Master Thesis
Environmental biology – Behavioural ecology

Rodent models of Autism Spectrum Disorder: strengths and limitations

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Abstract

Autism Spectrum Disorder (ASD) is a psychiatric disease that arises from a complex interplay between genetic and environmental background. Its symptomology comprises social interaction deficits, communication impairments and repetitive behaviours. However, ASD is currently diagnosed based on behavioural phenotypic traits, rather than neurobiological defects. Animal models have been used to gain insight into the aetiology of the disorder, in order to find a 'gold standard' to drive the diagnosis, and to search for treatments. Throughout history, lesion models, genetic models and models manipulating environmental influences have been developed. The effects on the behaviour of the animals are measured using a set of behavioural phenotyping assays. The question arises whether these models are validated through their behavioural symptoms or because of their resemblance with the mechanism that causes ASD. Furthermore, when using the phenotyping strategies, one should take into account the natural behaviour of the animals, thereby evaluating the ecological validity with respect to the human disorder. In this thesis, various types of models for ASD are critically reviewed for their use to mimic the complexity of the developmental biology of the disorder. Face, construct and predictive validity of these models are evaluated, as well as the behavioural phenotyping assays that are applied. Future directions point towards a multi-model approach, in which the body as one functioning system is emphasized. Animal models are always a compromise between mimicking the complexity of the actual disorder and designing practical experiments. However, these experiments should be designed in a way that the animal is minimally restricted in performing its natural behaviour. Combining knowledge, we narrow down the funnel towards not only a gold standard model, but also a gold standard behavioural phenotyping strategy, thereby increasing the translational capacity of the animal model.

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General introduction

Within the past few decades, much research has been done on the aetiology and pathology of Autism Spectrum Disorder (ASD). However, the clinical definition of ASD changes over time, as well as its diagnostic criteria. It is, therefore, crucial that animal models used in autism research are critically revised as well, since outcomes of fundamental laboratory research and application in clinical science are clearly interdependent. The problem with finding the perfect model, however, is that the aetiology of ASD comprises environmental and genetic influences, making it difficult to mimic the factors that predispose a child to develop ASD. Furthermore, when analysing the behavioural effects of the manipulations, the animal's natural behaviour is not always taken into account.

In this thesis the development of animal models is described throughout history of ASD research, thereby critically evaluating the current models for their use according to the animal's natural behaviour, and the continuous discussion on ASD diagnostic criteria. Furthermore, new models are suggested and the need for the development of innovative models is emphasized. Advise to the research field is to practise a multiple-model approach, covering as many symptoms and aetiological aspects as possible, in order to optimize face, construct and predictive validity. However, this approach should not neglect the fact that the body functions as one system, and that manipulations will have effects in areas unrelated to the disorder. Furthermore, behavioural phenotyping assays should respect the animal's natural behavioural repertoire; currently, this is often not the case.

In chapter 1, an introduction on animal models and ASD is provided, as well as an overview of relevant behavioural phenotyping assays used in studies with rodent models of ASD. In chapter 2, lesion models for ASD are discussed, in relation to the acquired knowledge on the neural substrates underlying the disorder. In chapter 3, genetic and epigenetic ASD models are discussed. Chapter 4 provides an evaluation of models in which environmental influences are manipulated in order to resemble ASD symptoms. In chapter 5, conclusions and recommendations for future research are provided.

1. Introduction

1.1. Introduction on animal models

1.1.1. Why use animal models?

Throughout history, animals have been used to investigate the functioning of the living organism, especially man. Documentations dating from 1628 show that animal experimentation has been performed for scientific purposes for centuries (Harvey, 1928). The use of animal models as always has been subject to discussion, which is often accompanied by moral objections (van den Brink, 1989). However, because of the lack of alternatives, animal models are indispensable for the time being. They play a vital role in both fundamental and applied research regarding human pathologies. However, since experiments involving models deal with an animal different from the target animal (humans, in most cases), which may differ in anatomy and ecology, experimenters should be careful with extrapolating their findings to the given human disorder. In order to assess the value of these models, several criteria should be met.

1.1.2. Criteria for an effective model

Effective animal models should meet at least three types of validity, which are; 1) *face validity*, which means that the model has strong analogies to the endophenotypes (i.e. quantifiable components in the genes-to-behaviours pathways (Gould & Gottesman, 2006) of the human syndrome; 2) *construct validity*, which states that the model has the same biological dysfunction that causes the human disease, such as a gene mutation or anatomical abnormality; and 3) *predictive validity*, meaning that the model has an analogous response to treatments that prevent or reverse symptoms in the human disorder (Petit-Demouliere *et al.*, 2005; Silverman *et al.*, 2010; Willner, 1984). Only when these criteria are met, a relationship can be deduced between the origin of the pathology and the behavioural phenotype resulting from it. However, another important criterion is that the phenotyping assays that are applied to assess the behavioural effects of the manipulations, take into account the animal's natural behaviour.

1.1.3. Rodent vs. other (primate) models

Rats and mice are preferable animal models for various reasons. First of all, an important common factor between rats and humans is that they are both species that live in complex, social groups. Therefore, they are equipped with an extensive socio-behavioural repertoire. Rats live in large groups in several (sub-)territorial compositions, and obtain an established dominance hierarchy. However, outside these territories, they are able to harmoniously share resources with other rat colonies. These circumstances demand complex social behaviour, since their survival relies for a large part on the success of group interactions. The rat's behavioural repertoire includes social play, which is known to be a distinct behavioural category and is often used in experiments to demonstrate the intrinsic motivation for social contact in many mammalian species (Vanderschuren *et al.*, 1997). Mice are less suitable subjects for modelling social aspects of human behaviour, since they restrict their social interactions within their own family, and tend to show inflexible territorial aggressiveness towards non-related conspecifics. Furthermore, their play behaviour is less conspicuous and comprises few interaction elements. However, the mouse genome is more easily

modified than that of the rat, which offers many opportunities for the genetic modelling of human disorders. Another important common factor between rats and humans is that they are both dietary generalists and opportunists. The social brain in particular is influenced by food preferences (Dewar, 2004; Galef Jr, 1993). Opportunistic eaters like rats require complex social behaviour in order to learn from conspecifics about the effects of food intake (Galef, 1996). Other advantages of having rodents as animal models are their size, which facilitates a large number of individuals to be kept at limited space, their rapid generation turnover, which facilitates the formation of particular strains, and the fact that they eat a large variety of food types of which they require small absolute amounts, which makes the animals less expensive to keep. Moreover, both species are considered to have an excellent learning capacity, which enables scientists to train them for paradigms that facilitate behavioural measures. The latter makes rats and mice particularly desirable models for behavioural research. Although primates have more genetic overlap with the human species, these animals are difficult to keep and are considered to be beyond the scope of this thesis.

1.2. Introduction on Autism Spectrum Disorder

1.2.1. The aetiology of ASD

Autism Spectrum Disorder (ASD) was first described in 1943 by Leo Kanner and is the most common of the pervasive developmental disorders, since it has a prevalence rate of 10:10.000 (Fombonne, 2003). According to recent definition, ASD is a collective term for several distinct disorders, i.e. autistic disorder, Asperger syndrome, and pervasive developmental disorder not otherwise specified (PDD-NOS). Although its symptoms are extensively described, the aetiology of ASD is still relatively unclear compared to many other psychiatric disorders (Murcia *et al.*, 2005; Silverman *et al.*, 2010). The reason why its origin is so difficult to determine, lies in the fact that it is caused by a combination of genetic, epigenetic and environmental factors (Persico & Bourgeron, 2006). Moreover, evidence points out that there are many independent genes likely to be involved (Murcia *et al.*, 2005; Silverman *et al.*, 2010), making it even more difficult to attribute specific endophenotypes that are essential for the diagnosis of ASD. The (epi)genetic components and environmental factors that are believed to be involved in the causation of the disorder are described in Chapter 3 and Chapter 4, respectively.

1.2.2. The diagnosis of ASD

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), the diagnosis of ASD is based on symptoms from three categories, often referred to as the 'triad of impairments'. These are; 1) abnormal social interactions; 2) communication deficits; and 3) repetitive behaviours (American Psychiatric Association, 2000; see **Appendix 1**). These three categories have long been an important lead in aetiological research. However, there is still debate on whether the distinction between these categories is appropriate. A recent view on ASD that receives much support is that the disorder should be seen as a continuum of autistic-like traits, ranging from minor personality traits to severe impairments in social cognition (Persico & Bourgeron, 2006). In 2013, the American Psychiatric Association will release a new version of the DSM (DSM-V), in which the diagnostic criteria for ASD are revised substantially (Wing *et al.*, 2011; Worley & Matson, 2012). One major revision is that, in the DSM-V, the 'triad of impairments' will be altered to two components, i.e. 1)

abnormal social interaction and communication deficits (which are from now on regarded as a conjoined impairment) and 2) repetitive behaviours. However, people from the research field of psychiatry have responded critically on this modification (Wing *et al.*, 2011), which illustrates the on-going discussion. In addition, the DSM-V will refer to the whole autistic spectrum as a single category of ASD, which will become the new clinical standard. This is why the term ASD is used throughout this thesis.

1.2.3. Rodent models for ASD

When using the DSM-IV criteria, an ideal model of ASD would show phenotypical characteristics of all three categories of the 'triad of impairments'. However, studies have shown that there are few aetiological correlations between these domains, both on the genetic level and environmental influences (Robinson *et al.*, 2012; Ronald *et al.*, 2006). This means that the gross part of ASD symptoms is caused by different genes and environmental cues, thereby suggesting that the domains can be studied independently. However, this contradicts the statement made earlier by the American Psychiatric Association, which state in the DSM-V that the first two of the 'triad of impairment' should be seen as one. As the view on the triad has changed over time, there is a need for a change of view towards animal models used in ASD research as well. Within animal behaviour, the conjugated character of the impairments in social interactions and in communication seems intuitively logical. After all, communication can be viewed as an instrument to emit social behaviour. Throughout this thesis, the association between these two domains will be discussed within the context of the chapter.

As referred to earlier, an ideal model should satisfy as many types of validities as possible in order to be valuable and relevant to the human disorder (Petit-Demouliere *et al.*, 2005). When developing an ideal model for ASD, then, there should be aimed for a model that includes symptoms from all three domains of the triad, with this resemblance based on as many types of validity (i.e. face, construct and predictive) as possible.

1.2.3. Restrictions in ASD modelling

One crucial aspect should be taken into consideration when using animal models for studying a human disorder like ASD, which is the fact that the conditions in which the animals are tested are far from natural. First of all, rats and mice are nocturnal species, whereas testing occurs primarily during daylight hours (Whishaw, 2004), thereby not regarding their natural circadian rhythm, which has substantial influence on its behavioural repertoire (Crawley, 2007; Whishaw, 2004). Furthermore, laboratory mouse and rat strains are pre-selected on many traits, which favours particular behaviours that are different from that of their wild conspecifics (Whishaw, 2004), thereby making it difficult to compare the effects of ASD specific genetic or environmental manipulations.

Another important restriction of these models is the lack of rodent analogies to several other social impairments associated with ASD in humans. For example, many children with ASD are believed to not have fully mastered the concept of theory of mind, which is the ability of a person to comprehend what another person is thinking or feeling (Baron-Cohen, 2000). Theory of mind has not been discerned in rodents, and complicated test paradigms are required to demonstrate this ability in non-human animals. Furthermore, several communication deficits associated with ASD, such as impairments in the interpretation of irony or sarcasm, and the literal use of language, are difficult to simulate in animal models (Silverman *et al.*, 2010). The fact that many, but not all ASD patients have

these deficits, complicates the situation even further. Thus, a distinction should be made between mimicking the different disorders that together form the spectrum of ASD.

As far as methodological considerations are concerned, effects of other body function deficits in the animal models should be excluded. If an animal has, for example, vision or motor deficits, this may inhibit performance in tasks, unrelated to social performance. For this reason, the number of animals needs to be large in behavioural studies (Silverman *et al.*, 2010). Furthermore, many behavioural tests that are used to validate models are one-dimensional, providing few options for the animal to choose between. These paradigms, therefore, test the behavioural outcome rather the actual function of the behaviour. In this thesis, the necessity of behavioural choice and the acquisition of multi-dimensional test paradigms are emphasized.

1.3. Behavioural phenotyping

In order to test whether an animal model applies to the different types of validity discussed earlier, the phenotypic characteristics of the animal have to be analysed. Phenotypes in general include biochemical, anatomical, physiological and behavioural characteristics. The main focus of this thesis, however, lies on analysis of the behavioural phenotype, since ASD is primarily associated with behaviour deficits and no consistent biological markers have been identified so far (Happé & Ronald, 2008). A variety of measure instruments have been developed to study behavioural phenotypes in rodents. Those relevant to models of ASD are described in this section.

1.3.1. 'Natural' behaviour of laboratory rats and mice

In order to quantify the effects of the treatment that the animal model received to resemble aspects of ASD, knowledge is required on the natural behaviour of the animal used. However, as mentioned in Section 1.2.3., a critical consideration here is that laboratory rat and mouse strains are bred specifically for scientific research (Whishaw, 2004). Within a laboratory setting, their behavioural response to the environment may have different consequences than it had in nature. Therefore, following the principle of natural selection, these behaviours may be altered over generations in captivity. Thus, the behaviour of these animals may differ from that of their free-living conspecifics (Whishaw, 2004). For example, laboratory rats are less fearful, less active and have less cognitive abilities (B.M. Spruijt, personal communication). Given this fact, it is important to acknowledge that within an experimental design, behavioural phenotypes of rats and mice should be assessed with respect to their laboratory-raised controls, rather than comparing their behaviour with the wild-type subspecies.

1.3.2. Measuring social interaction abnormalities

According to DSM-IV, symptoms that are associated with social interaction deficits in ASD are; 1) impairments in gestures to regulate social interaction, like eye-to-eye gaze, facial expression and body posture; 2) failure to develop peer relationships appropriate to developmental level; 3) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people; and 4) lack of social or emotional reciprocity, e.g. not actively participating in simple social play or games, preferring solitary activities, or involving others in activities only as tools or "mechanical" aids (American Psychiatric Association, 2000) (**Appendix 1**). Some of these symptoms do not have a

rodent equivalent. However, rats and mice are known to have an internal drive to explore their social environment (Crawley, 2007; Silverman *et al.*, 2010), whose deficit may be an equivalent of symptom 3 described above. Furthermore, social play is a common feature in healthy rats and mice, and has an important role in demonstrating the need for social contact (Crawley, 2007; Vanderschuren *et al.*, 1997). Impairments in social play could, therefore, be considered as an equivalent of symptom 4.

In order to test laboratory rodents on their motivation for engaging in interactions like social exploration or social play, many behavioural paradigms have been designed. Rats and mice are both social animals, and have an extensive repertoire of social behaviours (Crawley, 2007; Silverman *et al.*, 2010). Assays used to measure abnormalities in the social repertoire of these animals have been developed to assess the similarity to the symptoms of ASD in humans. An important test is the one that measures **reciprocal social interactions**, in which the behaviour is scored of either mice or rats grouped together. Important parameters are pushing past each other with physical contact, crawling over and under each other, chasing, mounting, wrestling, nose-to-nose sniffing and nose-to-anogenital sniffing (Bolivar *et al.*, 2007; McFarlane *et al.*, 2008). Rats show more elaborated play behaviour than mice; therefore, the frequency of pinning behaviour is often scored as a measure of social interaction in rats (Vanderschuren *et al.*, 1997; Wolterink *et al.*, 2001). The behavioural testing is often repeated on the same animals over time in order to assess behavioural change throughout different developmental stages (Silverman *et al.*, 2010). A simpler method to measure abnormal social interaction is by the **social approach test**; animals are placed in a three-chamber environment, in which they can choose between approaching either a novel conspecific or a non-social object. The subject animal is said to be more social if it spends more time with the novel conspecific than with the object (McFarlane *et al.*, 2008; Nadler *et al.*, 2004). Another simple paradigm is the **partition test**, in which the subject animal and a conspecific are divided by a partition that disables the opportunity for physical contact, although the animals can see, smell and hear each other. Time spent close to the partition is a measure of sociability (Arakawa *et al.*, 2008). This test can also be used to evaluate social memory, which is defined as time-delayed recognition of a familiar conspecific (Silverman *et al.*, 2010). Social memory can also be evaluated using the **social preference test**, in which the choice between partners is measured by the amount of time spent by the subject animal with each partner (Silverman *et al.*, 2010). The final test to measure social interaction abnormalities in mice and rats is through the ability to **socially transmit food preference**. In this test, the tendency of the subject animal (observer) to obtain meaningful information on a novel food item from a conspecific (demonstrator) is the measure of social interaction (Galef, 2003).

The advantage of the tests described in this section is that they enable researchers to quantify the animals' behavioural outcome in a linear fashion; that is, the degree to which an animal in the experimental group shows the behaviour to a more or lesser extent than their conspecifics in the control group. However, a drawback is that it can be difficult to distinguish between emotional and cognitive aspects of behaviour. For example, an animal model of ASD may be cognitively incapable of social contact, but may as well be less motivated to engage in social contact, or even fearful towards social contact. It is often a challenge to distinguish between the underlying causes of the behaviour, but it is crucial that this distinction is ensued in the light of developing the ideal ASD model. The distinction is possible, since emotional aspects of behaviour involve different neural substrates than cognitive aspects, and therefore models require different manipulations.

1.3.3. Measuring communication deficits

Symptoms associated with the second of the ASD triad, i.e. impairments in communication, are 1) delay in, or total lack of, the development of spoken language; 2) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others; 3) stereotyped and repetitive use of language or idiosyncratic language; 4) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level (American Psychiatric Association, 2000). The fact that rodents are believed not to possess a spoken language like humans do makes it difficult to see a clear rodent equivalent of these human symptoms. However, studies in the past decades have shown that rats use a variety of **ultrasonic vocalizations** to communicate their emotional state to other members of the social group (Brudzynski, 2013; Nyby & Whitney, 1978; Portfors, 2007). It has been found that rats distinguish between two categories of ultrasonic vocalizations; calls of 22 KHz that express a negative state, and calls of 50 KHz that express a positive state (Brudzynski, 2013; Portfors, 2007). Both calls are a product of distinct neural circuits and they have the ability to alter the receiver's affective state. An implication for ASD may be that rat models of ASD may not be able to distinguish between these different calls, since many patients have an impaired capacity to extract information on someone's mental state from their voice (Rutherford *et al.*, 2002).

In addition to ultrasonic vocalizations, both rats and mice communicate through olfactory cues (Keverne, 2002). The leading role of auditory and olfactory modalities is ethologically relevant, since both rodent species are nocturnal and spend much of their time in narrow habitats. Mice and rats both secrete **urinary pheromones** that are used as scent marks, which are an important subject of exploration in this species (Silverman *et al.*, 2010; Tirindelli *et al.*, 2009). A measurement of olfactory communication may, therefore, be the tendency of subject mice to explore the anogenital area of a novel mouse and investigate urinary scent marks in the environment (Arakawa *et al.*, 2008). Another test for social communication deficits is the mouse's ability to **habituate to novel olfactory cues**. Repeated presentation of the same odour through a cotton swab will, therefore, reduce the time spending on sniffing by healthy subjects. Mice that do not show this habituation are believed to be less able to discriminate between novel and familiar odours (Crawley *et al.*, 2007).

Since communication and social interaction are inescapably intertwined, deficits in these areas are likely to be intertwined as well. It may be argued that social interaction models, which already acquired substantial face, construct and predictive validity, may be further investigated for the communication deficits described here.

An important note here is the one already mentioned in Section 1.2.3., stating that when using these behavioural tests, the experimenters should rule out the possibility of impaired auditory or olfactory function as a cause for the abnormal behaviour.

1.3.4. Measuring repetitive and stereotyped behaviours

The third triad of impairments according to the DSM-IV comprises symptoms associated with restricted repetitive and stereotyped patterns of behaviour, interests and activities. These symptoms are; 1) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus; 2) apparently inflexible adherence to specific,

non-functional routines or rituals; 3) stereotyped and repetitive motor mannerisms; 4) persistent preoccupation with parts of objects (American Psychiatric Association, 2000). Considering these symptoms, several rodent analogies have been found. For instance, restricted patterns of interest may be expressed in a reduced motivation to explore a novel environment (Pearson *et al.*, 2011). This is measured as a reduced time spent in novel areas compared to familiar areas. Furthermore, spontaneous stereotyped behaviours have been observed in mice, such as circling, backflips, jumping and self-grooming (Ryan *et al.*, 2010), but can be induced deliberately by introducing the animal into a novel environment. Although rats do not show spontaneous stereotypies, these behaviours can be induced by prenatal exposure of the rats to the teratogen valproate (Reynolds *et al.*, 2012). The duration of self-grooming bouts is often a measure of exhibited stereotyped behaviour in rodents (Pearson *et al.*, 2011; Ryan *et al.*, 2010; Silverman *et al.*, 2010). Insistence on sameness, or behavioural inflexibility, can be tested by use of operant learning or reversal learning tasks, which measure the flexibility of the animal to switch from an established habit to a new habit (Silverman *et al.*, 2010). The Morris water maze and the T-maze are commonly used in experiments that measure the level of behavioural inflexibility in rodents. In these experiments, the animal first requires spatial discrimination training, in which it learns that food is always found in the left arm of the T-maze, for example. Subsequently, the food is transferred to the other arm, and rodent models of ASD are predicted to fail in learning this new location.

2. Lesion models of ASD

2.1. Introduction on lesion models

2.1.1. Function of brain lesion studies

A lesion is defined as an abnormality (i.e. damage) in organismal tissue. In neuroscience, brain lesioning (also referred to as ablative brain surgery) can be informative in understanding the function of the damaged area, by comparing the behaviour of individuals with lesions in an established area to the behaviour of healthy individuals. Ablative brain surgery was first introduced by Pierre Flourens, a French psychologist who removed parts of the nervous system in animals and subsequently studied the effects (Flourens, 1842). Since brain lesions are irreversible, animal models are used to artificially induce brain lesions in order to resemble the specific neuropathologies associated with a particular human disease or disorder. Such models may have a certain level of construct validity, since the same anatomical abnormality is causing the biological dysfunction in both the model and the patients. However, there is always an interpretation problem with lesion studies. The behaviour disappearing or appearing after the brain lesion may be the result of other brain structures, which are related to the lesioned area. Especially when lesioning regions of the limbic system, which is thought to play a crucial role in autism pathology, one should be careful with interpreting the behavioural results. The limbic system serves as an inhibitor on many other brain functions; therefore, lesions in this region may induce disinhibition of other behaviours, resulting in all kinds of behaviours that are no longer controlled. These behaviours are then incorrectly associated with the lesioned area. Furthermore, since artificial lesions are likely to be caused differently than lesions caused by accidents or diseases, lesioning is less helpful in providing insight into the first aetiological steps of a given disorder.

2.1.2. Methods to induce brain lesions

Several methods can be used to induce tissue damage in brain regions. Mechanical lesions (i.e. knife cuts or suctions), electrolytic and radio frequency lesions damage passing fibers and neural cell bodies, whereas excitotoxic or neurotoxic lesioning (i.e. the process of damaging nerve cells by overstimulation of neurotransmitters, like glutamate or NMDA (Glenn *et al.*, 2005) leave these fibers intact. When damaging passing fibers, connected areas in the nervous system that are not necessarily involved with the brain function of interest, may still be affected by the lesion. This may lead to side effects in the behavioural phenotype that are not related to the lesioned brain area. Lastly, temporary lesions can be induced, either by cooling, using anaesthetics or transcranial magnetic stimulation (TMS). Sham lesions in control groups provide the data to rule out potentially unwanted effects from the procedure on the behaviour of the subjects.

A comparative study, in which several lesion methods were used to damage the perirhinal cortex in rats, demonstrated that different lesion methods may result in different experimental outcomes (Glenn *et al.*, 2005), emphasizing that care should be taken in choosing the best suitable method. Furthermore, it should be emphasized that histological examination after the experiment is of high importance, especially when working with rodents. The size of the animals may complicate the accuracy of the lesion procedure. Determining the extent and locus of the lesion by sectioning the brain will increase the validity of the experiment.

2.2. The 'social brain' and the 'autistic brain'

2.2.1. Brain areas involved in human social behaviour

The question which brain areas are involved in human social behaviour has received much attention in past and present research. The function of the social brain seems clear-cut, and is elegantly described by (Frith, 2007); it enables us to interact with other humans, and, importantly, allows us to predict what the other person is going to do next. The better a person is able to make predictions, the more successful the interaction will be. The neurobiological substrates underlying this ability for social cognition and social perception are thought to primarily involve the amygdala, orbitofrontal cortex and temporal cortex (Brothers, 2002), as well as the extrastriate body area, the fusiform face area, the medial prefrontal cortex and the superior temporal sulcus area (Olexová *et al.*, 2012), which together are often termed 'the social brain' (Figure 1). The knowledge on the function of these brain areas in relation to social cognition is mainly based on lesion studies with primates, of which the review of Frith provides an overview (Frith, 2007).

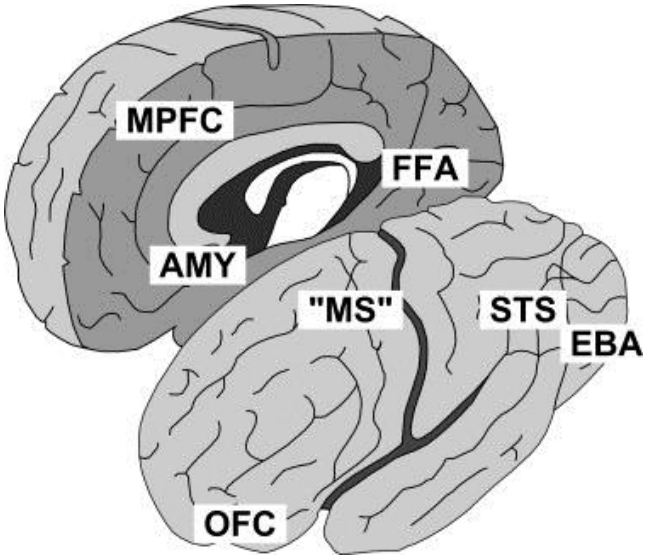

Figure 1. The human 'social brain', comprising areas involved in social perception and social cognition. Abbreviations: AMY = amygdala; EBA = extrastriate body area; FFA = fusiform face area; MPFC = medial prefrontal cortex; "MS" = mirror system – part of parietal lobe; OFC, = orbitofrontal cortex; STS = superior temporal sulcus area.

(Olexová et al, 2012)

2.2.2. Neuropathology of ASD

There is still no universal agreement on which brain structures are involved in ASD and which changes in brain structure or cell composition may attribute to the development of the disorder (Murcia *et al.*, 2005). Though various other psychopathological disorders have clear structural endophenotypes that drive the final diagnosis, ASD still lacks of such a 'gold standard' for identification of the disorder. Since ASD concerns a strictly socio-behavioural deficit, it is unlikely that its aetiology lies in independent neural structures. It is rather expected that ASD emerges through interplay between several brain regions during neurodevelopment, in particular those of the social brain (**Figure 1**), in combination with other factors. Several structural abnormalities have been found in parts of the cerebellum and limbic system in human ASD patients (Murcia *et al.*, 2005), although most outcomes are inconsistent when comparing multiple studies. For example, while some MRI studies on ASD patients found a structural decrease in volume and cell size of the amygdala (Aylward *et al.*, 1999; Herbert *et al.*, 2003), others claimed to have found no structural change (Bailey *et al.*, 1998) or even an increase in amygdala volume (Howard *et al.*, 2000). The same

inconsistencies hold for abnormalities found in the hippocampus (Aylward *et al.*, 1999; Bailey *et al.*, 1998). What does seem to be consistent across autopsy studies is the decreased number of Purkinje cells in the cerebellum of ASD patients (Bailey *et al.*, 1998; Duong *et al.*, 1986; Kemper & Bauman, 1993). However, the sample size of these studies is small, which emphasizes the need for valid animal models to further investigate neural substrates involved in the symptomology. The heterogeneity of structural abnormalities found in ASD patients, supports the earlier stated notion that several factors together cause the typical behavioural phenotype. Another explanation for the inconsistencies may be that the variety of disorders in the autistic spectrum may differ in their neuropathology, and therefore their aetiology should be viewed separately. For example, it is likely that non-mentally retarded ASD patients, like someone with Asperger syndrome, may have a different neuropathology than the ones with severe cognitive impairments. Considering this, the attitude towards animal models may need reconsideration as well, distinguishing different models for different disorders from the autism spectrum.

2.3. ASD lesion models

2.3.1. Models involving amygdala lesions

Although lesion studies were already performed in order to gain more insight into the neural substrates involved in social behaviour, one of the first rodent lesion studies that focused primarily on autism neuropathology was conducted in 2001 (Wolterink *et al.*, 2001). In this study, the effects of amygdala removal on social behaviour in rats were investigated. Amygdala damage was induced by the infusion of the neurotoxin ibotenic acid in male rat pups, either 7 or 21 days after birth (early and late-lesion groups). For behavioural testing, two rats with the same lesion condition were placed together for 15 minutes. Play behaviour was measured as the frequency of pinning (i.e. one animal lying on its back on the floor, with the other animal standing over it (Vanderschuren *et al.*, 1997)). For measurements of social interaction, a beforehand isolated rat was put together with a non-isolated stimulus rat for 10 minutes, in which exploration of the partner by sniffing or licking, approaching or following of the partner, social grooming, and crawling over the partner were scored. Lastly, to quantify exploratory behaviour, an open field test was conducted (Wolterink *et al.*, 2001). Juvenile play behaviour and social behaviour were disturbed compared to sham-lesions, indicating that the amygdala may play a critical role in the display of social behaviour (Wolterink *et al.*, 2001). Additionally, many rats continuously walked along the edges during the open field test, from which the authors concluded they displayed stereotype-like movement patterns after amygdala lesions. The disturbances were found in both early and late amygdala lesion-groups, although the early-lesion group the disturbances were more prominently in later life. In another study, performed by Diergaarde and colleagues, excitotoxic amygdala lesions in rats resulted in reduced behavioural flexibility, which was believed to be a result of disturbed neural connections between the amygdala and medial prefrontal cortex at an early stage in development (de Bruin *et al.*, 1994; Diergaarde *et al.*, 2005). Both studies of Diergaarde (2005) and Wolterink (2001) advocate that the timing of the lesion during the course of neurodevelopment is an important determinant of the effects on later brain function, since connections between different brain areas are formed at an early and delicate stage. This has implications for the development of mental disorders like schizophrenia and ASD (Daenen *et al.*, 2002; Diergaarde *et al.*, 2005; Wolterink *et al.*, 2001).

Animal models in which the amygdala is damaged are claimed to have both face and construct validity for human ASD (Wolterink *et al.*, 2001). The reduced social behaviour, behavioural inflexibility and the stereotype-like movement patterns exhibited by the rats indeed showed similarities with ASD symptoms, supporting face validity. However, as discussed in Section 2.2.2., abnormalities in the amygdala of ASD patients are inconsistent over different studies. Although many studies support the assumption that the amygdala is critically involved in social behaviour, the exact structural deficit (e.g. reduced or increase volume or cell size) that causes the behavioural abnormalities is not yet determined. Furthermore, the authors claimed that the continuous walking pattern of the lesioned rats was a form of stereotyped behaviour. However, this is not the customary parameter used to quantify stereotypies. Instead, the duration of self-grooming bouts is the commonly accepted measure (see Section 1.3.4.). The observed walking pattern along the walls of the compartment may be a normal strategy for an anxious rodent, since it reduces the chance to be exposed to and captured by predators. Therefore, this parameter does not provide ecological validity, and re-analysing of the videos is advised, thereby scoring the self-grooming behaviour of the lesioned rats. These outcomes, then, may provide either acceptance or rejection of continuous walking patterns as a parameter for stereotyped behaviour. Thus, these measurements are necessary to certify construct and predictive validity of these lesion models.

2.3.2. Models involving cerebellar lesions

As already described in Section 2.2.2., both autopsy and imaging studies on human ASD patients have found consistent abnormalities in the cerebellum. This has led to the construction of experiments with cerebellar lesions, in order to gain more insight into the role of this brain area in ASD neuropathology. In one of these studies, the effects of early midline cerebellar lesions on cognitive and emotional functions in the rat were investigated (Bobee *et al.*, 2000). The authors measured motor activity in an actimeter apparatus, during night hours. Lesioned animals showed spontaneous motor activity, interpreted as stereotype-like behaviour, most likely caused by disinhibition of the motor circuitry. Additionally, attentional capacities were measured by letting the animals explore an area with novel objects, and emitting an intermittent noise. This noise altered the exploration behaviour of control rats more than that of lesioned rats, indicating that rats with cerebellar lesions were less susceptible to environmental distractions. Furthermore, anxiety-like behaviour was measured in terms of the subjects' number of visits to the closed arms compared to the open arms of an elevated plus maze, together with quantifying its burying behaviour (burying an electrode in sawdust after the rat had received an electric shock). Lesioned rats spent relatively more time in the open arms, and more lesioned rats than intact rats buried the electrode after receiving an electric shock. These results indicate reduced anxiety levels as a consequence of the cerebellar lesion. However, as the authors already emphasize, the latter result may also be an effect of the decreased pain sensitivity in the lesioned rats, which is also characteristic of ASD patients. Lastly, a social discrimination task demonstrated that the lesioned animals were more neophilic than the control animals, indicating a reduction in anxiety. However, this is contradictive to an expected reduction in explorative behaviour, which is thought to be an important feature of the ASD phenotype (Balemans *et al.*, 2010). Furthermore, many studies also suggest an increased anxiety in the ASD behavioural phenotype, in children with ASD (Skokauskas & Gallagher, 2012) as well as in rodent models of ASD (Balemans *et al.*, 2010). Therefore, the decreased anxiety found in the lesioned animals in the present study does not implicate symptoms of ASD per se.

The role of the cerebellum in ASD pathology seems evident, giving the consistent abnormalities found in human ASD cases, and additionally the results of cerebellar lesion studies. However, the exact role of the cerebellum as a marker for ASD is still not fully understood. According to the authors of the reviewed study above, their experiments demonstrate face validity of the models, since stereotypies and reduced anxiety are common features in autistic patients. However, some of their results seem contradictive with other studies. This demonstrates that it is difficult to ascribe certain behavioural characteristics to damage in one brain area. Moreover, lesion studies may not provide the answer to this question, since cerebellar abnormalities are associated with many other disorders, like ataxia and the cerebellar cognitive affective syndrome (Schmahmann, 2004). A multiple-model approach would be advisory, in which animal models selected for other ASD traits are additionally tested on cerebellar defects.

2.4. Strengths and limitations of lesion models

Lesion models may provide information on which neural substrates underlie the phenotype of ASD, and may therefore provide construct validity for the disorder. The strength of these models lies in the fact that they aid in establishing the double dissociation between brain damage and behavioural abnormalities. An important limitation, however, is that when 'switching off' a specific part of the brain through lesioning, the central nervous system as a whole is likely to be influenced by this manipulation. Therefore, the causational relationship between the biological marker and the behavioural outcome cannot be easily deduced. Moreover, some lesion methods damage passing axon fibres, which may cause dysfunction of connected brain areas. This could result in an altered behavioural phenotype, not primarily caused by the lesioned area. Thus, when using lesion methods, fibre-saving methods like neurotoxic lesioning are preferred.

An important aspect to consider when discussing the validity of lesion models is the plasticity of the brain; when one area is damaged, another area may take over its functions. This holds especially for lateralized brain areas. This complicates the interpretation of lesion studies even further, since the exact function of a lesioned area cannot be ascertained once that function has been taken over by another area, and therefore no abnormal behaviour is observed in the animal. This may be the case especially in the early lesion models described in this chapter. Since the brain is still developing at the time the lesion is conducted, it may be possible that parts of the functions are taken over by other brain regions. However, lesions may have to be conducted early in neurodevelopment, since ASD is hypothesized to arise at a very early stage.

The behavioural tests that are used in the discussed lesion studies, are not always the once of procedural standard. Furthermore, contradictive results have been found. Standardizing procedures is important in this field of research, especially since many aetiological factors are involved and many behavioural parameters seem to be indicative of ASD development. Another limitation is the fact that within these models, brain damage is artificially induced. Therefore, they have no predictive value. However, the search for a consistent structural abnormality in ASD patients may be helpful in both the diagnosis of ASD and research on potential treatments. Although lesion models are still used for this reason, the accumulation of knowledge on genetics and the emergence of gene

knockout technologies caused the behavioural research field to switch to genetic models, which are discussed in the next chapter. However, lesion models should remain a part of the course of research, although they should always be combined with findings from human studies and other models, such as genetic ones, in order to bridge the gaps between face, construct and predictive validity.

3. Genetic models of ASD

3.1. Introduction on genetic models

3.1.1. Research strategies in behavioural genetics

Within the development of genetic models for human diseases, a distinction can be made between; 1) the behaviour to gene – or top-down – approach; and 2) the gene to behaviour – or bottom-up – approach. In the top-down approach, strains of animals that spontaneously show symptoms of a particular disease are studied in order to identify candidate genes that may be responsible for the pathological phenotype. In the bottom-up approach, strains are generated with mutations in these candidate genes, after which behavioural tests are used to evaluate the impact these genes have on the behavioural phenotype (Oddi *et al.*, 2013). In **Figure 2**, both research strategies are illustrated. Usually, the starting point is to identify candidate genes with the top-down approach, followed by studying the behavioural impact of these genes by modifying their expression, either by using germline (e.g. knock-out or transgenic) approaches or somatic technology (e.g. antisense, RNAi, and gene transfer)(Sousa *et al.*, 2006).

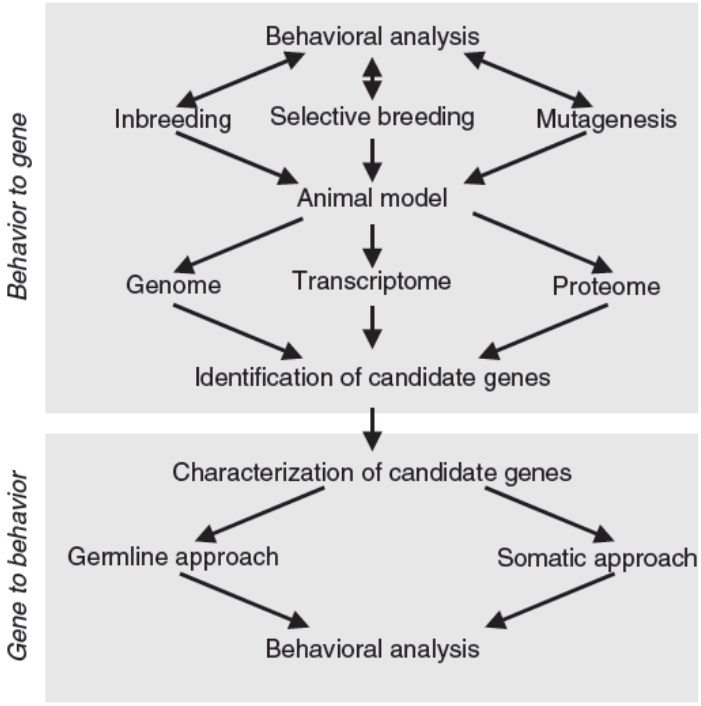

Figure 2. Research strategies in behavioural genetics.
In the 'behaviour to gene' strategy, animal models are acquired by selection on specific behavioural phenotypes, after which candidate genes responsible for the behaviour are identified. Once identified, the behavioural impact of these candidate genes can be studied by germline or somatic approaches (the 'gene to behaviour' strategy).

(Sousa et al., 2006)

3.1.2. The genetic basis of ASD

Although the neuropathology of ASD is not yet clear, twin and family genetic studies provide evidence that the disorder has a genetic component. However, with this technique, the same problem applies as in the lesion models discussed in the previous chapter; one cannot infer the role of a single gene in behaviour, since behavioural traits are mostly polygenic. Therefore, the modification of a single gene will unlikely lead to the (dis)appearance of that behaviour. To the most, it may influence the range of variation in which that behaviour can occur (Sousa *et al.*, 2006). Nevertheless, the fact that multiple genes are involved in the aetiology of ASD does not mean we

cannot identify them. Many candidate genes have been proposed, identified using linkage and association studies, genome scans, chromosomal rearrangement analysis and mutation screenings (Oddi *et al.*, 2013). The genetic component of ASD aetiology believed to comprise an interaction between multiple molecular defects during brain development. The products of the proposed candidate genes fall into distinct functional categories, which are; 1) chromatin remodelling and regulation of transcription (MeCP2, FMR1); 2) actin cytoskeleton dynamics (TSC1, TSC2, NF1); 3) synaptic scaffolding proteins (SHANK3); 4) neuro-receptors and transporters (GRIN2A, GRIK2, GABAR, SLC6A4, SLC25A13, OXTR, AVPR1); 5) second-messenger systems (PRKCB1, CACNA1C, NBEA); 6) cell adhesion (NLGN3, NLGN4); and 7) secreted proteins (RELN, LAMB1) (reviewed by (Persico & Bourgeron, 2006). These are many, but by far not all candidate genes proposed by the scientific community. This illustrates the genetic basis of ASD: strong heritability, but no single, easily identifiable, disease locus (Murcia *et al.*, 2005). Despite the polygenic character of ASD aetiology, however, mutations have been identified that can cause a large part of the autistic phenotype, while located in a single locus. These monogenic mouse models are, therefore, believed to be informative on the underlying mechanisms causing the development of ASD (Ey *et al.*, 2011). These single gene knock-out models, as well as polygenic models, are reviewed in Section 3.2.

3.1.3. Epigenetic factors influencing ASD

During the past decade, it has been proposed that the genetic basis of ASD and many other psychiatric diseases is further complicated by epigenetic contributors (Gould & Gottesman, 2006). A heterozygous mutation in the *Mecp2*-gene causes Rett syndrome in females, which causes an autistic phenotype, among other abnormalities. MeCP2 causes transcriptional silencing by binding to methylated promoters, and recruiting co-repressor and histone deacetylase complexes. The reduced MeCP2 activity causes transcriptional de-repression at specific promoters that are essential to brain development and plasticity, including those regulating levels of brain-derived neurotrophic factor (BDNF), which may result in the development of ASD (Chen *et al.*, 2003). In several studies, a mouse model of Rett syndrome (*Mecp2*$^{308/y}$) has been used to examine the role of MeCP2 activity in the acquisition of autistic symptoms in Rett syndrome (Moretti *et al.*, 2005; Shahbazian *et al.*, 2002). Social interactions were impaired in these animals, which became apparent from a social interaction test without physical contact. However, the *Mecp2*$^{308/y}$ mice did not differ from wild-type mice in behaviour during a resident-intruder test (Moretti *et al.*, 2005). In another experiment, the same mouse model was used to test whether Rett syndrome features were visible (Shahbazian *et al.*, 2002). The *Mecp2*$^{308/y}$ mice in this study were shown to display many symptoms that are similar to human Rett syndrome, but also characteristic of ASD, such as motor impairments, increased anxiety, stereotypic forelimb motions, and seizures (Shahbazian *et al.*, 2002). The methods used to find these results were a vertical pole test, a suspended wire test, a grip strength test, a rotating rod test, a dowel test, an open field test, a tube test, a resident-intruder test, a conditioned fear test and a Morris water task. However, the same 19 wildtype and 24 mutant mice were used for all these 10 tests. When such a high number of tests is used on a relatively small number of animals, it is to be expected that some results correspond to the hypotheses. No corrections were applied on the results, making the conclusions of this study quite debatable. Again, this stresses the need for not only a gold standard model, but also a gold standard behavioural phenotyping strategy.

Next to alterations in methylation processes, it is also hypothesized that mother-infant interactions, like maternal care, may influence brain and neuroendocrine development on an epigenetic level (Champagne & Curley, 2005). Although little is known about implications on this matter regarding ASD pathology, the fact that rats and mice provide maternal care to their pups may facilitate the development of models that can be used to investigate the role of maternal care in ASD aetiology.

3.2. Genetic models of ASD

3.2.1. *Mouse models of ASD*

The development of genetic models for ASD started with the emergence of single gene knock-out mouse models. An example is the oxytocin (OT)-KO and OT receptor (OTR)-KO mice. The candidate genes deleted in these models were identified through human studies, from which oxytocin appears to be an important factor in autism aetiology (Modi & Young, 2012). The OT-KO and OTR-KO mouse models are used to characterize the behavioural impact of genetic defects in oxytocin regulation, following the 'gene to behaviour' approach from Figure 2. Thus, in these types of models the mechanism of the disorder is leading, i.e. investigating whether these genes cause the behavioural defects, rather than the behavioural symptoms of the disorder. These mice displayed deviant ultrasonic vocalizations as infants, as reduced calling rates were observed in OT-KO pups compared to wild type pups when separated from their mother. A hypothesized explanation for this observation is that the OT-KO mice may be less sensitive to maternal separation, due to the lack of oxytocin (Winslow & Insel, 2002). Furthermore, the OT-KO mice exhibit social recognition deficits, observed as a decrease in olfactory investigation of cage mates. These behavioural abnormalities indicate a resemblance with autistic symptoms (Winslow & Insel, 2002). However, these KO mice may not be a good model for the human disorder, since it has been found that mice (in contrast to rats and humans) do not need oxytocin in order to exhibit normal sexual and maternal behaviour (Winslow & Insel, 2002). Thus, the behavioural mechanisms regulated by oxytocin may work differently in mice than in rats or humans, emphasizing the fact that ethological relevance is an important factor that should not be forgotten in the light of animal models. Most importantly, another difficulty here is that the principle of knock-out models (i.e. switching off a gene) underestimates the complexity of ASD development. To continue with the oxytocin example, ASD is not likely to be caused by the total lack of oxytocin production, for example, since human ASD patients have lowered but not an absence of plasma oxytocin levels (Modahl *et al.*, 1998). More likely, the regulation of oxytocin production or its functioning in distal pathways may be disrupted, partly causing the behavioural phenotype. Therefore, these models may provide face validity, but not construct validity. Furthermore, switching off a gene is quite rigorous, and will most likely influence other systems of the brain that are not involved in the pathology of ASD. The behavioural consequences may lead to invalidated conclusions on involvement of these brain areas in ASD aetiology. As a last objection, knockout experiments do not emphasize the plasticity of the brain. After a mutation, such as a deletion of a specific gene in the case of knockout models, it is likely that mechanisms are activated to compensate for the loss of gene activity (Routtenberg, 2002). Therefore, analysing the behaviour of knockout mice is above all an analysis of the animal's ability to cope with the deletion (i.e. its plasticity), rather than an analysis of the deleted gene's function (Sousa *et al.*, 2006). An alternative for these knockout models, therefore, are the nowadays commonly used conditional gene knockout models. This technology is relatively new, and comprises

the knockout of a target gene in a single organ or brain structure, rather than switching it off throughout the body. This allows for more sophisticated experiments that are more scientifically precise. An additional advantage is that conditional null-mice live longer than traditional null-mice. An example study that demonstrates the advantages of conditional over conventional knockout mouse models is provided by (Ferguson *et al.*, 2007), who engineered a mouse line with a conditionally inactivated β3 subunit of the γ-aminobutyric acid type A receptor ($GABA_A$-R), which has been identified as a candidate gene for Angelman syndrome and ASD (Ferguson *et al.*, 2007). Behavioural and other parameters were compared between global β3 knockout mice and forebrain selective β3 knockout mice. Measurements relevant to ASD were spontaneous locomotor activity assessment using an automated open field test apparatus, and operant learning through a passive avoidance task. Both groups of knockout animals displayed more locomotor activity and impaired passive avoidance learning compared to the control mice (Ferguson *et al.*, 2007), indicating that; 1) conditional knockouts have the same behavioural phenotype as global knockouts, but do not have the unwanted features of global knockouts; 2) absence of the β3 gene is partially responsible for the emergence of autistic-like symptoms in mice.

Other mouse models that are the most frequently used in ASD research are the BTBR, C58/J, BALB, and Fmr1-KO mouse strains. These are all inbred strains that display behavioural traits with face validity to one or more of the diagnostic criteria of ASD (social or communication deficits, display of repetitive behaviours), and experimental data on these mouse models and others are abundant (for reviews, see (Provenzano *et al.*, 2012; Silverman *et al.*, 2010). Many of these strains are developed specifically for modelling other pathologies, like fragile X syndrome (*Fmr1*) or Rett syndrome (*Mecp2*), but tend to display the same behavioural abnormalities that are typical for ASD. They are thus thought to contribute to understanding the coherent molecular mechanisms underlying these disorders. **Table 1** provides an overview of these models, their behavioural characteristics relevant to ASD, and the behavioural tests that were used to validate the presence of these behaviours. Considering the ASD triad of impairments, the following can be concluded: 1) social interaction deficits are most frequently studied using social interaction tests, social approach tests, and/or a three-chambered choice paradigm. These assays are often one-dimensional, in which the animal has a small amount of behavioural choices to make; 2) communication deficits are primarily observed as abnormal ultrasonic vocalizations, whereas the processing of social olfactory information is rarely studied; 3) repetitive behaviours are often observed as increased self-grooming and motor stereotypies.

Table 1. Most frequently used genetic mouse models and behavioural phenotyping assays in ASD research.

Mouse model	**ASD-like behaviours** (Adopted from Silverman *et al.*, 2010)	**Behavioural tests**
BTBR	• Reduced reciprocal social interactions • Low sociability • Increased repetitive self-grooming • Reduced social transmission of food preference (STFP) • Ultrasonic vocalizations elevated in pups and reduced in adults • Unusual ultrasonic vocalization call categories in pups and adults	• Social interaction and social approach test • Scoring spontaneous grooming behaviour • STFP test • Classification of SUVs with ultrasonic microphone (McFarlane *et al.*, 2008; Scattoni *et al.*, 2008; Yang *et al.*, 2007)

C58/J	• Low sociability • High level of repetitive motor stereotypies • Increased repetitive self-grooming	• Three-chambered choice test • STFP test • Social test box • Morris water maze • Scoring repetitive behaviours (Moy *et al.*, 2008; Ryan *et al.*, 2010)
BALB	• Low sociability • No genotype differences in preference for social novelty • Reduced reciprocal social interactions • Reduced ultrasonic vocalizations in adolescent same-sex social interaction • Reduced place-conditioned social reward • Reduced social learning during social distress	• Social interaction test, social behaviour test • Recording with ultrasonic microphone • Social conditioned place preference (Moy *et al.*, 2007; Panksepp & Lahvis, 2007; Panksepp *et al.*, 2007)
Fmr1 KO	• Increased social approach • Reduced reciprocal social interactions • No genotype differences in sociability • No genotype differences in preference for social novelty • Low sociability dependent on genetic background • No genotype differences in preference for social novelty	• Social interaction test • Three-chambered choice test (McNaughton *et al.*, 2008; Moy *et al.*, 2009; Spencer *et al.*, 2008)

3.2.2. *The emergence of ASD rat models*

Although the rat was the first mammalian species that was used for research purposes specifically, and has quickly become the most widely used animal model in behavioural neuroscience (Suckow *et al.*, 2005), the animal has been less suitable for genetic manipulations than mice. Therefore, mice have long been the standard model for genetic manipulations in behaviour sciences. However, as mentioned earlier in Chapter 1, rats show more complex social behaviour then mice do, and many behavioural phenotyping assays (e.g. the Morris water maze and the elevated plus maze) are originally based on rat behaviour. Therefore, with the emergence of knock-out mouse models, mouse behaviour is often analysed as if they were rats, attributing social and cognitive capacities to them that they normally would not possess. Nevertheless, until recently, the use of mouse models was the only way in which the genetic components of behaviour could be investigated.

Within the past years, however, there have been new developments in the generation of rat knock-out models, which are promising for future research within the field of behavioural genetics. A lab developed a target knock-out rats that are commercially available for autism research, including *Fmr1*, *Mecp2*, and *mGluR5* knockout rats (www.sageresearchmodels.com). Apart from their increased socio-behavioural similarity to humans, there are more advantages to using rats instead of mice as genetic models for ASD. For instance, the metabolic physiology of rats is closer to humans than in mice. This makes the rat a more suitable animal to study the molecular metabolic processes involved in ASD aetiology. In addition, their larger size facilitates surgical manipulations to be carried out more accurately. All in all, it is plausible that the rat will recapture its place in behavioural research in the future. However, as discussed in Section 3.2.2., knockout models are not the most

optimal genetic models to work with, especially regarding the complexity of ASD as a behavioural disorder with its aetiology based on an interaction between multiple genes. Therefore, more developments in rat genetic manipulations are necessary in order to conduct an ideal rat model for ASD.

3.3. Strengths and limitations of genetic models

Genetic rodent models are used to study the validity and impact of candidate genes that are postulated to play a role in ASD susceptibility. Although single knockout mice still dominate the research field, they do not reflect the complexity of the behavioural disorder for several reasons. First, the deletion of a gene may result in alternations in protein activity elsewhere in the body, which are not related to the aetiology of ASD, but may be interpreted as such, when they affect the behavioural phenotype of these animals. The use of conditional knockout models, which affect target genes only in specific areas, may attribute to the face validity of these models. Second, ASD development is likely to be caused by an interaction between multiple genetic defects, which cannot be modelled in a single gene model. Polygenic models may, therefore, contribute to the acquirement of knowledge on ASD aetiology, since it accentuates its complexity. Third, given the fact that mouse models are the current standard in behavioural genetics instead of rats, the species-specific behavioural differences should be taken into account. The socio-behavioural and communication repertoire of mice is less sophisticated compared to that of rats and humans. Consequently, the currently used behavioural phenotyping assays that were primarily designed to test behavioural capacities of rats, may not be sufficient to extrapolate mouse behaviour to human behaviour. Furthermore, in many behavioural assays, such as the three-chambered choice test, the subject animals do not have many behavioural options to choose from. This may affect the conclusions that can be drawn from behavioural choice, as the natural behaviour of the animal is not fully appreciated in these situations. The new technological developments enable scientists to genetically engineer knockout rats. Since rats reflect human social behaviour more than mice do, this is a promising development in behavioural genetics, and can attribute to revealing the puzzling aetiology of ASD.

Putting these considerations altogether, genetic rodent models of ASD are of great value in gaining knowledge on the biological mechanisms underlying the aetiology of the disorder. However, it remains a fact that the body works as a system, and the consequences of manipulating one part of that system are not easily identified. The use of genetic models alone will, therefore, not be sufficient in unravelling the aetiology of ASD completely, which is thought to comprise genetic, epigenetic and environmental influences. Furthermore, genetic models will only provide face validity, but not construct validity for the disorder, since the mutations lead to biochemical disturbances seen in ASD, but do not cause the structural, neuropathological abnormalities. In order to take into account the multi-dimensional character of the disorder, a multi-model approach is necessary, which combines not only genetic models, but also models that illustrate the epigenetic factors that drive the development of ASD. However, the latter models yet have to be developed. Finally, these models need to be combined with models illustrating the influence of the environment on the aetiology of ASD, which are discussed in the next chapter.

4. Models for environmental factors

4.1. The 'chemistry' of ASD

By far the largest group of ASD patients have idiopathic ASD, which means that its aetiology is unknown. However, 10% of the patients have 'syndromic' ASD, which means that the symptoms attributed to autism are either secondary to a known genetic disorder, such as fragile X syndrome or tuberous sclerosis, or caused by exposure to teratogens early in development, such as valproate (Christianson *et al.*, 1994) or thalidomide (Strömland *et al.*, 1994). These findings have led to the hypothesis that ASD susceptibility is not only determined by (epi)genetic defects, but that the disorder can also arise in the first stages of embryonic development (Murcia *et al.*, 2005).

When constructing models that mimic the development of syndromic ASD, the behavioural symptoms are the point of departure in validating these models, rather than the resemblance of the underlying mechanism. This means that face validity is often provided, but construct validity is hardly ever provided using these models. The most widely used models to demonstrate the influence of environmental factors on the aetiology of ASD are; 1) prenatal exposure to neurotoxins, like valproic acid (VPA), thalidomide or sevoflurane; and 2) prenatal or perinatal exposure to viral agents, like Borna virus, Rubella virus, and influenza. These models are often used to further explore the neurochemistry of ASD. For example, the inflammations in the brain caused by prenatal exposure to viral agents are believed to cause immune dysfunction, which may be similar to the mechanism that causes ASD. Finally, a growing body of evidence suggest that alterations in neuropeptide mechanisms, such as the opiate or glutamatergic system, are associated with ASD aetiology. The models developed for studying the role of these factors are discussed in the remainder of this chapter. Simultaneously, **Table 2** provides an overview of the most frequently used models and behavioural assays regarding environmental manipulations.

4.2. Constructing a model through environmental manipulations

4.2.1. Prenatal exposure to neurotoxins

Valproic acid (VPA, or valproate) is a chemical agent that is used primarily for the treatment of epilepsy, bipolar disorder, and migraine. However, when taken during pregnancy, the embryo has an increased risk of developing autistic symptoms, which is referred to as the fetal valproate syndrome (Christensen *et al.*, 2013). This effect has led to the use of prenatal exposure to VPA as an animal model for ASD (Bristot Silvestrin *et al.*, 2013; Kataoka *et al.*, 2011; Kim *et al.*, 2013; Markram *et al.*, 2007; Markram *et al.*, 2007; Markram *et al.*, 2007; Markram *et al.*, 2007; Persico & Bourgeron, 2006; Schneider & Przewlocki, 2004). The agent is thought to be particularly neurotoxic during a specific time in embryonic development, that is, during the closure of the neural tube. Among other defects, the neural damage causes cognitive impairments that are associated with autistic symptoms. Studies are abundant in which rats prenatally exposed to VPA displayed autistic-like behavioural alterations. Social interaction tests demonstrated lower sociability in VPA rats (Kim *et al.*, 2011; Markram *et al.*, 2007; Schneider & Przewlocki, 2004). Furthermore, the rats displayed impaired exploratory behaviour during an open field test (Schneider & Przewlocki, 2004). Additionally, a decreased number of visits to the open arms in an elevated plus maze indicated that rats exposed to VPA

displayed increased anxiety levels (Markram *et al.*, 2007). VPA rats were less sensitive to painful stimuli (Markram *et al.*, 2007; Schneider & Przewlocki, 2004), but more sensitive to non-painful stimuli (Schneider & Przewlocki, 2004). Lastly, repetitive behaviours were increased in VPA rats, which was demonstrated by a spontaneous alteration paradigm comprising a Y-maze (Markram *et al.*, 2007) and by registration of three consecutive photobeam breakages (Schneider & Przewlocki, 2004). Prenatal VPA treatment in mice gave similar results, indicating from the behaviour of subjects during a social interaction test, open field test, and an elevated plus maze (Kataoka *et al.*, 2011). However, the exact mechanism through which valproate exposure results in the behavioural abnormalities observed in ASD is still obscure. A recent study suggests that VPA-induced histone hyperacetylation plays a key role in the formation of the abnormal phenotype (Kataoka *et al.*, 2011).

Thalidomide was introduced as a drug in the 1950s, after it was discovered to reduce morning sickness in pregnant women. However, it has been withdrawn in 1962, after it was found to have damaging effects on the embryonic development. Offspring of women who used thalidomide during pregnancy had an increased risk of developing morphological abnormalities and symptoms of ASD (Strömland *et al.*, 1994), similar to the effects of VPA. However, thalidomide seems to not have the same teratologic effects in rats than it has in primates (Schumacher *et al.*, 1972), which is why rodent models involving prenatal thalidomide exposure may be not valuable for extrapolating the neurotoxic effects on rodents to the aetiology of human ASD. Thus, VPA-exposed rodents are the most widely used models to demonstrate environmental influences on ASD development.

4.2.2. Prenatal exposure to viral agents

Besides exposure to neurotoxins, ASD symptoms can also arise from exposure to viral infections during crucial stages in neurodevelopment (Atladóttir *et al.*, 2010; Libbey *et al.*, 2005). There is a growing body of evidence that maternal infection by these viruses lead to early immune dysregulation, which may be involved in the development of ASD (Patterson, 2009). The dysregulation in the brains of autistic patients comprises microglial and astrocyte activation, as well as cytokine up-regulation in the brain and the cerebrospinal fluid (Pardo *et al.*, 2005; Vargas *et al.*, 2005). Prenatal exposure to certain viral agents lead to activation of the maternal immune response (Patterson, 2009). Children with congenital rubella, for example, are known to display higher levels of autistic symptoms than healthy children (Chess *et al.*, 1978). In the same way, exposure to the cytomegalovirus during early development may induce ASD in some cases (Yamashita *et al.*, 2003). However, literature on rodent models involving infection with these viruses is scarce. A virus infection that is commonly used to model ASD in rats is a prenatal infection with the Borna disease virus (BDV). BDV rats display some autistic-like behaviours, and are therefore often used as an animal model for ASD (Lancaster *et al.*, 2007; Pletnikov *et al.*, 2002). For example, BDV infected rats showed a decreased number of social behaviours during a social interaction test (Lancaster *et al.*, 2007). However, there are no case studies available of human ASD patients associated with prenatal BDV infection, which is why this model does not provide predictive validity. Nevertheless, the fact that the animals and autistic patients share the same symptomology indicates that face validity is provided. Therefore, this model may be helpful in understanding these and other underlying environmental mechanisms that can lead to ASD development.

4.2.3. Juvenile isolation

Early social experiences are essential for the development of normal social behaviour. The consequences of juvenile isolation for later social interactions have been studied widely in rats (for review, see (Hall, 1998)). Socially deprived rats display decreased social behaviour, dependent on the developmental stage in which the period of deprivation took place (Hall, 1998). An experiment comprising a social interaction test with early isolated rats demonstrated that socially deprived rats display a decrease in social approach, anogenital sniffing and social exploration, in addition to a longer latency until the first social interaction. However, the structure of these behaviours was unaffected by the treatment (Van Den Berg, Caroline L *et al.*, 1999). Studies have shown that the consequences of juvenile isolation cannot be reversed by rehousing with socially reared rats (Hol *et al.*, 1999). This emphasized the importance of social interactions at a young age, in which the rats display high levels of social play behaviour (Vanderschuren *et al.*, 1997). In a study in which amygdala lesioning was combined with social deprivation in rats, it was found that juvenile isolation resulted in a decreased duration of social sniffing. Furthermore, after a combination of amygdala lesioning and juvenile isolation, the total duration of social interactions decreased (Diergaarde *et al.*, 2004). Apart from describing the necessity of social stimuli for normal development, this study demonstrates the results of using a multi-model approach, combining two potentially successful models of ASD. However, one should be careful with using multiple models that have not yet been fully validated. In this situation. The neurodevelopment of the model animals is manipulated at multiple levels, making it difficult to ascribe behavioural abnormalities to a particular cause. Moreover, when manipulating more than one aspect, it is likely that both defects together result in even more complicated phenotype alterations. Therefore, the combination of models should be carefully applied.

4.3. Models involving alterations in neuropeptide mechanisms

4.3.1. Models involving opiates

In 1979, Panksepp proposed that the social behaviour deficits characteristic for autism are caused by endogenous overactivity of the opiate system in the autistic child's brain (Panksepp, 1979). The fundamental theory of his study was based on the assumption that similar brain mechanisms are responsible for both narcotic addiction and the drive to seek the company of others. In his early experiments, Panksepp administered doses of opiate drugs to animals, and found autistic-like alterations in their behaviour. The animals were less motivated to seek social contact, which became apparent when rats who received morphine chose food items over a social stimulus in a T-maze (Panksepp *et al.*, 1981). Additionally, these animals were less sensitive to painful stimuli, and displayed unusual learning effects, characterized by extreme persistence of behaviour, without being presented any external reward (Panksepp, 1979). His findings have led to the use of models involving alterations in the opiate mechanism to mimic ASD symptoms in rodents. However, these models do not provide predictive validity, since ASD patients do not clearly benefit from opiate antagonist therapies with naloxone or naltrexone (Tordjman *et al.*, 2007). Nevertheless, the earlier findings of Panksepp resulted in more detailed studies on the underlying mechanisms involved. Other studies found as well that the opiate system mediates social reward in rats (Trezza *et al.*, 2011; Vanderschuren *et al.*, 1995; Vanderschuren *et al.*, 1997). The authors found that morphine administration increased play behaviour in rats, which is a behaviour that is believed to have a high social reward value in these animals. Alternatively, administration of opioid antagonist naloxone

reduced play behaviour (Trezza *et al.*, 2011). This indicates that manipulating an animal's need for social reward by administering opiate drugs has the potential to be a suitable model for ASD.

4.3.2. Models involving glutamate

Although the exact mechanism is not clear, ASD is believed to involve disturbance in excitation or inhibition of neurotransmitters during crucial stages of neurodevelopment (Rubenstein & Merzenich, 2003). Since glutamate is the main excitatory neurotransmitter in the nervous system, it is proposed to play a role in ASD aetiology. Therefore, animal models involving manipulation of these neurochemicals may be potentially valid models for studying ASD. Within the past years, astrocytes are thought to be important as well, since they maintain the neuronal microenvironment and modulate excitatory synapses (Bristot Silvestrin *et al.*, 2013). A post-mortem study, which examined the brains of 10 autistic patients, found abnormalities in the AMPA-type glutamate receptors and glutamate transporters in the cerebellum. These abnormalities were suggested to be directly involved in ASD pathology (Purcell *et al.*, 2001). Additionally, in a recent study proton magnetic resonance spectroscopic imaging was used to unveil significantly higher blood and brain glutamate levels in patients with ASD, compared to healthy controls (Hassan *et al.*, 2013). The raised levels of glutamate found in ASD patients in this study indicate that ASD may be a hyperglutamatergic disorder. In contrast, a few years earlier, it was suggested that the development of ASD may be caused by a hypoglutamatergic defect, based on findings that NMDA antagonist administration induces ASD-like symptoms in healthy subject mice (Carlsson, 1998). Mice were injected with NMDA antagonist reserpine, which stimulated their motor activity, according to the measurements with an electronic motility meter (Carlsson & Carlsson, 1989). However, the NMDA antagonists are not known to have an effect on sociability and communication features. These results altogether indicate that the role of the glutamatergic system in ASD pathophysiology is rather complex, and does not only consider an excessive or decreased amount of the excitatory neurotransmitter. Other studies examined the possibility of glutamatergic pathways to function as a pharmacological treatment for ASD (Burket *et al.*, 2011; Silverman *et al.*, 2009). Both studies used genetic mouse models of ASD in order to assess the effects of glutamate antagonist MPEP on the ASD-like behavioural repertoire of these animals. Silverman and colleagues used BTBR mice (see Chapter 2), and examined the effect of MPEP administration on sociability and self-grooming. They used a three-chambered choice test to assess social approach, and measured the cumulative time spent grooming to assess the tendency for repetitive behaviours. The authors found a reduction in self-grooming, but sociability in the BTBR mouse strain did not improve (Silverman *et al.*, 2009). Burket and colleagues used BALB/c mice, another mouse model of ASD (see Chapter 2), and tested the effect of MPEP on their sociability, using a three-chambered choice test. The authors conclude that although some aspects of social behaviour were improved, like an increased time spent sniffing the stimulus mouse, others were unaffected, like the time spent in the compartment containing the stimulus rat (Burket *et al.*, 2011).

Although the latter experiments indeed suggest that the glutamatergic system may be involved in ASD development, the role of glutamate antagonists as a treatment for the symptoms is not yet clarified. Moreover, the fact that the authors used genetic mouse models to test the effect of glutamate antagonists may not be pragmatic, since these models themselves still do not provide face, construct and predictive validity. Therefore, results from these studies should be interpreted with caution.

4.3.2. Models involving oxytocin

Oxytocin (OT) plays an important role in social processing and social bonding. Autistic children are found to have lower levels of OT (Modahl *et al.*, 1998). Furthermore, intranasal OT administration in ASD patients results in an increase in social interaction, trust and attention to socially informative stimuli (Andari *et al.*, 2010). These findings suggest that animal models with reduced OT production or abnormal OT processing are believed to be valid models of ASD (for review, see (Modi & Young, 2012). Although genotype based models of OT, like the OT-KO and OTR-KO mice, are already discussed in the previous chapter, environmental manipulations of the OT system are also used as a model. For example, intracerebroventricular administration of oxytocin in rats is demonstrated to lead to more social interactions between adult rats (Witt *et al.*, 1992). In this study, osmotic pumps were implanted in male rats, which delivered OT to their system. The measured behavioural parameters were the latency, duration and frequency of sexual interactions with a female stimulus rat, and exploratory behaviour in an open field. It was found that OT-males engaged in physical contact twice as long as controls did, even in the absence of sexual interactions. The OT-males additionally displayed more anogenital sniffing and autogrooming, but their sexual behaviour appeared to be unaffected by the OT treatment. The authors propose that oxytocin increases non-sexual social interactions by altering olfactory and somatosensory processing (Witt *et al.*, 1992). This may have implications for ASD, since OT increases sociability, also in humans (Andari *et al.*, 2010). However, the rats in this study displayed increased self-grooming, which is often interpreted as stereotyped behaviour. Therefore, the model does not give consistent results regarding the ASD triad of impairments.

Studies on oxytocin administration in rodents in relation to ASD are not abundant. However, several studies can be found in which other animals, like primates or pigs, or humans were used to examine the effects of oxytocin on social interactions. However, these are beyond the scope of this thesis.

Table 2. Most frequently used models and behavioural tests illustrating environmental influences on ASD aetiology

Rodent model	Model type	ASD-like behaviours	Behavioural tests
VPA	Neurotoxin exposure	• Lower exploratory behaviour • Lower sensitivity to painful stimuli • Decreased social interactions • Increased repetitive behaviours • Increased anxiety	• Small open field with holes in wall • Tail flick and thermal paw withdrawal test • Infants: pinning behaviour. Adults: social interaction test • Measure breakage of photobeams, spontaneous alteration paradigm • Elevated plus maze (Kim *et al.*, 2011; Markram *et al.*, 2007; Markram *et al.*, 2007; Schneider & Przewlocki, 2004)
Borna disease virus	Viral agent exposure	• Decreased social interactions	• Social interaction open field test (Lancaster *et al.*, 2007; Pletnikov *et al.*, 2002)
Juvenile isolation	Housing strategy	• Reduced social exploration • Reduced anogenital sniffing • Reduced approach/following	• Social interaction test (Van Den Berg, Caroline L *et al.*, 1999)

Opiate	Neuropeptide administration	• Reduced nee for social contact • Less sensitive to painful stimuli • Extreme persistence of behaviour	• T-maze, play behaviour (Panksepp *et al.*, 1981; Trezza *et al.*, 2011)
Glutamate antagonist	Neuropeptide administration	• Slightly improved sociability in BALB/c mice • Reduced self-grooming in BTBR and BALB/c mice	• Three-chambered choice test • Cumulative time spent grooming (Burket *et al.*, 2011; Silverman *et al.*, 2009)
Oxytocin	Neuropeptide administration	• Increased non-sexual social interactions • Increased autogrooming	• Social interaction test with female • Open field test (Witt *et al.*, 1992)

4.4. Strengths and limitations of environmental models

Like many other psychiatric disorders, ASD is most likely caused by an orchestration of genetic and environmental factors. Although a child may be predisposed to ASD due to its genetic background, prenatal and postnatal experiences may have substantial influence on its course of neurodevelopment. The models mimicking these experiences might be a valuable way to gain insight into the perinatal processes underlying ASD pathophysiology. Additionally, external manipulations, like infusing neuropeptides or inducing inflammations, are more easily achieved than genetic manipulations. This is especially the case in rats, since their genome is more difficult to manipulate than that of mice. Since we have already established that rats are a more suitable model for ASD than mice, the advantage of using environmental models instead of genetic ones seems evident. However, given the gene x environment character of ASD, environmental models will not be sufficient in providing all knowledge on the developmental biology of the disorder. Moreover, the fact that these models mimic the development of syndromic rather than idiopathic ASD, depicts that it is not the mechanism of ASD that is used for validating these models, but rather the resemblance of behavioural symptoms of the rodents compared to children with ASD. This suggests that the models will not provide construct validity, although face validity is provided.

Within the category of models manipulating environmental factors to examine aspects of ASD aetiology, one model is sometimes used as a basis to test another model. For example, Silvestrin (2013) used rats prenatally exposed to VPA as a model to examine the role of glutamate receptor alterations in the neurobiology of ASD. Additionally, BTBR and BALB/c mouse strains were used to examine the effects of glutamate antagonist administration on the autism-like behaviours these mice display. Although a multi-model approach is encouraged in general, one should be careful with interpretations of the results. After all, a model should have high face, construct and predictive validity, before it can be used as a model to test other predictions.

5. Conclusion

5.1. Towards an optimal model for ASD

5.1.1. Further development of genetic rat models

The future of ASD modelling should be focused on rat models, since they have a rich socio-behavioural repertoire compared to mice. Moreover, their communication system has a higher complexity than that of mice, as they clearly distinguish between ultrasonic vocalizations that represent either a positive or a negative state. This has implications for ASD, since a valid rat model of ASD would hypothetically not be able to make this distinction, as their ability to distinguish positive emotions from negative emotions would be impaired. Although mice are currently the standard for genetic models, new developments on genetic knock-out technologies have made it possible to engineer rat knockout models, making them suitable as genetic models (Wöhr & Scattoni, 2013). However, when using knockout models, the complexity of the disorder is not fully appreciated, since it can be established that a psychiatric disease like ASD is not caused by the dysfunction of a single gene. Besides, knocking out a gene throughout the body will have effects elsewhere, unrelated to the disorder phenotype. Therefore, further development of genetic technologies are necessary in order to engineer conditional knockout rats, that make it possible to shut down a gene on a target location. When this tool is combined with polygenic models, appreciating the multiple genes involved in ASD aetiology, we are on the way to engineer an optimal rat model. Although the future focus in genetic models should lie on rats, mice are a more convenient species to use with respect to stereotypies. In mice, spontaneous stereotypies are often observed, whereas it is difficult to induce stereotypies in rats. This suggests that mouse research on this subject can still make an important contribution to understanding the full behavioural repertoire characteristic for ASD.

5.1.2. Integrate genetic and environmental factors

There is a substantial body of evidence indicating that a child is predisposed to ASD development due to complex and dynamic interplay between genetic and environmental influences (Olexová *et al.*, 2012). Thus, animal models should reflect this combined action between nature and nurture. The models that are currently used for ASD research are nearly all either based on genetic modifications, or manipulation of environmental factors. More attention is needed for the development of a combined-model approach, in which the multiple-trait character of ASD is represented. In these models, multiple candidate genes can be cross-tested under environmental conditions hypothesized to influence development of the disorder. A prior advantage of this approach is that genetic models provide construct validity, as they mimic the mechanism that leads to ASD development, whereas environmental models provide face validity, as they primarily mimic the behavioural symptoms common in ASD patients. A combination of both may, therefore, result in a stronger model. For example, a conditional oxytocin knockout mouse (or yet better, a rat) may be tested for its susceptibility to develop ASD-like phenotypic characteristics after being prenatally exposed to valproic acid, compared to animals not exposed to neurotoxins and compared to wild type animals.

An important additional advise is that one should aim for a detailed mapping of all (endo)phenotypic side-effects of a manipulation, either of genetic or environmental kind. This is necessary to gain insight into the functioning of the body as a whole, instead of examining one part isolated from the other. After all, it is not possible to isolate one part of the nervous system without affecting other areas. Thus, experiments should be designed keeping this in mind. In that way, more detailed relationships can be described between manipulation and response. A downside, however, is that within this approach, the effects of brain plasticity may be overlooked; if loss of function is somehow compensated by another gene or area, the manipulation will not have a net effect on the total system (Sousa *et al.*, 2006). This is why a combination is necessary of both approaches.

A final remark on aiming for the perfect model for ASD is that we may have to consider different models for the different disorders of ASD. For example, the Asperger phenotype differs substantially from the Rett syndrome phenotype. Therefore, it is unlikely that one model for both should suffice. Although it may be useful for diagnostic purposes to group these disorders together as ASD, this categorization may be less advantageous with respect to the development of a model.

5.2. Considerations for behavioural phenotyping

5.2.1. Gold standard for phenotyping and multi-dimensionality

Currently, there is lack of a gold standard in behavioural phenotyping research. This is illustrated by the observation that many behavioural tests are used to measure the same parameter. In the case of ASD, stereotypies are most often measured in duration of self-grooming, although some studies think of a different way to quantify this behaviour. As we have seen in Chapter 2, a continuous walking pattern along the edges of the compartment during an open field test was considered stereotype-like behaviour according to the authors of the study (Wolterink *et al.*, 2001). However, since this behaviour is ethologically relevant in the (probably) anxiety-inducing test situation, this parameter does not provide ecological validity. Furthermore, the fact that a different parameter is used here, makes it difficult to compare the results of this experiment to quantifications of auto-grooming in other studies. Another example is the study discussed in Chapter 3, in which a set of 10 behavioural tests were used to measure stereotypies and anxiety in a relatively small group of mice (Shahbazian *et al.*, 2002). Some of these tests were never reported before, and no corrections were suggested to take into account the low power of the study. These are both examples in which a validated gold standard phenotyping test for quantifying behavioural parameters is essential for behavioural research.

However, this gold standard does not necessarily imply standardization; the problem with standardized experimental procedures is often that they are not easily generalized to the world outside the laboratory (Würbel, 2002). Especially since a phenotype is influenced by both genetic and environmental background, this natural variability should be taken into account when assessing an animal's behaviour. Only then can the model obtain external validity. An important comment on current behavioural phenotyping assays in this context is the one-dimensionality of many tests. For example, we have seen that the three-chambered choice test is a common instrument to assess an animal's need for social interaction. However, the rats and mice subject to this test generally have three options to choose from; visiting either a social stimulus (conspecific), a non-social stimulus (e.g. a food item) or stay put. In this situation, the natural behaviour of the animal is not fully

appreciated for several reasons. For example, the rat or mouse cannot choose to flee from the situation in total. Additionally, there is usually only one food item to choose from, as well as one social stimulus. The animal may have personal preferences, which are part of its behavioural ecology. These aspects are not taken into account in this test situation. Therefore, it is advised to design these paradigms in a multi-dimensional way, facilitating variation and thereby increasing external and ecological validity.

5.2.2. Promising behavioural parameters

Rat ultrasonic vocalizations can be a very useful indicator of communication deficits. As described earlier, rats distinguish between two frequency categories, representing negative or positive emotions. A rat model of ASD would be impaired in its ability to distinguish between these different communication signals of conspecifics, as patients with ASD often fail to read emotional states from other people's voice (Rutherford *et al.*, 2002). This property facilitates implications for the use of ultrasonic vocalizations in behavioural phenotyping assays. For example, when manipulated rats do not respond to warning calls like their controls, they may have deficits similar to ASD patients. Obviously, one should control for the possibility of auditory defects causing the behavioural abnormalities, instead of higher brain function defects.

Another important parameter that has not been used frequently is the use of olfactory cues in the form of scent marks, in order to assess rat behaviour in relation to ASD. As already depicted in Chapter 1, olfactory cues are used by both rats and mice in many different social contexts, which explains the relevance to the social deprivation disorder ASD. This feature has been applied often in mice (Wöhr & Scattoni, 2013), but literature on rat studies using scent marks is scarce. It has been hypothesized that an interaction takes place between olfactory information processing and ultrasonic vocalizations, which makes these especially interesting parameters to investigate further (Silverman *et al.*, 2010).

We have proceeded far along the road towards an ideal model of ASD. By critically reviewing both the models that are currently used, and the behavioural phenotyping assays that are applied, future directions are pointed out. Animal models are always a compromise between mimicking the complexity of the actual disorder and designing practical experiments. Combining knowledge, we narrow down the funnel towards not only a gold standard model, but also a gold standard behavioural phenotyping strategy, thereby increasing the translational capacity of the animal model.

Appendix I

Diagnostic Criteria for Autistic Disorder according to DSM-IV

(I) A total of six (or more) items from (A), (B), and (C), with at least two from (A), and one each from (B) and (C)

(A) qualitative impairment in social interaction, as manifested by at least two of the following:

1. marked impairments in the use of multiple nonverbal behaviours such as eye-to-eye gaze, facial expression, body posture, and gestures to regulate social interaction
2. failure to develop peer relationships appropriate to developmental level
3. a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people, (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)
4. lack of social or emotional reciprocity (note: in the description, it gives the following as examples: not actively participating in simple social play or games, preferring solitary activities, or involving others in activities only as tools or "mechanical" aids)

(B) qualitative impairments in communication as manifested by at least one of the following:

1. delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
2. in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
3. stereotyped and repetitive use of language or idiosyncratic language
4. lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(C) restricted repetitive and stereotyped patterns of behaviour, interests and activities, as manifested by at least two of the following:

1. encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
2. apparently inflexible adherence to specific, nonfunctional routines or rituals
3. stereotyped and repetitive motor mannerisms (e.g hand or finger flapping or twisting, or complex whole-body movements)
4. persistent preoccupation with parts of objects

(II) Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years:

(A) social interaction

(B) language as used in social communication

(C) symbolic or imaginative play

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