between January 2006 and May 2009 at the Rintveld Center for Eating Disorders in Zeist (Altrecht Mental Health Institute). All treatments were covered by Dutch health insurance and were without additional costs for patients and their families. Rintveld is a specialist center for eating disorders and one of the two top clinical centers in The Netherlands. This center offers multidisciplinary in- and outpatient treatment for adolescent (<18 years) and adult patients. In addition, if necessary, medical treatment is provided by the nearby Meander Hospital in

Amersfoort. The staff of the adolescent treatment program consists of psychiatrists and other physicians, dietitians, family therapists, psychomotor therapists (Probst et al., 2010), creative therapists, psychologists, and nurses. An integrated approach (aimed at recovery of weight, eating pattern, and body attitude, as well as normalizing family relations and developmental and social skills) was offered in a system-oriented stepped and matched care model. Additional treatments were assigned to patients according to their needs and adjusted during treatment as appropriate (e.g. atypical neuroleptics, SSRI's, additional psychotherapy or psychomotortherapy). Patients attended school at home or at the center whenever possible. Weight gain was targeted at 0.5–1.0 kg/week in accordance with clinical guidelines.

Measurements

Demographic information collected during the first visit included a physical examination, psychiatric interview, and an assessment of eating disorders and comorbid psychopathology. A trained psychologist assessed the presence of AN characteristics based on DSM-IV criteria using the eating-disorder examination. Additionally, Morgan and Russell Outcome Assessment Schedule (MROAS), Child Depression Inventory (CDI), Comprehensive Psychopathological Rating Scale (CPRS-S-A), body weight, body fat percentage (fat%) and serum leptin levels were measured at initial assessment and the end of study.

Recovery definition. For analysis purposes, we used MROAS to divide the patient population into a recovered (MROAS score ≥ 9) and a non-recovered group (MROAS score < 9). The MROAS is often used in AN research and provides a quantitative measure (scores 0–12) of current outcome state divided over five subscales: nutritional state, menstrual function, mental state, psychosexual adjustment, and socio-economic status. The composite MROAS score can also be used to evaluate patient's outcome state in three categories: Good (composite score ≥9), Intermediate (≥4 and ≤8), and Poor (<4) (Lee et al., 2003). In the current sample, 22 patients obtained a MROAS score of 9 or more at the end of study and were classified as recovered. Remaining 22 patients were classified as non-recovered.

Normalized BMI and body weight. We used BMI normalized (sdBMI) on the basis of the standard growth curves for the Dutch population (Growth Analyser 3.5, Dutch Growth Foundation, Rotterdam, NL). The use of the sdBMI values instead of BMI values was motivated by the fact that this study involved adolescent patients (13.5 – 17 years of age) and because we analyzed longitudinal data of each patient (premorbid BMI, at intake and the end of study). The average BMI changes

significantly over puberty and may reach low values (according to standard cut-off points) which are in a healthy range taking under consideration the standard growth curves for the age and gender. Adolescent patients should gain weight appropriate for their age. The amount of body weight they loose during illness is potentiated by the fact that they drift away from the growth curve normal for their age. Therefore, sdBMI is more precise description of the body growth for the adolescents than BMI.

Patients were also asked about their highest body weight prior to illness which was considered the highest premorbid body weight. Patients provided also their height at the same time point and this information was used to calculate highest premorbid sdBMI. Finally, we calculated the change in sdBMI between premorbid state and acute phase of the illness (delta sdBMI = the difference between the self-reported maximum sdBMI and the sdBMI at initial assessment).

Physiological parameters. Body weight and body composition (body fat percentage, fat%) were measured using a TANITA Body Composition Analyzer TBF-300 (Tanita Corporation, Tokyo, Japan). Blood samples for leptin levels analysis were obtained by a trained nurse by venipuncture in the morning between 8 and 10am. Subjects were not asked to be in a fasted state. Blood samples were centrifuged at 1,200×g for 15 min at 10°C and plasma was stored at −80°C prior to determination. Leptin was measured using a sensitive Radio-Immuno-Assay (RIA) (Sensitive Human Leptin RIA Kit, Linco, St. Charles, Missouri, U.S.A.; with this kit sensitivity of 0.05 ng/ml for 100 μl sample), intra-assay CV of 5.63%, inter-assay CV of 5.66%. Samples were analyzed in duplicate. Finally, in order to express changes in leptin levels evoked by the therapy we calculated an additional variable (delta leptin = difference in plasma leptin levels between initial assessment and the end of study).

Data analysis

Independent samples Student's t-test was used to compare recovered and non-recovered patients at initial assessment and the end of study. For comparison of leptin levels at the end of study in recovered and non-recovered patient we used Mann-Whitney U test. Spearman's rho correlation coefficients were used to evaluate the correlations between fat% and plasma leptin levels as well as the correlation between delta sdBMI, delta leptin and leptin at the end of study. Repeated measures ANOVA was used to assess changes in fat%, sdBMI and plasma leptin levels as a consequence of the therapy (between the initial assessment and the end of study). The assumptions of repeated measures ANOVA were met in case of fat% and sdBMI values. In case of leptin data, the values of plasma leptin levels were not normally distribute in the non-recovered group. Therefore, leptin levels at the end of study

were log transformed for this analysis. Effect size for repeated measures ANOVA was calculated using partial Eta^2 ($\eta^2_{partial}$). All analyses were performed with SPSS 20.0 (IBM), and data are presented as mean ± standard error of the mean. A *P*-value of 0.05 was used throughout the paper as a significance threshold.

Table 8.1. Basic characteristics of non-recovered (nonRec) and recovered (Rec) groups. M = mean, SD = standard deviation of the mean, n = count per group, fat% = body fat percentage, MROAS = Morgan and Russell Outcome Assessment Schedule, CDI = Child Depression Inventory, CPRS-S-A = Comprehensive Psychopathological Rating Scale, sdBMI (normalized BMI), EDE = Eating-Disorder Examination, max sdBMI = highest premorbid sdBMI, delta sdBMI – difference between highest premorbid sdBMI and sdBMI at initial assessment. Independent sample Student t-test showed no difference between the groups for any of the variables measured.

	nonRec			**Rec**			**t-test**	
	Mean	SD	n	Mean	SD	n	t(df)	*P*
Age	15.23	1.25	22	15.26	1.18	22	-0.073 (42)	0.942
IQ	101.31	12.65	21	108.75	11.99	20	-1.931 (39)	0.061
Fat%	10.52	5.76	20	12.06	6.44	21	-0.806 (39)	0.060
Leptin	5.82	5.48	18	5.82	4.41	16	0.000 (32)	1.000
MROAS	5.41	1.45	22	6.23	1.81	22	-1.669 (42)	0.103
CDI	21.70	9.11	20	20.77	9.14	22	0.33 (40)	0.744
CPRS-S-A	26.73	11.85	19	21.68	9.85	22	1.50 (39)	0.142
BMI	16.04	1.33	18	15.97	1.66	19	0.143 (35)	0.887
sdBMI	-2.39	1.33	18	-2.36	1.30	19	-0.060 (35)	0.953
EDE	3.69	1.26	22	3.6	1.28	22	0.225 (41)	0.800
- restraint	3.33	1.55	22	3.11	1.74	22	0.424 (41)	0.674
- eating concerns	3.28	1.29	22	2.95	1.34	22	0.817 (41)	0.418
- weight concerns	3.71	1.62	22	3.97	1.56	22	-0.549 (41)	0.586
- shape concerns	4.48	1.28	22	4.36	1.32	22	0.303 (41)	0.763
max sdBMI	-0.14	0.76	10	0.36	0.76	12	-1.581 (21)	0.129
delta sdBMI	-2.58	0.39	10	-2.52	1.02	12	0.037 (21)	0.971

Results

Basic characteristics

Participants (n=44) were females, aged 13 to 17.5 years at the first assessment (mean=15.18, SD=1.21) and sdBMI between -6.6 and -0.6 (mean=-2.54, SD=1.26). We conducted Student's t-test (or Mann-Whitney U test) to compare results of recovered

and non-recovered patients at initial assessment (age, IQ, fat%, leptin, MROAS, CDI, BMI, sdBMI, EDE total and subscales, highest premorbid sdBMI, delta sdBMI) and the end of study (fat%, leptin, CDI, BMI, sdBMI); please note that MROAS score at the end of study was not compared as this was the variable defining the two groups. Recovered and non-recovered patients did not differ significantly at initial assessment (Table 8.1) for any of the parameters measured. At the end of study, the two groups significantly differed for leptin levels, CPRS-S-A and CDI (Table 8.2). We also compared the menstrual status at the initial assessment and the end of study between the two groups. At initial assessment, almost all patients were characterized by amenorrhea (Table 8.3) and therefore the two groups did not differ in menstrual status. At the end of study, two-thirds of the recovered patients and only one of non-recovered patients regained their menstrual cycle (Table 8.3).

Table 8.2. Characteristics of non-recovered (nonRec) and recovered (Rec) groups at the end of the study. Fat% = body fat percentage, MROAS = Morgan and Russell Outcome Assessment Schedule, CDI = Child Depression Inventory, CPRS-S-A = Comprehensive Psychopathological Rating Scale, sdBMI (normalized BMI), delta leptin = difference in plasma leptin levels between initial assessment and the end of study. Independent sample Student t-test showed no difference between the groups for fat%, BMI, sdBMI and delta leptin. Mann-Whitney U test showed significant difference between recovered and non-recovered patients in leptin levels at the end of study.

	nonRec			**Rec**			**t-test**	
	Mean	SD	n	Mean	SD	n	t (df) / U(z)	*P*
Fat%	19.39	6.16	21	22.61	4.01	21	-1.998 (34.73)	0.054
Leptin	9.01	8.93	19	14.26	8.02	20	218.00 (2.95)	0.002*
MROAS	7.37	0.91	22	10.30	0.86	21	-	-
CDI	19.23	8.35	22	12.05	8.67	22	2.79 (42)	0.008*
CPRS-S-A	18.00	11.95	20	10.33	7.61	21	2.435 (32.03)	0.021*
BMI	18.81	2.01	22	19.26	1.43	21	-0.837 (41)	0.407
sdBMI	-0.94	1.18	22	-0.52	0.79	21	-1.382 (36.85)	0.175
delta leptin	3.53	10.88	18	7.20	7.73	16	-1.118 (32)	0.272

*Mann-Whitney U test

Treatment effects on body fat percentage, sdBMI and plasma leptin levels

We tested the effect of treatment on fat% sdBMI and plasma leptin levels in the recovered (n=22) and non-recovered (n=22) groups. It is of note that recovery was defined on the basis of MROAS composite score (including other characteristics than only sdBMI). Repeated measures ANOVA for fat% showed that there was a

Table 8.3. Number of patients with regular menstruation or lack of menstruation a the initial assessment and the end of study. nonRec – non-recovered patients, Rec – recovered patients, anticonc. pill – use of anticonceptive pills.

	Admission		End of study	
	nonRec	Rec	nonRec	Rec
regular menses	0	1	1	12
amenorrhea	15	16	15	6
premenarche	3	1	2	0
anticonc. pill	0	1	0	1

Table 8.4. Spearman's rho correlation coefficients for correlations between body fat percentage (fat%) and leptin levels in recovered and non-recovered patients. Significant results are in bold; * *P*<.05. Number in brackets indicate the sample size in the given correlation analysis. InAs = initial assessment, EoS = the end of study.

		Fat%		Leptin	
		InAs	EoS	InAs	EoS
Recovered					
Fat%	InAs		.075 (19)	**.593*** (15)	-.162 (17)
	EoS			.181 (13)	**.495*** (17)
Leptin	InAs				.363 (13)
	EoS				
Non-recovered					
Fat%	InAs		**.541*** (16)	.384 (14)	.172 (15)
	EoS			.472 (15)	.284 (16)
Leptin	InAs				.264 (15)
	EoS				

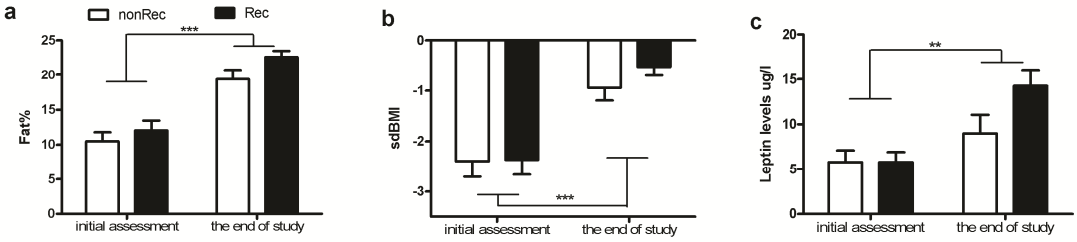

Figure 8.2. Improvement of fat% (panel A), sdBMI (panel B) and plasma leptin levels (panel C) in recovered (Rec) and non-recovered (nonRec) patients as a consequence of the treatment. Repeated measures ANOVA showed significant effect of time; marked as follows: ***P*<0.01, ****P*<0.001. fat% = body fat percentage, sdBMI = normalized BMI

significant increase in the fat% in both, recovered and non-recovered, groups between the initial assessment and the end of study (Figure 8.2a). The analysis showed a significant effect of time and non-significant effects of the recovery group classification and interaction on fat% (time: $F(1, 38)=82.233$, $P<0.001$, $\eta^2_{partial}=0.684$; recovery: $F(1, 38)=2.835$, $P<0.10$, $\eta^2_{partial}=0.069$; interaction: $F(1, 38)=0.895$, $P<0.350$, $\eta^2_{partial}=0.023$). Repeated measures ANOVA for sdBMI showed that there was a significant increase in sdBMI in both (recovered and non-recovered) groups between initial assessment and the end of study (Figure 8.2b). The analysis showed a significant effect of time and non-significant effects of the recovery group classification and interaction on sdBMI (time: $F(1, 35)=45.980$, $P<0.001$, $\eta^2_{partial}=0.568$; recovery: $F(1, 35)=0.976$, $P<0.332$, $\eta^2_{partial}=0.027$; interaction: $F(1, 35)=1.224$, $P<0.276$, $\eta^2_{partial}=0.034$). Finally, repeated measures ANOVA for plasma leptin level changes during the treatment showed that there was a significant increase in the plasma leptin levels in both, recovered and non-recovered, groups between the initial assessment and the end of study (Figure 8.2c). The analysis showed a significant effect of time and interaction and non-significant effects of the recovery group classification on plasma leptin levels (time: $F(1, 26)=9.814$, $P<0.004$, $\eta^2_{partial}=0.274$; recovery: $F(1, 26)=1.818$, $P<0.189$, $\eta^2_{partial}=0.065$; interaction: $F(1, 26)=5.595$, $P<0.026$, $\eta^2_{partial}=0.177$).

Obtained results showed that both, recovered and non-recovered, groups regained some body mass and fat tissue as a consequence of clinical intervention. However, only the recovered patients were characterized by an increase of leptin plasma level.

Leptin and fat percentage correlations

We tested whether the high plasma leptin levels observed in the current sample in the recovered patients can be explained by the restored fat%, and if there are differences between recovered and non-recovered patients for this correlation. In the recovered group, plasma leptin levels correlated with fat% at initial assessment and the end of study (Table 8.4). In the non-recovered group leptin did not correlate with the fat% at any data point (Table 8.4).

Leptin correlates with sdBMI changes during progression of illness

Finally, we tested whether high levels of leptin at recovery are related to changes in the sdBMI caused by the progression of the illness. Thus, we investigated whether leptin levels at the end of study depend on the amount of body weight (BW) loss between the pre-morbid period and the acute phase of the illness. It is of note that only 19 patients had data on both, leptin at the end of study and premorbid body

weight. Therefore, only these patients were included in this particular analysis. None of the patients included for this analysis was diagnosed with EDNOS.

Spearman correlation analysis was conducted in recovered (n=10) and non-recovered (n=9) patients for leptin levels at the end of study and delta sdBMI between the highest sdBMI and lowest sdBMI (Figure 8.3). We detected a strong significant correlation, in the predicted direction, for the recovered group (r_s= 0.839, *P*<0.002), namely, the more profound the body weight loss, the higher the leptin levels at recovery. No correlation was found for the non-recovered group (r_s= - 0.371, *P*<0.325). This was observed despite the fact that there was no difference between recovered and non-recovered group for delta sdBMI (difference between the highest sdBMI and lowest sdBMI) (Table 8.1).

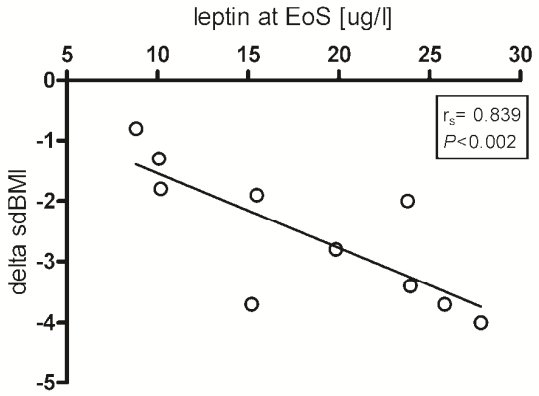

Figure 8.3. Spearman's correlation (r_s=Spearman's rho) of the plasma leptin levels at the end of study (EoS) with the delta sdBMI defined as difference between the premorbid maximum sdBMI and minimum sdBMI during the illness in recovered patients. sdBMI = normalized BMI

The above mentioned correlation is confirmed when delta leptin (between initial assessment and the end of study) is used in the analysis instead of leptin levels at the end of the study. Namely, there is a strong significant correlation for the recovered group (rs= 0.857, *P*<0.014) and no correlation in the non-recovered group (rs= - 0.385, *P*<0.306).

Leptin does not correlate with sdBMI changes during recovery

Another explanation for plasma leptin levels seems more intuitive. Namely, that high levels of leptin at recovery are correlated to increased sdBMI caused by the body weight recovery (Hebebrand et al., 1997). We tested the hypothesis in our sample and

according to our definition of recovery. The analysis revealed that there was no correlation between leptin levels at the end of study with change in sdBMI between initial assessment and the end of study (non-recovered group: r_s =0.003, *P*<0.992; recovered group: r_s =0.358, *P*<0.159).

Discussion

In the current sample, leptin levels in recovered adolescent AN patients were strongly correlated with the amount of body weight loss due to the progression of the illness and not with the amount of body weight gain during the recovery process. This correlation was not observed for the non-recovered patients. Furthermore, only in recovered patients leptin levels correlated with body fat percentage at initial assessment and the end of the study. Recovered and non-recovered patients differed at the end of study for some indicators of psychopathology. Finally, leptin levels were higher in recovered than non-recovered patients at the end of study although both groups did not differ significantly at the end of study for the sdBMI and fat%. These group differences cannot be accounted for by initial differences as recovered and non-recovered patients did not differ at the initial assessment for any parameter tested. Furthermore, the differences were observed despite the fact that patients from both, recovered and non-recovered, groups lost comparable amount of body weight due to illness.

It may be surprising that we observed differences in plasma leptin levels at the end of the study despite the lack of clear differences in body composition and body weight restoration between the recovered and non-recovered patients. However, recovered and non-recovered patients differed in four important aspects. First, the majority of recovered patients regained their menstrual cycle at the end of study while this happened only in case of one non-recovered patient. Second, plasma leptin levels in non-recovered patients did not increase during the treatment. Third, leptin levels correlated with body fat% at initial assessment and the end of study only in recovered patients. Four, recovered patients had lower scores on the Child Depression Inventory as well as Comprehensive Psychopathological Rating Scale. These physiological and psychological differences between recovered and non-recovered patients may underlie the difference in the MROAS score obtained at the end of treatment. They may also explain why in non-recovered patients there was no correlation of leptin levels at the end of treatment with body weight loss during progression of illness (delta sdBMI). Although we cannot speculate on the

mechanism underlying these observations, our data suggest that recovered and non-recovered patients differ significantly in their leptin physiology before and after treatment.

Current analysis suggests, in agreement with previous observations (Eckert et al., 1998) that plasma leptin levels are not fully explained by an increase in sdBMI and body fat percentage due to recovery. We found no correlation between leptin levels at the end of study with change in sdBMI between initial assessment and the end of study. This seems to stay in contradiction with a previous report which showed a strong correlation between leptin in weight recovered patients, and the increase in BMI (Hebebrand et al., 1997). The discrepancy may be accounted for in three ways. Firstly, in the cited study, patients regained body weight in approximately three months from intake while in the current study median time in treatment was 12 months. Secondly, in the mentioned study, delta BMI correlated with the highest measurement of leptin. In the current study, we did not use the maximum leptin levels but leptin levels at the time point when patients are considered recovered on the basis of MROAS composite score. Thirdly, in the current study the premorbid body weight was assessed through patients' reports. As this self-reported measure may be not accurate, one should keep in mind that this constitutes a limitation of the current study.

As mentioned previously, leptin levels at recovery cannot be fully explained by the amount of fat tissue restoration (Eckert et al., 1998; Hebebrand et al., 1997; Hebebrand et al., 2007). It was suggested that leptin's role changes during starvation (Prentice et al., 2002) and its most important function is to regulate the gonadal, adrenal and thyroid axes (Ahima et al., 1996) to prevent further weight loss (Haas et al., 2005). In order to fulfill this function, leptin levels may uncouple from body fat percentage during long term hypocaloric diet (Haas et al., 2005; Hebebrand et al., 2007; Polito et al., 2000; Scholz et al., 1996) as well as during body weight restoration (Eckert et al., 1998; Hebebrand et al., 1997; Hebebrand et al., 2007; Mantzoros et al., 1997; Wabitsch et al., 2001). In these states of altered energy intake, leptin may be regulated, without change in body fat mass, by various other factors such as pro-inflammatory cytokines (Grunfeld et al., 1996; Sarraf et al., 1997), corticosteroids (Berneis et al., 1996) and growth hormone (Berneis et al., 1996). Levels of all these factors are severely altered in AN patients before treatment (Argente et al., 1997; Boyar et al., 1977; Pomeroy et al., 1994).

Nevertheless, the correlation of leptin at recovery with body weight loss due to the progression of AN, which was observed in the current study, may partly depend on

the dynamics of body fat tissue restoration. It has previously been shown that during refeeding, patients foremost regain their abdominal fat and not extremity fat (Grinspoon et al., 2001; Scalfi et al., 2002) and that there is more profound increase in subcutaneous abdominal fat than of visceral abdominal fat (Zamboni et al., 1997). As more leptin is secreted by subcutaneous fat than by visceral fat (Minocci et al., 2000; Van Harmelen et al., 1998) body weight restoration may have different effects on leptin levels depending on the amount and placement of body fat tissue restored. In the current sample, there was no difference between recovered and non-recovered patients in terms of the fat% at the end of study. However, the TANITA instrument used in this study measures fat tissue in the lower limbs. Thus, the use of this methodology might have underestimated the visceral fat% which might have been restored differently between recovered and non-recovered patients.

Additionally, an indirect argument supports the hypothesis that the fat tissue restoration differs between recovered and non-recovered patients. The correlations of leptin levels with fat% have been reported multiple times in individuals with AN (Grinspoon et al., 1996; Haas et al., 2005; Hebebrand et al., 1997; Mathiak et al., 1999) (see also (Hebebrand et al., 2007) for an extensive review). Interestingly, in the current sample, leptin levels correlated with the fat% at initial assessment and the end of study only for the recovered patients. In contrast, in non-recovered patients, there was no correlation of leptin with fat% at any time point. This was observed, despite the fact that sdBMI and fat% increased in both groups as an effect of the treatment. There are two possible explanations for this observation. First, this may suggest that for patients who did not recover, plasma leptin levels were uncoupled from the body fat%. Second, limitations of fat% measurements using TANITA instrument (as mentioned earlier) may partly explain the differences in correlations between recovered and non-recovered patients supposing that there are differences in the amount of visceral fat tissue regained between the two groups.

To conclude, plasma leptin levels of patients who recently recovered from anorexia nervosa partly reflect the increased body fat percentage and strongly depend on the amount of body weight loss due to illness. While we are aware that this is a pilot study that should be replicated by additional studies, recovered patients (according to MROAS score) showed a remarkable strong correlation between these parameters (r_s= 0.839, P<0.002). Namely, the higher the amount of body weight loss during the illness, the higher the observed leptin levels at recovery. This relation can be seen if one takes under consideration the BMI changes that are expressed in relation to the growth curves typical for a given age and gender (sdBMI). This is the case, because in

adolescent patients, occurrence of AN and body weight loss intersects with the body weight and height growth which should be normally observed at a given age. Based on previous findings which showed that high leptin levels at recovery may be a risk factor for a relapse, we speculate that patients who loose relatively the highest levels of body weight due to illness, may be at higher risk for relapse once they are considered recovered.

Perspective Paper: The Use of Mouse Models to Unravel Genetic Architecture of Physical Activity

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9

Abstract:

The discovery of genetic variants that underlie a complex phenotype is challenging. One possible approach to facilitate this endeavor is to identify quantitative trait loci (QTL) that contribute to the phenotype and consequently unravel the candidate genes within these loci. Each proposed candidate locus contains multiple genes and, therefore, further analysis is required to choose plausible candidate genes. One of such methods is to use comparative genomics in order to narrow down the QTL to a region containing only a few genes. We illustrate this strategy by applying it to genetic findings regarding physical activity (PA) in mice and human. Here, based on a review of studies which point to candidate genetic regions for PA in humans (genetic association and linkage studies) or use mouse models of PA (QTL studies), we identify candidate genetic regions that overlap between species. Based on a large variety of studies in mice and human, statistical analysis reveals that the number of overlapping regions is not higher than expected on a chance level. We conclude that the discovery of new candidate genes for complex phenotypes, such as PA levels, is hampered by various factors, including genetic background differences, phenotype definition, and a wide variety of methodological differences between studies.

Introduction

The genetic basis of complex phenotypes is difficult to unravel due to the high number of genetic and environmental factors that shape these phenotypes across different developmental stages. Multiple genetic loci influence complex phenotypes. The influence of each one of these loci is further modulated by genetic background, gender, age and environment. Furthermore, most of these genetic loci (called Quantitative Trait Locus or QTL) have small and pleiotropic effects on multiple (complex) phenotypes (Flint 2003; Flint and Mott 2008; Garland, Jr. et al., 2011b). One may think of genetic loci contributing to the occurrence of a complex phenotype more as 'modifiers' than causal factors, as each of them has only a small effect size (Botstein and Risch 2003). Animal models are often used to facilitate the process of discovering novel QTLs and candidate genes, because they enable the study of complex phenotypes in a controlled environmental and genetic background conditions (Schughart et al., 2012). Multiple QTLs are discovered in this way, all pointing with some confidence interval to possible candidate genetic regions which contain one or more candidate genes for the phenotype. However, choosing a probable candidate gene remains difficult (DiPetrillo et al., 2005). To narrow down a

candidate gene region to the level of a few possible candidate genes one may use various methods, including comparative genomics. This method makes use of interspecies genetic homology between QTLs coming from various species (DiPetrillo et al., 2005). We evaluate this method of discovering possible candidate genetic regions by using an example of studies regarding the genetic basis of physical activity (PA) in humans and mice.

With the increasing concerns over effect of low PA on health of individuals (Casazza et al., 2013; Haskell et al., 2007; Manson et al., 2004; Mokdad et al., 2004; Myers et al., 2002), and observations that many people in Western societies do not engage in sufficient amount of exercise (Kelly and Pomp 2013; Marcus and Forsyth 1999) numerous studies addressed the question of the genetic basis of PA. Hopefully, better prevention programs may be created if we increase the knowledge of factors contributing to the appropriate amount of PA (Rowland 1998). Undoubtedly, an array of psychological, cultural and other environmental factors has an influence on the levels of PA. However, there are also premises which suggest that habitual PA is strongly regulated by evolutionary conserved genetic factors (Rowland 1998). One of the possible ways to unravel the biological pathways involved in the regulation of PA is to investigate the genetic basis of this heritable phenotype (Bray et al., 2009; de Vilhena e Santos DM et al., 2012; Kelly and Pomp 2013; Stubbe et al., 2006).

As with other complex phenotypes, the expression of PA levels is under the influence of multiple genetic loci, modified by genetic background, gender, age and environmental factors (for details please see Thesis Introduction). Taking under consideration this complex network of factors influencing PA, one may wonder whether it is possible to use animal models to unravel the genetic basis of this complex trait in humans (Garland, Jr. et al., 2011b). In the Thesis Introduction, we discuss which animal models may be applied in an attempt to model complex genetic architecture of human PA. In this perspective paper, we consider the possibility of using already available results of mouse and human studies to point to plausible candidate genes for this complex phenotype. In order to address this question, we use a review of studies which pointed to candidate gene regions for PA in mice (QTL studies) and humans (genetic association and linkage studies) (please see the Thesis Introduction). We assess the degree of overlap between candidate genetic regions. Finally, we assess whether the potential overlap is greater than expected on the basis of random sampling from a limited pool of genetic regions. In case of statistically reliable overlap, it would be possible to narrow down the candidate gene regions and possibly point to candidate genes influencing PA levels.

Methods

Literature search

We conducted a literature search in order to find all human linkage and gene association studies of candidate genes or genetic regions that may be involved in the regulation of intensity or duration of PA and physical inactivity in humans (Tables 1.1 and 1.2 in the Thesis Introduction). We included studies which addressed the genetic basis of voluntary exercise (VE) as well as of spontaneous physical activity (SPA). Please refer to the Thesis Introduction section ('Operationalization of physical activity in humans') for the exact definitions of VE and SPA.

In order to include all mouse QTL studies that identified candidate genetic regions associated with PA, we conducted a literature search with a combination of terms: QTL, mouse, mice, locomotor activity, home cage activity, and running wheel activity (RWA). We also followed references from identified articles. We included all papers that ever reported a significant QTL for any of the phenotypes related to PA in mice. In total, we included 6 mouse studies identifying 16 QTLs for the locomotor activity in the home cage (HC) (Table 1.3 in the Introduction) and 7 mouse studies identifying 31 QLTs for the RWA (Table 1.4 in the Thesis Introduction).

Syntenic genetic regions

As we were interested in the possible refinement of genetic candidate regions for PA, we aimed at unraveling the amount of overlap between regions reported up to now from mouse and human studies. Therefore, we translated the obtained mouse QTL regions to syntenic human regions (using UCSC Gene Browser; Human Feb. 2009 dataset; GRCh37/hg19; http://genome.ucsc.edu/cgi-bin/hgGateway). The translation was conducted for 7 QLTs for HC, and 24 QTLs for RWA, in the following manner:

1) for mouse QTL regions with defined borders we translated the whole QTL into syntenic region in humans;
2) for QTLs for which only a peak marker was given (either Mit marker, SNP, locus name or JaxLab marker), we used the MGI database (http://www.informatics.jax.org/) or NCBI map viewer (http://www.ncbi.nlm.nih.gov/projects/mapview/) to check the exact location of this marker:
    a) if the marker was a gene or was placed within a gene, we translated the location of that particular gene;
    b) in other cases, we checked whether there was a gene not further than 50kbp away from the marker location. This region between

the marker and the gene (including the gene) was used for the translation.

Simplified annotation of candidate genetic regions

We expressed the location of obtained hypothetical human genetic regions as a chromosomal regions defined on the basis of chromosomal bands (e.g. 20q11). This was done in order to define a finite number of possible genetic regions that could have been pointed by association or linkage studies. Based on the human chromosome banding this number was set to 850 possible genetic regions (Kosyakova et al., 2009). The exact explanation of the chromosomal binding can be found below. Finally, the significance of the number of overlapping regions between the mouse QTL and human gene association studies was estimated by conducting a Monte Carlo simulation (MATLAB).

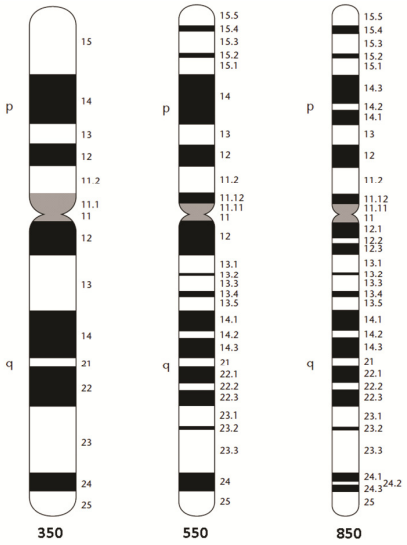

Figure 9.1. Ideogram of G-banding of the human chromosome 11 for 350, 550 and 850 resolution. White and black lines indicate light and dark bands obtained during G-staining; p – the short arm of the chromosome, q – the long arm of the chromosome; numbers on the right of the chromosome ideograms indicate the band number for each of the different banding resolutions. This figure is adapted from (Bickmore 2001).

Karyotypes – chromosomal banding

The karyotype is a number and appearance of chromosomes that is typical for eukaryotic cell of a given species. It may be examined by conducting a staining, for example G-staining (Giemsa staining) (Kosyakova et al., 2009). During the procedure,

a suitable dye is applied to cells which are arrested in mitosis by a solution of Colcemid (Bickmore 2001). The method is used for identification of chromosomes, for discovering chromosome number aberrations and for confirmation of evolutionary relationships between species (Bickmore 2001). The exact number of bands will depend on the base content, the stage of mitosis, and the chromatin organization (Bickmore 2001). The bands of the chromosome are numbered separately for the short (p) and the long (q) arm of the chromosome, consecutively, starting from the centromere. Examples of band resolution for human chromosome 11 are shown on Figure 9.1.

Results

Overlap of genetic findings between species

Two distinct genetic regions were identified independently by human and mouse studies. Firstly, a QTL on chromosome 7 associated with an average distance run and time spent on RWA maps to 3 different syntenic regions in humans: 11p15.4-p15.1, 16p12.3-12.1 and 11q13.4 (Kelly et al., 2010). The first of these QTL encloses a previously reported locus on 11p15.2, which in a study by Simonen and colleagues was associated with a total time spent on PA when assessed by a questionnaire (Simonen et al., 2003b). Secondly, Umemori and colleagues discovered a broad QTL (13Mbp) on mouse chromosome 2 associated, with activity in a HC which explained 8.9% of the phenotypic variance (Umemori et al., 2009). This region encloses a syntenic human region (20q12) associated with physical inactivity when measured with a questionnaire (Simonen et al., 2003b).

Evaluating the significance of the interspecies overlap

The number of regions overlapping between species is low despite the high total number of candidate regions pointed in both species. This is most probably caused by the methodological discrepancies and genetic background influence, as mentioned before in this paper. There are various possible conclusions that may result from this observation. It may be hypothesized that the regions which overlap are very significant QTLs with a robust effect on PA levels (thus occurring multiple times despite the potential discrepancies). In this case, the overlapping regions could be used in the comparative genomics approach. It is, however, also possible that the observed overlap is solely caused by sampling multiple times from a limited pool of possible genetic regions. We wanted to test this hypothesis and assessed the probability of obtaining this QTL overlap by chance. In order to do so, we conducted

a simulation which aim was to assess the expected overlap levels in case of a given number of sampling events (experiments).

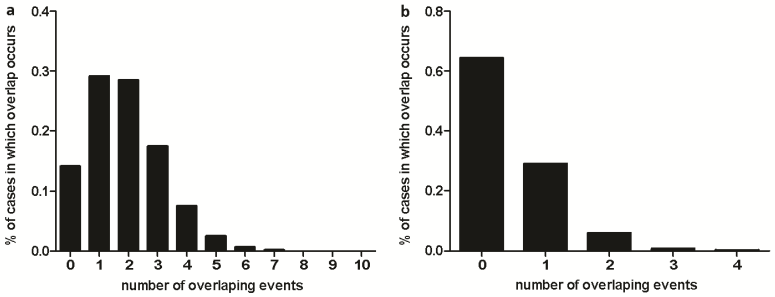

Figure 9.2. Results of the Monte Carlo simulation regarding chances of obtaining an X number of overlapping genetic regions when sampling two times from a limited pool of genetic regions (where the sampling is a model of conducting human or mouse studies). The y axes depicts the percentage of times (if the sampling is repeated 1000000 times) when one would obtain X times an overlap due to chance. (a) In this simulation the number of chromosomal regions identified in human and mouse studies is taken under consideration. On the basis of literature review we discovered one region which was indicated by both, mouse and human studies. The graph shows that this event is expected in 29.16% of the samplings. Therefore, obtained overlap does not exceed the chance level. (b) In this situation, the number of QTLs identified in human and mouse studies is taken under consideration. On the basis of literature review we discovered overlap between one QTL from human studies and one QTL from mouse studies. The graph shows that this event is expected in 29.03% of the samplings. Therefore, obtained overlap does not exceed the chance level.

In order to proceed with the statistical analysis of the interspecies overlap, the following choices were made. First, as most human genetic studies considered VE and not SPE (only one study), we decided to conduct the simulation only for the human VE studies in combination with RWA mouse studies. Second, as PA and physical inactivity in humans may have different genetic basis, we included only findings regarding PA in humans. Third, in order to make the analysis possible, we approximated a finite number of possible candidate regions (850 chromosomal regions; see Methods section).

As a result, we chose the following number of studies into the final analysis. Eight studies on PA in humans pointed out to 15 candidate regions which mapped to 28 different chromosomal regions for VE. Furthermore, studies using RWA pointed to 25 QTLs, out of which all could be translated into 64 human chromosomal regions. When these results are taken under consideration, only one region overlaps between mouse and human studies of VE.

A Monte Carlo simulation was conducted to model the following situation. A pool of 850 genetic regions was used twice in a sampling with replacement; in the first experiment 28 regions, and in the second experiment 64 regions were picked randomly. This operation was simulated one million times. We asked the question, how likely would it be to find the same region in experiments 1 and 2. The Monte Carlo simulation (Figure 9.2a) showed that one could obtain a double overlap in 29.16% of cases due to a random sampling. The result suggests that the overlap obtained while using the real data of mouse and human candidate regions is not higher than predicted on a chance level.

There are limitations of this simulation that should be addressed. These limitations result from several assumptions and conditions necessary to conduct this thought experiment. The most significant initial step was the translation of all the regions into chromosomal regions. It was conducted in order to obtain a finite number of all possible regions that may be identified as candidate regions. The simulation would not be possible when using simply numbers of discovered QTLs. It is caused by the fact that it is not possible to establish a final number of QTLs that can be obtained. As a consequence, two properties of this simulation emerged which may work as a disadvantage for the comparative genomics approach. First, we neglected the fact that QTLs have different sizes and, therefore, harbor different amount of genes. However, the size of the majority of the QTLs could not have been established due to a lack of confidence intervals reported. As a consequence, we have, in fact, increased the chance of a theoretical overlap by including also the QTLs for which only the peak value was known. Secondly, we neglected the fact that some of the bands are not independent as they are included by one QTL. A way to circumvent this in the simulation method would be to consider each QTL as pointing to only one candidate region. To test this we conducted additional simulation in which a pool of 850 genetic regions was used twice in a sampling with replacement; in the first experiment 15 regions, and in the second experiment, 25 regions were picked randomly. This operation was simulated one million times. We asked the question: how likely would it be to find the same region in experiments 1 and 2? The Monte Carlo simulation showed that one could obtain a double overlap in 29.03% of cases due to a random sampling (Figure 9.2b). Thus, even in this case the obtained overlap could still be very likely obtained by chance.

It is of note that the inclusion of all 850 bands as the final number of all possible candidate regions worked in advantage of the comparative genomics approach in this thought experiment. The number of all possible candidate regions should be

lower than 850 because, otherwise, it would suggest that each of the possible regions harbors a candidate gene. In this case, the further studies aiming at finding regions contributing to a complex phenotype, would be pointless. The use of this high number of possible candidate regions increased the chance of considering the existing overlap to be significant. Therefore, despite the fact that this simulation has limitations, one may conclude that caution should be taken when interpreting the meaning of any single overlap of the genetic regions for VE activity between human and mouse studies when the comparison is made on the basis of currently available findings. The current analysis shows that it is not feasible to distinguish a significant overlaps from random ones when using data coming from studies with methodological discrepancies.

Finally, it is of note that inclusion of the findings described in Chapter 2 of the thesis (entitled 'A Candidate Syntenic Genetic Locus is Associated with Physical Activity Levels in Mice and Humans.') does not change the conclusion of the Monte Carlo analysis. Furthermore, even including the findings of Chapter 2 by increasing the number of overlapping regions to 2 does not result in a significant overlap (according to the Monte Carlo simulation). In case of the first analysis (Figure 9.2a) one could obtain a double overlap in 28.5% of cases due to a random sampling. In case of the second analysis (Figure 9.2b), this would be the case in 6% of random sampling experiments.

Discussion

Data gathered in this chapter, as well as in the Thesis Introduction, point to the difficulties that are encountered when comparing QTLs for a complex phenotype, such as PA levels. There are various sources of these challenges in the field of PA genetics. First, various definitions and methodologies may be used to assess the phenotype of interest in one species. Although these methods are considered to measure one phenotype, divergent phenotypes may be in fact measured. Consequently, genetic regions identified by these studies may regulate various phenotypes instead of one phenotype. Second, accurate translation of complex phenotypes between species may be difficult. As a consequence, results obtained using an animal model of a complex phenotype may point to the candidate genetic regions for another phenotype than initially intended. Thirdly, genetic background has a complex modulating influence on the contribution of any genetic region to the occurrence or the level of expression of a complex phenotype. Therefore, comparison

between various human populations or various mouse strains may be difficult. As a consequence, only some of the QTLs identified in different species may be comparable. Finally, other modifying factors such as sex and age of the study subjects may have a profound influence on the expression of the phenotype and on its genetic architecture.

Taking under consideration the above mentioned facts, the low level of between species overlap of genetic regions associated with PA is not surprising. However, this observation has consequences for the interpretation of the overlap when it occurs. Care needs to be taken when interpreting functional significance of an overlap between regions coming from different experiments because this overlap may be based on a chance level. Therefore, it may be difficult to use the comparative genomic strategy to narrow down candidate genetic regions for PA on the basis of currently available findings.

It is probable that the increasing number of mouse and human studies would lead to an increased number of observed overlap; however, an increase in the number of conducted studies results also in an increased number of regions which would overlap by chance. Therefore, it may be necessary to evaluate the exact setup of HC and RWA experiments more strictly, which would enable their use as models of the SPA and the VE, respectively. The consideration should regard the duration of HC or RWA experiment and the time after which the behavioral outcome is considered stable, representative and not influenced by novelty. One could question whether HC and RWA are adequate models of SPA and VE and whether we should develop more adequate models of these complex phenotypes. We hoped that this question could be solved on the basis of genetic overlap between human and mouse studies. However, due to the methodological limitations, this kind of comparison is not informative when using the currently available data. Therefore, we propose to consider more focus on phenotype definitions and on the phenotype assessment methodology within and between species in order to optimize translational research for complex traits.

Acknowledgements

We would like to thank Wouter Koning for conducting the simulation in MATLAB.

General Discussion

10

Overview and discussion of the main findings

1.1. On the biological basis of physical activity

Unraveling the biological basis of PA is a tremendous task which is beyond the scope of one PhD thesis. In the current set of research, we attempted to add an additional "brick" into the cathedral of knowledge regarding the biological basis of PA. In order to accomplish this task, we looked for possible novel genetic factors that regulate physical activity levels. We used behavioral genetics methodology combined with a translational genetics approach in order to propose genetic candidate regions as well as candidate genes for this complex phenotype in humans (Chapter 2 and 3) and mice (Chapters 2, 3, and 4).

QTL studies and translational genetics approach

First, we applied a QTLs analysis on a panel of consomic mouse lines (CSS panel) in order to point to candidate genetic regions contributing to a phenotypic variance in the running wheel activity (RWA) test – an animal model of voluntary physical activity in humans. We could indeed show that QTLs on a mouse chromosome 2 (Chr2; **Chapter 2**) and on chromosome 19 (Chr19; **Chapter 3**) contain genetic variants that contribute to RWA levels. The two regions together explained 42.4% of the observed phenotypic variance. Encouraged by these significant results, we asked whether the syntenic human genetic regions could be treated as candidate genetic regions for the voluntary physical activity levels in humans. We tested this hypothesis by conducting a gene association analysis on these narrow candidate regions from the mouse study. We used human data from two large population-based studies (TwinsUK and ALSPAC). We could detect a significant association of voluntary PA with a genetic variance in human region syntenic to mouse region on Chr2. However, no association was detected for the region syntenic to the mouse candidate region on Chr19.

The abovementioned analysis requires an assumption that the operationalization of voluntary PA in mice is an adequate model of voluntary PA in humans. This assumption may be disputed based on difficulties arising from a comparison of complex phenotypes between species. Furthermore, subjective methods of PA assessment were mostly used in the two population-based studies that were applied in this comparative genomic approach. It is known that objective methods for PA assessment are more reliable for this research aim. For the detailed discussion of these methodological limitations, see Part 3 of the General Discussion. Taking these

limitations under consideration, we concluded that a single significant association (of a genetic region syntenic between species) found in a human sample should not be considered reliable. Therefore, in Chapter 2, we decided to replicate the detected significant association (TwinsUK) in an independent population (ALSPAC).

The methodological limitations of the translational approach may account for the fact that no significant association was found in the human sample in Chapter 3 (in genetic region syntenic to mouse QTL on Chr19). However, successful detection of a significant association in two independent human samples (described in Chapter 2) might be considered an argument for the applied translational approach. The discrepancy in the success of the applied approach in Chapters 2 and 3 may pose questions on the expected success rate of this methodological approach. I would like to discuss this problem in the following paragraph.

PA is a complex phenotype with hundreds of genetic loci potentially influencing its levels. The strength of the contribution of each of these loci is modulated by various factors such as age, gender, and also genetic variance in other genetic regions (so-called genetic background). The use of a mouse model of PA enabled us to detect significant QTLs under genetically and environmentally controlled conditions. These narrow genetic regions could be tested in a genetically heterogeneous human population in which no environmental control could be applied. The approach enabled a detection of a human genetic candidate region (on human chromosome 20, HSA20) with a small contribution to the observed complex human phenotype. The advantage of this strategy was that the region on HSA20 would remain undetected if a genome-wide association study was applied in the TwinsUK or ALSPAC sample. This reasoning implies that a detection of the association between phenotype and a (syntenic) genetic region in two different species may be considered an argument for the significance of this association (especially if replicated in an independent sample). This significance is not informing us about the effect size of the particular genetic variant on the complex phenotype tested. However, when no association is detected for a syntenic human locus, this may be accounted for by the small contribution of this locus to the expression of the phenotype. This small contribution may be evoked by species and population specific effects of the genetic background. All in all, we cannot predict the expected success rate of the translational approach used in Chapters 2 and 3. However, we speculate that this success rate may be low due to the various factors modifying the genetic architecture of the complex phenotypes as well as due to the challenges embedded in the modeling of complex

human phenotypes in animals and the accuracy of the assessment methods used to objectively measure PA in humans.

Even if the mouse candidate genetic regions for RWA found in Chapters 2 and 3 are not useful to unravel the genetic basis of PA in humans, they still remain interesting and significant loci associated with a complex mouse phenotype. At the current stage of the analysis, we are not able to indicate the causal genes that could influence the RWA levels in the CSS panel. Few candidate genes may be, however, mentioned. First, as far as the region on mouse Chr2 is concerned, two genes are likely candidates for the regulation of RWA in CSS panel: 1) melanocortin 3 receptor (*Mc3r*) (*Mc3r* deficient mice express reduced voluntary running wheel activity levels (Butler et al., 2000)); and, 2) cytochrome P450, family 24, subfamily A, polypeptide 1 (*Cyp24a1*), as it is responsible for the degradation of vitamin D, which in turn is essential for proper muscle functioning. Second, the most interesting candidate gene from the genetic region on mouse Chr19 is myoferlin (*Myof*), which mediates myoblast fusion during muscle development.

Candidate gene approach

Nfatc2

In **Chapter 4** we examined the specificity of an interesting candidate gene, namely the nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2 (*Nfatc2*) gene. In order to do so, we phenotyped genetically modified mice with a targeted knock-out (KO) mutation in the *Nfatc2* gene. The mutation created by Xanthoudakis and colleagues (1996) results in a deletion of the Nfatc2 protein fragment, which is essential for the binding of this protein to the DNA. Consequently, Nfatc2 cannot fulfill its function as a transcription factor. Furthermore, the mutated protein is either not produced or degraded as it cannot be detected with an immunobloting. Our results showed that these particular *Nfatc2* deficient mice are characterized by a specific reduction in the RWA levels. These effects could not be accounted for by changes in other elements of the energy balance regulation because body weight, food intake, and adiposity did not differ between the knockout, heterozygous, and wild-type mice. Furthermore, we could show that the decrease in RWA was not mediated by the changes in muscle strength, sensory-motor coordination, or the alterations in natural reward sensitivity.

A distinctive *Nfatc2* KO line created by Horsley and colleagues (2001) showed a slightly reduced number of type 1 (slow-twitch) muscle fibers. These muscle fibers are essential for prolonged physical activity and are highly resistant to fatigue.

Therefore, we speculate that genetic deletion of the *Nfatc2* gene could result in decreased exercise capacity and consequently result in reduced total time spent running in a running wheel. This suggestion is further supported by previous observations of Courtney and Massett (2012), who demonstrated that 32.6% of exercise capacity variance in inbred mice can be explained by one single nucleotide polymorphism in an intron of *Nfatc2* gene.

The influence of *Nfatc2* gene deletion on muscle fiber proportion is a likely mechanism which could explain the phenotypic observations. However, we cannot exclude other possible ways in which *Nfatc2* deficiency could result in diminished running wheel activity levels. Interestingly, binding sites for *Nfatc2* are present on the leptin gene promoter. Considering the role of leptin the regulation of energy balance, it is probable that alterations of *Nfatc2* function (e.g. expression levels, affinity to the DNA binding sites) result in altered leptin signaling and subsequently cause changes in energy expenditure levels. It is of note that changes in leptin signaling should also lead to altered energy intake. This effect was, however, not observed by us in the KO line tested.

Last but not least, we cannot exclude that *Nfatc2* deficiency caused by the gene knockout does not illustrate fully the role of Nfatc2 protein in a wild-type organism. Nfatc2 is a transcription factor with a broad spectrum of functions including regulation of development of different tissue types. Developmental changes caused by gene modification may mask the subtle functional consequence which might be caused by naturally occurring genetic variance.

Ptpn1

Additionally (in **Chapter 5**), we discovered a gene possibly regulating another complex murine phenotype measured by a running wheel, namely food anticipatory activity (FAA; an increase in RWA of mice in response to food restriction). Based on results of a set of experimental procedures, we first proposed protein tyrosine phosphatase, non-receptor type 1 (*Ptpn1*) as a candidate gene for FAA. Subsequently, we showed that manipulation of *Ptpn1* gene expression in a murine hippocampus leads to a significant decrease in FAA.

Regulation of leptin signaling is a potential mechanism by which *Ptpn1* may influence FAA levels. Ptpn1 is an enzyme that dephosphorylates tyrosyl phosphoproteines such as Jak2 (Tonks and Neel 2001). After binding of leptin to a leptin receptor, Jak2 phosphorylates itself and the leptin receptor, leading to an activation of leptin receptor signaling pathways (Zabolotny et al., 2002).

Consequently, Ptpn1, by dephosphorylating Jak2, acts as a negative regulator of leptin signaling (Johnson et al., 2002). Therefore, downregulation of *Ptpn1* may lead to strengthening of leptin signalling. During limited food access, leptin levels drop, which then is a signal of limited energy intake. However, when food deprivation is combined with *Ptpn1* downregulation, leptin signalling may remain high (despite low leptin levels). This could mimic satiety signals despite actual negative energy balance.

Additional arguments support this hypothetical cascade of events. First, *Ptpn1* deficient mice have been characterized as having decreased body weight and food intake, increased total locomotor activity, and, most importantly, enhanced leptin sensitivity (Bence et al., 2006). All the observable behavioral consequences of *Ptpn1* KO may be attributed to this changed leptin sensitivity. Second, various observations point to the possible role of leptin in FAA. Namely, FAA is enhanced in a rat strain (so called Zucker rats) characterized by point mutation in leptin receptor, which leads to reduced leptin binding and obesity (Mistlberger and Marchant 1999). Furthermore, increase in leptin levels due to obesity (Persons et al., 1993) or due to exogenous leptin administration (Exner et al., 2000; Hebebrand et al., 2003) lead to a decrease in FAA.

The choice of hippocampus (hipp) as a target brain area in the current set of experiments may seem surprising. However, there are various arguments supporting our choice. First, hipp has been implicated to play a role in the utilization of hunger and satiety signals (Tracy et al., 2001) and the involvement of hipp in regulation of FAA has been suggested (Wakamatsu et al., 2001). Second, leptin receptors, as well as *Ptpn1,* are well expressed in the hipp (Shioda et al., 1998; Zabolotny et al., 2002). Finally, leptin infusion in the hipp facilitates the induction of NMDA receptor mediated plasticity (Shanley et al., 2001), which indicates that leptin may modulate the acquisition of association between environmental cues and meal arrival.

Taken together, all these arguments support the possible role of *Ptpn1* in regulation of FAA by modulating leptin signaling in murine hippocampus. This hypothesis should be validated in further experiments.

1.2. The impact of physical activity on the course of eating disorder pathology

HLPA as a risk factor to develop ED

In **Chapter 6**, we used a population based study (TwinsUK) to assess whether self-reported participation in sporting (PA) is a risk factor for the development of ED. The analysis showed that a life-long probability of developing ED is 2.6 times higher for women who exercise 5 or more hours per week in comparison to those who exercise less than 5 hours per week. However, as this was a cross-sectional study, we cannot discuss the direction of the relation between the two variables. On the one hand, it is likely that people diagnosed with ED have been highly physically active before the development of the disorder and this was one of the triggering factors for the occurrence of ED. On the other hand, it is also possible that in some cases, the high level of physical activity (HLPA) is an aftermath of the psychological or physiological changes resulting from the development of ED. A longitudinal study starting in children and continuing to early adulthood is needed to assess the proportion of ED cases in which HLPA occur before the onset of ED and the number of ED sufferers for whom HLPA is a symptom that developed together with the development of ED. Finally, it remains an open question whether, in case of ED sufferers who were highly physically active before the onset of ED, one should expect a decrease in PA as a consequence of treatment and recovery.

Some additional limitations of the study should be discussed. It is of note that the TwinsUK population study is based on self-reported measurements of PA as well as self-reported diagnosis of ED. This is a methodological drawback for the findings presented in Chapter 6 (see also Part 2 of the General Discussion). It is hard to predict whether similar findings would be obtained using objective PA measurement methods in combination with medical history data (including ED diagnosis established by professionals).

In Chapters 6 and 7 of the thesis, we used an arbitrary chosen cutoff point of 5 hours of sporting per week to split the study participants into those with high levels of physical activity (HLPA) and those with low levels of physical activity (LLPA). The threshold value was chosen on the basis of previous studies described in the literature. We would like to stress that sporting for at least 5 hours per week is not pathological or unhealthy. Even more, sporting may have positive influence on body image and self-esteem and therefore protect some individuals from developing ED. Nevertheless, our results show that in the level of the general population, HLPA rather contributes to the risk of developing an ED than serves as a protective factor. Although HLPA is not pathological per se, in combination with other factors, it may

become one of the triggering factors of ED. For example, intensive exercising in combination with high motivation to loose weight and regain control over one's body may be particularly dangerous.

The results described in Chapter 6 point also to binge and purge eating habits (as measured by Bulimia Scale of EDI-III questionnaire) as an important contributor to the ED pathology. However, one may argue that the presence of binge eating/purging behaviors is already a symptom of ED pathology and not a risk factor. The discussion of this issue is beyond the scope of this thesis. Let us only mention that this issue is rooted in the unresolved discussion on the definition of mental illness and borders between mental health and illness. These two states are not separate categories but may be seen as two continua (Keyes 2002), or even one continuum (Satcher 2000).

HLPA during the course of ED

As mentioned above, HLPA may be present before the onset of ED or occur as a response to semi-starvation and neuroendocrine changes evoked by this extreme energy deficit state. In the first case, one should not expect permanent decrease in PA after successful therapy. On the contrary, in the second case, recovery should be accompanied by measurable decrease in PA. As clinical reports were inconsistent in this matter, we assessed levels of PA in adolescent anorexia nervosa patients at the acute state of the illness, at recovery, and at one year follow-up (**Chapter 7**). In our sample, successful recovery was associated with marked and prolonged reduction in PA in patients who were characterized by HLPA at the acute phase of the illness. This finding supports the notion that the HLPA observed in ED patients may be to some extent considered starvation induced hyperactivity.

This notion may seem to contradict the findings described in Chapter 6. However, we should bear in mind that the population of ED patients is very heterogeneous. It is not surprising that, within this group, there are various pathways leading to the development of pathology. Consequently, the role of HLPA in the course of ED may differ between subpopulations of ED patients.

Regardless of its exact role, HLPA might have prolonged consequences for ED patients during the progression of and recovery from ED. For example, we could show in Chapter 7 that patients who were characterized with HLPA at the acute phase of the illness had significantly higher amounts of adipose tissue at recovery in comparison to the LLPA group. Similar direction of the change was observed for plasma leptin levels. This may have potentially positive or negative influence on the

risk of relapse. However, on the basis of our data, we were not able to predict the direction of the influence. In our sample, none of the groups was at higher risk of developing a relapse.

Increase in leptin levels at recovery from ED

An increase in plasma leptin levels after a (partial) restoration of body weight is an expected consequence of successful treatment of ED. In case of some of the ED patients, this increase may be, however, higher than expected on the basis of the amount of the fat tissue (the main source of leptin). It was hypothesized that the overshoot of leptin may be an important risk factor for a relapse. Although, we did not observe a negative influence of high leptin levels on the recovery rate or a chance of relapse in our sample (Chapters 7 and 8), we were still curious about the source of the high leptin levels in recovered patients. We could show (in a limited sample of AN patients) that the leptin levels at recovery were inversely related to the amount of body weight loss during progression of AN (**Chapter 8**).

Interestingly, in our sample, high leptin levels were rather related to a positive treatment outcome. Although recovered and non-recovered patients did not differ significantly in the amount of body weight and adipose tissue recovery, the recovered patients had significantly higher leptin levels. Furthermore, recovered patients had also much better psychological (lower anxiety levels) and physiological (restoration of menstrual cycle) treatment outcomes. This is not surprising as an increase of leptin levels above a certain threshold is a necessary condition for the restoration of the function of other neuroendocrine systems including hypothalamus-pituitary-adrenal axis as well as gonadal hormones. Given the current state of knowledge, we cannot explain the apparent difference in the amount of leptin secretion per unit of body fat tissue between the recovered and non-recovered AN patients.

1.3. Comparative genomics approach in research regarding the genetic basis of PA

QTL studies often result in genetic candidate regions containing dozens of possible candidate genes. Consequently, it is difficult to choose a likely candidate gene for further research. Multiple strategies may be used to diminish the size of the QTLs. For example, in comparative genomics, QTLs coming from different species and associated with a given complex phenotype are aligned based on the synteny between these species. The overlapping genetic region is considered very likely to contain the causal gene for this complex trait. In **Chapter 9,** we wondered whether it

is possible to apply this approach to the currently available data regarding the genetic basis of physical activity in mice and humans in order to discover novel candidate genes for this phenotype. We were motivated to conduct this analysis because it is common practice to consider an overlap between a novel QTL and some previously reported QTLs as a strong argument supporting the significance of the novel finding. It is of note that in some cases, this reasoning is used even if the two QTLs were associated with phenotypes which should not be compared (e.g., running wheel activity and home cage ambulation). Furthermore, sometimes a practical significance of the newly discovered QTL is derived from its co-localization with a QTL for a related complex human phenotype. This is done despite difficulties in conducting an exact translation between the phenotypes measured in the two species.

The statistical analysis (Chapter 9) conducted on the currently available mouse and human genetic association studies of PA revealed that, in the current state of knowledge, an overlap with previously discovered QTLs cannot be treated as a confirmation of a novel QTL. It is so because by multiple sampling from a limited pool of possible genetic regions, we create a situation in which it is very likely to obtain this type of an overlap. Consequently, the currently occurring overlap of regions coming from various studies is very low and does not surpass the overlap expected purely on the basis of random sampling. This observation can be accounted for by various factors including genetic background differences (within species), difficulties with phenotype definition, and a wide variety of methodological differences between studies.

This analysis does not indicate that any occurring overlap has no biological bases. It solely shows that, based on the current data, we cannot distinguish the overlaps occurring by chance from those with an actual biological significance. Therefore, it would not be justified to apply the comparative genomics approach to currently available results regarding genetic regions associated with voluntary PA.

Methodological considerations

Several decisions and assumptions were required for some of the research approaches described in the thesis. In some cases, we were also faced with methodological limitations. Some of the considerations of the assessment of physical

activity in humans, translational value of animal model, as well as sample size limitations are discussed below.

Operationalization of the PA in humans

As mentioned in the General Introduction (Chapter 1), there is a trade-off between the feasibility and reliability of objective (such as accelerometer) and subjective (such as questionnaires) PA measurement methods (Shephard 2003). The correlation between the results obtained with the use of the objective and subjective methods is rather low as shown by various authors (de Vilhena e Santos DM et al., 2012; Dishman 2008; Garland, Jr. et al., 2011b) as well as in Chapter 2 of this thesis. This observation points to the fact that the two PA operationalization methods may in fact measure different phenotypes related to the PA participation (de Vilhena e Santos DM et al., 2012). The TwinsUK study, used in Chapters 2, 3, and 6, also applied questionnaires to estimate the amount of time spent on exercise participation. This was most likely caused by the practical difficulties related to the use of objective PA measurement methods in large population based samples (Dishman 2008). The use of subjective methods of PA assessment should be accounted for as a methodological limitation of the studies described in the above-mentioned chapters. However, it is of note that the reliability of the results presented in Chapter 2 is partly supported by the observation that a significant association was found between genetic region on human chromosome 20 and subjectively assessed PA in two independent human populations.

Choosing a threshold value for the HLPA

As mentioned before, the choice of 5 hours of sporting per week was arbitrary. It was based on previously published studies. We decided to follow the established threshold to enable comparison between different studies. This choice may evoke concerns as the results may be artifacts resulting from this arbitrary threshold. However, when using a cutoff of, for example, 4.5 hours of sport per week, one can obtain similar results with effects in the same direction as when using a cutoff value of 5 hours of sporting per week.

The sample size in the clinical studies

The longitudinal study used for the analyses described in Chapters 6 and 7 was characterized by relatively high drop-out of AN patients between initial assessment, the end of study, and follow-up. This resulted in a small headcount in some of the sub-groups used for the analyses. Consequently, these two studies should be treated as pilot studies and replicated in an independent AN patients population.

Translational value of RWA as a model of PA

As mentioned in the General Introduction (Chapter 1), voluntary RWA can be considered a suitable rodent behavior to model human voluntary PA (Eikelboom 1999; Garland, Jr. et al., 2011b; Kelly et al., 2010; Rezende et al., 2009). It is likely that both behaviors (RWA and voluntary PA) are ethologically comparable and originate from the evolutionarily conserved physical (e.g. muscle strength) and mental (e.g. motivation) capability to stay active in order to survive in demanding environments (e.g., obtain food despite pain, effort, and potential danger). Nevertheless, it is of note that there are arguments against this hypothesis. These arguments should be always kept in mind when interpreting findings coming from translational studies of PA. However, animal models are constantly used with the aim of unraveling the biological basis of complex phenotypes such as PA. It is hoped that these research efforts will deliver some novel findings, which may be applied for the benefit of human health and quality of life. Taking under consideration the challenges encountered while applying the translational approach, we attempted to bridge the gap between mouse and human studies (Chapters 2, 3). These findings illustrate the challenges resulting from phenotype definitions as well as complex phenotype modeling. However, we hope that findings described in Chapter 2 would be a valuable example of a critical approach to translational research regarding the genetic basis of a complex phenotype.

In light of challenges regarding animal models of human PA, in Chapter 9 we addressed the questions of: 1) translational value of RWA behavior as a model of PA; and, 2) translational value of genetic findings coming from mice studies using a PA model. To conclude, as a result of numerous methodological discrepancies, the use of currently available genetic mouse and human studies did not prove helpful in settling the question of the translational value of RWA (Chapter 9).

Concomitantly, the findings described in the above-mentioned chapters point to the limitations of the translational approach. We hope that these issues will be further critically assessed by the scientific community. Hopefully, the effort of bridging the gap between animal and human studies will lead to more applicable research.

The Monte Carlo simulation

The Monte Carlo simulation presented in Chapter 9 is a thought experiment with specific limitations that resulted from the following practical limitations. First, we attempted to conduct an analysis of an overlap with the use of exact candidate genetic regions reported in mouse and humans studies. This approach would enable us to consider the size of the genetic regions as well as the number of genes pointed

in particular studies. However, it was not possible to use this approach because many of the mouse studies did not report the approximate span of the candidate genetic region. Furthermore, majority of the human studies also only approximately revealed the location of the genetic candidate region expressed in a simplified 'chromosomal address' based on Giemsa staining (e.g. 20q11). Second, the analysis required an approximated finite number of possible candidate regions. Therefore, we decided to express all the reported human and mouse candidate genetic regions as the 'chromosomal address' based on the Giemsa staining (and syntenic human chromosomal locations). The results of this staining method differ in resolution depending on the exact conditions of the staining. In our analysis, we used the resolution of 850 possible genetic regions. This is a standard resolution that was also used by the UCSC genome browser (http://genome.ucsc.edu/) – a tool we used in order to establish human genetic regions syntenic to the reported mouse candidate genetic regions (from the QTL studies).

The approach we chose may be criticized for its apparent simplification. We argue that the assumptions, which were made in order to enable the analysis, work in favor of the translational approach; however, the results of the analysis were, nevertheless, negative. We acknowledge the limitations of the thought experiment and therefore, we do not defend the exact values obtained through the Monte Carlo simulation as absolute threshold values. We, nevertheless, consider them to be valuable approximations.

The analysis of the between-species overlap (using the Monte Carlo simulation) was conducted for two reasons: 1) to show a simple validation method regarding the significance of a genetic overlap when it occurs (in research examining genetic basis of any complex phenotype); and, 2) to show that care needs to be taken when using genetic overlap between species (exactly due to methodological problems in human and animal studies, as well as the translational challenges). We believe that the chosen analysis method, although simplified, was appropriate for this purpose.

Clinical implications

Despite the abovementioned limitations, the current thesis contains several findings of clinical relevance in relation to the ED pathology as well as findings regarding possible biological regulators of physical activity in the healthy population.

Eating disorders

First, our data support the potentially negative contribution of HLPA to the development of ED. On the one hand, we could demonstrate that HLPA is indeed a risk factor for the development of ED when assessed in a general population. On the other hand, in case of individuals who suffer from ED, HLPA may mask the severity of the illness. As a consequence, those ED sufferers who are also highly physically active may be sick for a considerable time before searching for professional help.

Second, our data demonstrate that the presence of HLPA in the acute state of the illness does not have a negative consequence for the recovery rate. This may be the case for those ED sufferers who manage to diminish their levels of PA during the treatment. Furthermore, our data suggest that the presence of HLPA as a symptom of ED in acutely ill AN patients influences the adipose tissue restoration and plasma leptin levels. Both factors can have a substantial impact on a short- and long-term risk of relapse. It is of note that in our study (Chapters 7 and 8), despite previously reported findings, higher leptin levels were not a risk factor for a relapse. On the contrary, leptin levels at recovery were inversely related to the signs of ED pathology. These findings indicate that, if the HLPA and high leptin levels are effectively managed during treatment, they may have a positive effect on the recovery from AN. This needs to be, however, further replicated in an independent population of AN patients.

Third, the findings reported in Chapter 8 suggest that leptin dynamics at recovery may depend on the amount of body weight loss during the progression of AN. Importantly, the body weight change, if assessed in adolescents, should be corrected for gender- and age-specific body weight norms. Taking under consideration the versatile (positive and negative) possible effects of plasma leptin level changes on recovery and relapse, it could be recommended to assess this relative body weight change in clinical practice. Further research is, however, required to confirm our findings which were based on a small patient population.

Last but not least, the data presented in Chapter 7 seem to support the hypothesis that HLPA in ED patients is a biological consequence of starvation and therefore, one should expect a decrease in HLPA after successful treatment and body weight restoration.

Physical activity

With the extensive characterization of the *Nfatc2* deficient mice, we confirmed the role of this gene in the regulation of physical exercise levels (as indicated by previous

studies involving mice and humans (Courtney and Massett 2012; Rico-Sanz et al., 2004)). We could demonstrate a specific reduction in the running wheel activity levels, which may be attributed to decreased acute exercise capacity. These results suggest an important, evolutionarily conserved role of *Nfatc2* gene (and biological pathways regulated by this transcription factor) in the determination of levels of voluntary physical activity.

Future directions

Research brings answers, but also inevitably gives rise to further questions. In addition to some suggestions placed within specific chapters of this thesis, I would like to propose some new interesting research directions.

The research on physical activity and plasma leptin levels in AN patients is dominated by considerations of their negative influence on the course of ED (Epling and Pierce 1992; Hebebrand et al., 1997; Hechler et al., 2005; Holtkamp et al., 2003a; Holtkamp 2004; Pierce et al., 1994). Based on the essential role of leptin in the regulation of neuroendocrine system as well as the positive psychological effects of exercise, one could also consider a possible positive contribution of leptin and physical activity to the recovery process. Some of the findings placed in Chapters 7 and 8 seem to support these suggestions. More research is needed regarding the relation between leptin dynamics, physical activity, and psychological as well as physiological recovery.

Additionally, we believe that treatment procedures will benefit from an increase in the understanding of the mechanisms which underlie the fluctuations of leptin beyond levels expected on the basis of body weight and fat tissue content. What is the precise impact of speed and extent of body weight loss (and of the body weight recovery) on plasma leptin levels at recovery? Perhaps the different phases in plasma leptin fluctuation reflect different stages of slow recovery of neuroendocrine functions. In this case, the plasma leptin fluctuations at different time points during recovery process could be influenced by distinctive events which took place during illness development, maintenance, and treatment. Hopefully, this knowledge could help us to predict which patients are likely to develop hyperleptinemia (high leptin levels). In combination with a detailed analysis of leptin function (proposed in the paragraph above), this knowledge could help to manage the factors which increase the risk of relapse after recovery. It could also give rise to new guidelines for

pharmacological interventions targeting psychological (e.g., anxiety), neuroendocrinological (e.g., amenorrhea), and physical activity-related symptoms.

Further research is also needed to test whether the *Nfatc2* gene affects exercise capacity in humans. First, it should be determined whether there are genetic variances (single nucleotide polymorphisms, copy number variance, epigenetic modifications) which detectably influence *Nfatc2* function or expression levels in humans. Second, candidate gene association studies could be conducted to test the association of these genetic variants with exercise capacity-related phenotypes. Furthermore, it is of high value to unravel the pathways by which *Nfatc2* gene influences physical activity levels and exercise capacity. As *Nfatc2* has a broad spectrum of functions, it remains to be determined whether *Nfatc2* exerts its influence by modifying the skeletal muscle development or by the modulation of leptin functioning. Hopefully, this knowledge could open new frontiers for the research of diseases involving, for example, muscle dystrophy.

Finally, according to Theodor Garland Jr. (2011b), 'if animal models are to be used to elucidate the human condition, then we need to make claims – and eventually support them with data on underlying mechanism – regarding how behavioral phenotypes equate across species'. Certainly, not every research needs to have immediate or predictable applicability. However, if combined research efforts are about to deliver some innovations for therapies and treatments, the science community needs to establish methods of critical validation of translational value of the used methodologies. More research and critical consideration is needed to support a good quality translational research of PA and other complex phenotypes. The attempt taken by the authors in Chapter 9 is just an example of a (simplified) possible approach to this issue. More refine and advance methods of translational research validation will emerge once an open discussion of the issue takes place.

Concluding remarks

In summary, in the studies gathered in this thesis, we aimed at discovering new factors underlying the biological basis of PA, as well as at further elucidating the role of PA in the course of ED pathology. We could pinpoint new genetic factors contributing to the observable levels of physical activity in mice and human. Furthermore, we delivered additional support for a complicated role of PA in the etiology, maintenance of as well as the recovery from ED. Last but not least, we aimed at providing a critical evaluation of translational approaches to study the genetic basis of PA. This work will hopefully serve as a basis for extensive future studies.

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Addendum

Nederlandse samenvatting

Inleiding

De mate van lichaamsbeweging is een belangrijke factor die bijdraagt aan het behouden van een goede gezondheid. Te lage niveaus van lichaamsbeweging kunnen bijdragen aan obesitas, cardiovasculaire ziekten en andere gezondheidsklachten. Te hoge niveaus van lichaamsbeweging zouden kunnen bijdragen aan de negatieve effecten van psychologisch en fysiek welzijn, zoals wordt waargenomen in excessieve lichaamsbeweging bij sommige patiënten met een eetstoornis.

De mate van lichaamsbeweging heeft een sterke invloed op het verloop van eetstoornissen. Lichaamsbeweging wordt zelden objectief gemeten in klinische praktijken en wetenschappelijke studies. Dit heeft als gevolg dat kennis over de ontstaanswijze van hoge niveaus van lichaamsbeweging in eetstoornispatiënten nagenoeg ontbreekt. Het verkrijgen van meer kennis over de factoren die bijdragen aan lichaamsbeweging zou kunnen leiden tot meer gerichte behandeling en preventie van dit ziektebeeld. Het identificeren van genetische factoren die een belangrijke bijdrage aan de mate van lichaamsbeweging zou kunnen bijdragen aan het ontrafelen van het biologisch substraat dat de basis vormen van deze erfelijke eigenschap. Het identificeren van deze genetische factoren is een uitdaging, aangezien andere factoren de mate van lichaamsbeweging ook kunnen beïnvloeden (bijvoorbeeld psychologische, culturele, en omgevingsfactoren).

Met het oog op de complexiteit die ten grondslag ligt aan de regulatie van lichaamsbeweging, en de potentiele relevantie voor de mate van lichaamsbeweging voor eetstoornispathologie, richt dit proefschrift zich op de volgende onderzoeksdoelen:

1) ontrafelen van een deel van de biologische basis van lichaamsbeweging;
2) begrijpen hoe lichaamsbeweging bijdraagt aan het verloop van eetstoornispathologie.

Methode

Met betrekking tot onderzoeksdoel 1 zijn verschillende methoden toegepast om nieuwe kandidaatgenen en genoomregio's te identificeren die bijdragen aan de individuele mate van lichaamsbeweging. In het geval van de studies met proefpersonen zijn er genetische associatiestudies uitgevoerd waarbij de relatie tussen genetische variaties in kandidaatgenoomgebieden en mate van lichaamsbeweging werden onderzocht. De resultaten hiervan zijn gepresenteerd in **hoofdstukken 2** en **3** van dit proefschrift. Daarnaast zijn studies bij muizen uitgevoerd, waarbij de relatie tussen genetische variaties in het genoom en loopwielactiviteit zijn onderzocht. In de hoofdstukken 2 en 3 is een panel van inteelt muizenstammen (zogenaamde chromosoom-substitutiestammen) gebruikt om in een

genoomwijde analyse op zoek te gaan naar genoomregio's die gekoppeld zijn aan de mate van loopwielactiviteit. Daarnaast is in **hoofdstuk 4** onderzoek gedaan met een gen 'knock-out' muizenlijn. In deze muizenlijn is een specifiek gen (te weten *Nfatc2*) permanent verwijderd, zodat de functie van dit gen kan worden onderzocht. In **hoofdstuk 5** is genexpressie in het brein beïnvloed door gebruik te maken van een genetische 'knockdown'-technologie. Met deze technologie blijft het DNA van het gen intact, maar wordt het boodschapper-RNA van het gen afgebroken door een ander gen te introduceren (te weten een shRNA) in hersencellen met behulp van een virale vector. Tot slot, wordt er in de **hoofdstukken 2** en **3** een poging ondernomen om de bevindingen in muis en mens te integreren.

Met betrekking tot onderzoeksdoel 2, zijn twee verschillende populaties onderzocht. Allereerst is een Britse tweelingpopulatie gebruikt (genaamd: TwinsUK) om vast te stellen of de mate van lichaamsbeweging een risicofactor is voor de ontwikkeling van een eetstoornis (**hoofdstuk 6**). Voor dit onderzoek werden de individuele niveaus van lichaamsbeweging van de deelnemers vastgesteld aan de hand van vragenlijsten. Ten tweede, in **hoofdstuk 7**, werden de gevolgen van hoge niveaus van lichaamsbeweging in de acute fase van een eetstoornis en tijdens de herstelfase onderzocht in adolescente anorexianervosapatiënten. In een relatief kleine groep van patiënten zijn objectieve metingen van lichaamsbeweging vastgelegd met behulp van een versnellingsmeter.

Resultaten

In **hoofdstuk 2** werd een translationele methode toegepast om mogelijke kandidaatgenen voor vrijwillige lichaamsbeweging bij mensen te identificeren. Om dit te bewerkstelligen is er eerst een genoomregio gevonden voor vrijwillige lichaamsbeweging bij muizen door loopwielactiviteit te meten bij chromosoom-substitutiestammen. Op basis van deze analyse is een genoomregio op chromosoom 2 geïdentificeerd en hebben we vervolgens, op basis van syntenie tussen muis en mens, deze genetische bevindingen bij de muis gebruikt voor de analyse bij de mens. In deze studie testten we de hypothese in twee onafhankelijke populaties bij wie de mate van lichaamsbeweging was vastgesteld op twee verschillende manieren (aan de hand van een vragenlijst en met behulp van een versnellingsmeters). Uiteindelijk werd een genetische associatie in beide onafhankelijk populaties gevonden.

Deze methode werd nogmaals toegepast in **hoofdstuk 3** van dit proefschrift, waarbij een genoom regio op muischromosoom 19 werd gevonden. Deze regio verklaarde 26% van de variatie in de mate van loopwielactiviteit bij de muis. In dit geval werd geen significante associatie gevonden tussen een genetische variatie in het syntene gebied bij de mens (op chromosoom 10) en de mate van vrijwillige lichaamsbeweging.

In de volgende twee hoofdstukken is onderzoek gedaan naar kandidaatgen bij muizen. In **hoofdstuk 4** werden de fenotypische gevolgen in relatie tot

lichaamsbeweging onderzocht in muizen waarbij het *Nfatc2* gen specifiek was verwijderd. Het bleek dat de mate van vrijwillige loopwielactiviteit verlaagd was in muizen waarbij het *Nfatc2* gen is verwijderd. We hebben verder aangetoond dat de verlaging van loopwielactiviteit in deze muizen niet veroorzaakt werd door verstoorde motorische coördinatie of door verandering in gevoeligheid voor beloning. In **hoofdstuk 5** werd een ander kandidaatgen, te weten *Ptpn1*, onderzocht in relatie tot loopwielactiviteit. In dit hoofdstuk werd onderzocht of de expressie van *Ptpn1* in de hippocampus bij muizen bijdraagt aan zogenaamde anticipatiegedraag. Dit werd gedaan in een paradigma waarbij er dagelijks op een vast tijdstip een beperkte hoeveelheid voedsel werd aangeboden. Als reactie hierop werden dieren in de opvolgende dagen meer actief in de periode voorafgaand aan het moment van het beschikbaar worden van eten (anticipatiegedrag). De expressie van *Ptpn1* werd verlaagd door gebruik te maken van een shRNA vector die werd geïnjecteerd in de hippocampus van muizen. Deze vector zorgt ervoor dat het RNA van het gen wordt afgebroken waardoor er minder genproduct wordt aangemaakt op de plaats waar het geïnjecteerd was (hier in de hippocampus). In deze studie hebben we laten zien dat een verlaging van *Ptpn1* gen expressie in de hippocampus significant en specifiek de ontwikkeling van anticipatiegedrag verminderd bij muizen.

In de hoofdstukken 6, 7 en 8 is er onderzoek gedaan op basis van gegevens van mensen die verzameld zijn vanuit de Britse bevolking en bij anorexianervosapatiënten. In **hoofdstuk 6**, testten we de vraag of excessieve lichaamsbeweging een risicofactor is voor de ontwikkeling van een eetstoornis in de algemene populatie. Hiervoor hebben we gebruik gemaakt van bestaande gegevens uit een tweelingstudie vanuit Groot-Brittannië (TwinsUK). Van vrouwelijke tweelingen binnen dit cohort hebben we gebruik gemaakt van de volgende fenotypische informatie: 1) een eventuele diagnose voor een eetstoornis; en 2) de hoeveelheid tijd die iemand per week aan sport besteedt. Onze resultaten suggereren dat excessieve lichaamsbeweging een belangrijke risicofactor voor de ontwikkeling van een eetstoornis zou kunnen zijn. Door methodologische beperkingen was het op basis van deze gegevens niet mogelijk om de richting van causaliteit tussen excessieve lichaamsbeweging en eetstoornis vast te stellen.

In **hoofdstuk 7** hebben we onderzoek gedaan op basis van een uitgebreide en longitudinale klinische dataset van adolescente anorexianervosapatiënten om vast te stellen of een hoge mate van lichaamsbeweging afneemt tijdens de herstelperiode. Bovendien hebben we onderzocht of patiënten die excessieve lichaamsbewegingsatronen in de acute fase van de ziekte hadden mogelijk een verschillend profiel in herstel van lichaamsvetpercentage en plasma leptin hormoon niveaus hebben in vergelijking tot minder actieve patiënten. In **hoofdstuk 8**, en op basis van dezelfde dataset, konden we aantonen dat de circulerende leptine niveaus van herstelde anorexianervosapatiënten afhangt van de mate van relatieve lichaamsgewichtverlies tijdens de progressie van de ziekte. Dit is van klinische relevantie, aangezien leptine een hormoon is die direct door het vetweefsel wordt

afgegeven. Circulerende leptine niveaus fluctueren vaak in anorexianervosapatiënten en dit gebeurt vaak buiten de normaalwaarden voor dit hormoon. Deze fluctuaties zouden de therapeutische pogingen kunnen belemmeren, aangezien hoge leptine niveaus weer kunnen leiden tot verlaagde voedselinname.

Als laatste wilde we een kritische blik werpen op translationele methoden die gebruikt worden ter opheldering van complexe gedragsfenotypen (**hoofdstuk 9**). De ontdekking van genetische variaties die ten grondslag liggen aan een complex fenotype is een behoorlijke uitdaging voor het hedendaagse biomedisch onderzoek. Een mogelijke benadering is gebaseerd op de identificatie van een kandidaat genetische regio voor een kwantitatieve eigenschap (Eng. quantitative trait locus). In elk gevonden locus bevinden zich meerdere genen, en daarvoor is verdere analyse nodig om de meest waarschijnlijke kandidaatgenen hieruit te selecteren. Dit kan bijvoorbeeld door de gebruikmaking van een vergelijkende genomics analyse (tussen species) waarbij de grootte van het locus mogelijk verkleind kan worden. In hoofdstuk 9 illustreren we deze methode door gebruik te maken van data van muizen en mensen. Gezien de grote variatie in studies naar muizen en mensen (bijvoorbeeld door de verschillende manieren van fenotypering), is het niet mogelijk om data over de genetische basis van lichaamsbeweging van verschillende diersoorten te vergelijken. Het is daarom belangrijk om voorzichtig te zijn met de gebruikmaking van deze manier van analyse voor complexe fenotypen.

Samenvattend hadden de studies in dit proefschrift tot doel om nieuwe factoren te identificeren die ten grondslag liggen aan de biologische basis van lichaamsbeweging, en om de rol van lichaamsbeweging in de ontwikkeling van een eetstoornis beter te begrijpen. In dit proefschrift hebben we nieuwe genetische factoren geïdentificeerd die bijdragen aan lichaamsbeweging in muizen en mensen. Daarnaast hebben we additionele evidentie aangedragen voor een complexe rol van lichaamsbeweging in de oorzaak, instandhouding, en het herstel van eetstoornissen. Ten slotte hebben we geprobeerd een kritische evaluatie te presenteren over translationele benaderingen om de genetische basis van lichaamsbeweging te kunnen bestuderen. Dit onderzoek zal hopelijk bijdragen aan toekomstige studies op dit gebied.

Streszczenie w języku polskim

Uzasadnienie oraz cel badań

Właściwy poziom aktywności fizycznej jest niezbędny do zachowania dobrego zdrowia w sensie fizycznym i psychicznym. Z jednej strony zbyt niska częstotliwość i intensywność aktywności fizycznej są wymieniane jako jedna z możliwych przyczyn epidemii otyłości, chorób układu naczyniowego oraz innych schorzeń. Z drugiej strony zbyt wysoki poziom aktywności fizycznej może mieć negatywny wpływ na zdrowie, tak jak w przypadku podgrupy pacjentów cierpiących na zaburzenia odżywania (anorexia nervosa i bulimia nervosa), u których w przebiegu choroby występuje także nadmierne zaangażowanie w aktywność fizyczną. Chociaż wysoki poziom aktywności fizycznej może w wieloraki sposób modulować przebieg zaburzeń odżywiania, symptom ten jest rzadko mierzony w praktyce klinicznej oraz w badaniach naukowych. Z tego powodu wiele pytań dotyczących etiologii wysokiego poziomu aktywności fizycznej u chorych na zaburzenia odżywiania pozostaje bez odpowiedzi.

Motywacją do prowadzenia kolejnych badań w tej dziedzinie jest przekonanie, iż wiedza dotycząca etiologii oraz modulatorów wysokiego poziomu aktywności fizycznej może zostać wykorzystana do tworzenia bardziej efektywnych terapii oraz programów prewencyjnych zaburzeń odżywiania. Silny wpływ na poziom aktywności fizycznej wywierają czynniki genetyczne, dlatego dotycząca ich wiedza może zostać wykorzystana do odkrycia biologicznych podstaw tego dziedzicznego fenotypu. Badania w tym kierunku są jednak utrudnione, ponieważ wiele innych czynników (takich jak czynniki psychologiczne, kulturowe oraz środowiskowe) wywiera wpływ na poziom aktywności fizycznej.

Biorąc pod uwagę wyżej wymienione fakty postawiliśmy przed sobą następujące cele badawcze:

1) odkrycie nowych czynników biologicznych mających wpływ na poziom aktywności fizycznej;
2) próbę pogłębienia wiedzy dotyczącej wpływu, jaki aktywność fizyczna ma na przebieg zaburzeń odżywiania.

Metody

W odniesieniu do pierwszego celu badań wykorzystaliśmy różnorodne metody badawcze w celu zidentyfikowania nowych genów oraz regionów genetycznych mających wpływ na poziom aktywności fizycznej. W przypadku badań na ludziach przeprowadziliśmy analizę asocjacji pomiędzy poziomem aktywności fizycznej a znanymi wariantami genetycznymi w zawężonych regionach genetycznych. Wyniki tych badań są przedstawione w **rozdziałach 2** oraz **3**. W przypadku badań u myszy, skupiliśmy się na badaniu genetycznych podstaw aktywności fizycznej w kołowrotku. W **rozdziałach 2** oraz **3** wykorzystaliśmy panel mysich szczepów (tak

zwany panel CSS), który został wyhodowany w celu wykrycia nowych potencjalnych regionów (tzw. regionów-kandydatów) wywierających wpływ na poziom występowania złożonych fenotypów (takich jak aktywność fizyczna). Dodatkowo, w **rozdziałach 2** oraz **3** podjęliśmy próbę częściowego zintegrowania wyników pochodzących z badań na myszach i ludziach.

W **rozdziale 4** przeprowadziliśmy badania na myszach transgenicznych, u których jeden gen (w tym przypadku *Nfatc2*) jest całkowicie usunięty. Tego typu myszy są hodowane w celu odkrycia roli genu poprzez badanie konsekwencji jego usunięcia. W **rozdziale 5** wykorzystaliśmy bardziej zaawansowaną metodę modulowania ekspresji genu (tak zwany knock-down genu). W tym przypadku DNA kodujące gen pozostaje nienaruszone, jednak mRNA zostaje częściowo zniszczone, co prowadzi do znaczącego obniżenia poziomu ekspresji genu. RNA jest niszczone dzięki wprowadzeniu do komórki innego genu (np. shRNA) przy użyciu wektorów wirusowych.

W odniesieniu do drugiego celu badawczego, wykorzystaliśmy dane pochodzące z dwóch różnych ludzkich populacji. Po pierwsze, wykorzystaliśmy reprezentacyjną próbę ogólnej populacji kobiet w celu określenia czy wysoki poziom aktywności fizycznej może zostać uznany za czynnik ryzyka dla zaburzeń odżywiania (**rozdział 6)**. Aktywność fizyczna w tej próbie została zmierzona przy użyciu kwestionariuszy samoopisowych. Po drugie, w **rodziale 7**, zbadaliśmy konsekwencje, jakie pociąga za sobą wysoki poziom aktywności fizycznej u chorych na zaburzenia odżywiania. W tym przypadku możliwe było obiektywne zmierzenie aktywności fizycznej przy użyciu akceleromierzy.

Wyniki

W **rozdziale 2** zastosowaliśmy podejście translacyjne w celu odkrycia nowych genów mających wpływ na aktywność fizyczną u ludzi (tzw. genów-kandydatów). W pierwszym etapie badań zidentyfikowaliśmy genetyczny region zasocjowany z aktywnością fizyczną myszy. Aktywność fizyczna myszy była mierzona przy pomocy aktywności fizycznej w kołowrotku (zachowania uznanego za model aktywności fizycznej u ludzi). W konsekwencji wskazaliśmy na region na mysim chromosomie, 2 jako region mający wpływ na poziom aktywności fizycznej. Na podstawie tego wyniku wysnuliśmy hipotezę, że analogiczny (genetycznie zakonserwowany) region genetyczny u ludzi będzie miał regulujący wpływ na poziom aktywności fizycznej u ludzi. Przetestowaliśmy tę hipotezę w dwóch niezależnych populacjach, w których aktywność fizyczna była mierzona subiektywnie (kwestionariuszem) lub obiektywnie (akceleromierzem). Udało nam się wykazać istnienie asocjacji pomiędzy genetycznym regionem a poziomem aktywności fizycznej.

To samo podejście zastosowaliśmy w **rodziale 3** i w konsekwencji wykazaliśmy, że na mysim chromosomie 19 znajduje się genetyczny region-kandydat dla aktywności

fizycznej myszy. Region ten wyjaśnia 26% zmienności fenotypowej w badanej populacji myszy. Analogiczny ludzki fragment genomu (na chromosomie 10) nie posiada jednak wykrywalnego wpływu na poziom aktywności fizycznej u ludzi.

W pozostałych dwóch rozdziałach niniejszego doktoratu zawierających modele zwierzęce zastosowaliśmy podejście polegające na testowaniu genów-kandydatów. Po pierwsze w **rodziale 4** zbadaliśmy konsekwencje delecji genu *Nfatc2* na poziom aktywności fizycznej myszy. Wykazaliśmy, że usunięcie *Nfatc2* prowadzi do obniżenia aktywności fizycznej myszy (w kołowrotku). Ten mierzalny spadek aktywności fizycznej jest specyficzny, ponieważ nie może zostać wytłumaczony poprzez zaburzenia koordynacji ruchowej ani zmiany wrażliwości na nagrodę naturalną. Po drugie, w **rozdziale 5** zastosowaliśmy metodę zmiany ekspresji genu (tzw. knock-down) w celu zbadania roli jaką odgrywa gen *Ptpn1* w regulacji aktywności fizycznej. Przetestowaliśmy hipotezę, zgodnie z którą ekspresja *Ptpn1* w mysim hipokampie ma wpływ na wzrost aktywności fizycznej obserwowany na krótko przed dostępem do pokarmu u myszy z ograniczonym dostępem do pokarmu (tzw. aktywność antycypacyjna). Eksperyment został przeprowadzony na myszach ze stałym dostępem do kołowrotka i z dostępem do pokarmu ograniczonym do dwóch godzin dziennie. Poziom ekspresji *Ptpn1* został zmodyfikowany dzięki wprowadzeniu do mysiego hipokampu wektora wirusowego kodującego shRNA. Zademonstrowaliśmy, że obniżenie poziomu ekspresji *Ptpn1* w hipokampie myszy prowadzi do specyficznego obniżenia aktywności antycypacyjnej w badanym modelu.

Kolejne trzy rozdziały niniejszej pracy opisują badania przeprowadzone na ogólnej populacji oraz w grupie osób cierpiących na jadłowstręt psychiczny (anorexia nervosa). W **rodziale 6** przetestowaliśmy hipotezę, zgodnie z którą wysoki poziom aktywności fizycznej jest czynnikiem ryzyka dla rozwoju zaburzeń odżywiania. W tym celu użyliśmy danych pochodzących z ogólnodostępnej próby z populacji brytyjskiej (tzw. TwinsUK), dla której dostępne są dane dotyczące zachorowalności na zaburzenia odżywiania oraz czasu poświęcanego na aktywność fizyczną. Wyniki sugerują, iż wysoka aktywność fizyczna może być ryzykiem dla rozwoju zaburzeń odżywiania. Niestety, z powodu ograniczeń metodologicznych, nie jest możliwe wyciągnięcie wniosków dotyczących kierunku relacji między zaburzeniami odżywiania i aktywnością fizyczną.

W dalszym toku badań posłużyliśmy się danymi podłużnymi pochodzącymi z populacji osób cierpiących na anoreksję. W **rozdziale 7** sprawdziliśmy czy wysoki poziom aktywności fizycznej u chorych na anoreksję ulega zmniejszeniu wraz ze wzrostem wagi ciała. Dodatkowo przetestowaliśmy, czy pacjentki charakteryzujące się w trakcie choroby wysokim poziomem aktywności fizycznej różnią się od pacjentek nieaktywnych pod względem ilości tkanki tłuszczowej i poziomu leptyny w osoczu. Z kolei w **rozdziale 8** wykazaliśmy, że u pacjentek, u których wystąpiła poprawa symptomów, poziom leptyny w osoczu zależy od ilości wagi ciała, jaką

utraciły w wyniku postępu choroby. To odkrycie może być istotne w praktyce klinicznej, ponieważ leptyna jest hormonem wydzielanym przez tkankę tłuszczową. Fluktuacje jej poziomu w osoczu są jedną z konsekwencji postępu anoreksji oraz procesu odzyskiwania normalnej wagi ciała. Wahania te, które często przekraczają wartości normalne dla danego wieku oraz wagi ciała, mogą przeciwdziałać wysiłkom terapeutycznym, ponieważ wysoki poziom leptyny prowadzi do ograniczenia spożywania pokarmu.

W ostatnim rozdziale (**rozdział 9**) podjęliśmy próbę ewaluacji podejścia translacyjnego w badaniach dotyczących genetycznych podstaw złożonych fenotypów. Jedną z metod stosowanych w celu identyfikacji nowych czynników genetycznych jest tak zwana analiza locusu cechy ilościowej (quantitative trait loci, QTL), w której wskazuje się na nowy region genetyczny, który staje się regionem-kandydatem, który prawdopodobnie zawiera gen wpływający na poziom badanego fenotypu. Większość QTL zawiera jednak wiele genów i dlatego identyfikacja jednego genu- kandydata wymaga skomplikowanych analiz. Przy użyciu udanych dostępnych w literaturze możliwe jest czasem zawężenie otrzymanego QTL poprzez porównanie wyników analiz QTL pochodzących z różnych gatunków. W niniejszym rozdziale zastosowaliśmy to podejście do danych dostępnych analiz QTL dla aktywności fizycznej myszy i ludzi. Na podstawie szeregu danych wykazaliśmy, że porównanie wyników pochodzących z różnych gatunków nie jest możliwe w przypadku badań nad genetycznymi podstawami aktywności fizycznej. Wynik ten sugeruje, iż translacyjne podejście do genetycznych badań tego złożonego fenotypu nie jest wskazane.

Podsumowując, badania zgromadzone w niniejszym doktoracie miały na celu: 1) odkrycie nowych genetycznych czynników regulujących poziom aktywności fizycznej; oraz 2) pogłębienie wiedzy dotyczącej wpływu wysokiej aktywności fizycznej na przebieg zaburzeń odżywiania. W rozdziałach od 2 do 5 wskazaliśmy nowe czynniki genetyczne wpływające na poziom aktywności fizycznej myszy i ludzi. Dodatkowo, dzięki badaniom zamieszczonym w rozdziałach od 6 do 8 udało nam się pogłębić wiedzę dotyczącą skomplikowanej roli, jaką aktywność fizyczna odgrywa w przebiegu zaburzeń odżywiania. Wreszcie, podjęliśmy próbę ewaluacji podejścia translacyjnego do badania genetycznych podstaw aktywności fizycznej. Mamy nadzieję, iż wyniki niniejszej pracy zostaną wykorzystane jako podstawa kolejnych, bardziej szczegółowych badań.

List of abbreviations

A	A/J inbred mice
ABA	Activity-based anorexia model
AGRP	agouti-related peptide
AN	anorexia nervosa
ALSPAC	Avon Longitudinal Study of Parents and Children
ANOVA	analysis of variance
B6	C57BL/6J inbred mice
BDNF	brain-derived neurotrophic factor
bp	base-pairs
BMI	body mass index
BN	bulimia nervosa
CA	cornus ammonis
Chr	mouse chromosome
cM	centi-Morgan (unit of genomic distance)
CSS	chromosome substitution strain
CSS2	chromosome substitution strain with a chromosome 2 of an A/J mouse on the C57BL/6J background
CSS19	chromosome substitution strain with a chromosome 19 of an A/J mouse on the C57BL/6J background
Dok5	docking protein 5
DG	dentate gyrus
DSM	Diagnostic and Statistical Manual of Mental Disorders
ED	eating disorders
EDI-2	Eating Disorder Inventory
EDI-III	Shortened version of the Eating Disorder Inventory
EE	excessive exercise
F_1	Final 1 hybrid generation
F_2	Final 2 hybrid generation
FAA	food anticipatory activity
fat%	body fat percentage
Grm8	glutamate receptor, metabotropic 8
h^2	heritability
HC	home cage
hipp	hippocampus
HLPA	high levels of physical activity

HSA	human chromosome
KO	knockout
LLPA	low levels of physical activity
LOD	logarithm (base of 10) of odds
Mc3r	melanocortin 3 receptor
Mc4r	melanocortin 4 receptor
Myof	myoferlin
Nfatc2	nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 2
nonEE	non-excessive exercise
nonRec	non-recovered
NPY	neuropeptide Y
OFT	open field test
PA	physical activity
PI	physical inactivity
POMC	proopiomelanocortin
Ptpn1	protein tyrosine phosphatase, non-receptor type 1
QTL	quantitative trait locus
Rec	recovered
RWA	running wheel activity
sdBMI	body mass index corrected for age and gender
SEM	standard error of the mean
SHIRPA	SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
SIH	stress induced hyperactivity
SNP	single nucleotide polymorphism
SPA	spontaneous physical activity
SSRI	selective serotonin reuptake inhibitors
TwinsUK	Saint Thomas Twin Register
VE	voluntary exercise

List of publications

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Curriculum Vitae

Elżbieta Kostrzewa was born on the 10th of March 1983 in Kraków, Poland. From 2002 till 2007she studied applied psychology at Jagiellonian University in Kraków. This is when she developed a strong interest in biological basis of behavior and neuroscience. In order to follow up on this fascinating topic, she did an internship in 2007 at Rudolf Magnus Institute of Neuroscience (currently known as Brain Centre Rudolf Magnus). After obtaining her master of science title, she started a research career at Institute of Pharmacology, Polish Academy of Sciences in Kraków. During this time, she took part in research projects in Bordeaux and Barcelona. After two years of research experience, she decided to enroll for a PhD program in Clinical and Experimental Neuroscience at Utrecht University, The Netherlands. The experience and knowledge gathered over the four year period (2009-2013) are materialized in the form of the present PhD thesis.

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