THE IMPORTANCE OF RAT SOCIAL BEHAVIOR FOR TRANSLATIONAL RESEARCH

An ethological approach

Suzanne M. Peters

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HET BELANG VAN SOCIAAL GEDRAG VAN RATTEN VOOR TRANSLATIONEEL ONDERZOEK

Een ethologische benadering
(met een samenvatting in het Nederlands)

Proefschrift

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CHAPTER 1
General introduction

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SOCIAL BEHAVIOR: A SHORT HISTORICAL SUMMARY

Humans, as many other animal species, are social beings who live together and actively seek each other's contact. Also, a great part of the behavior of many animals consists of social behavior and it is essential for survival of the individual. In humans, having low quality social relationships has been related to increased mortality rates (Holt-Lunstad et al., 2010). However, in the past this has not always been the dominant view. Around the 1930-ties, young children in orphanages were severely neglected due to a lack of human warmth and bonding, causing a variety of psychological problems in adulthood or even the death of these children (Blum, 2002). It reflected a complete unawareness of the importance of social contact, probably also maintained by the fear of spreading diseases through contamination with viruses and bacteria by touch. The 'behavioristic' view was that too much physical comfort of a caregiver would lead to unadapted spoiled adults and the association between a mother (or any other caregiver) and a child was merely an association of food. During the 60ties and 70ties, the famous studies from Harry Harlow and his colleagues at the Wisconsin University were pivotal to show the detrimental consequences of growing up without a mother's care and affection (Blum, 2002; Seay et al., 1962; Suomi and Harlow, 1972) See also: https://www.youtube.com/watch?v=_O60TYAIgC4 for a short video about the work of Harlow and his monkeys. In these experiments infant rhesus macaques were housed with barren, made from steel, surrogate mothers. When these animals grew up, they behaved very abnormally, for example showing severe social deficits and displaying increased anxiety (Harlow et al., 1965). Additionally, when the infant monkeys were provided with a choice between a feeding 'cold' mother from steel or a non-feeding but 'warm' mother from cloths, they chose the warm clothed mother over the feeding mother. Fortunately, these now controversial experiments are seen as unethical, but they still illustrate the enormous importance of being social. In fact, social bonding is nowadays regarded as an essential ethological need, as strong as, for example, the need for food. Ethological needs are defined as behaviors that are crucial for survival and because of that, evolution has made the performance of them rewarding, despite risks of injury and high energetic costs of displaying them.

Denying the importance of play behavior, the ontogenetic precursor of social behavior, was another remarkable misconception of the first half of the previous century. It is hard to imagine nowadays, but social play behavior was regarded

to be a useless activity by some researchers, because to them it was a meaningless behavior (Schlosberg, 1947). Schlosberg, an American psychologist, argued that play should be investigated only in stimulus-response terms instead of wondering why animals play, a typical behavioristic point of view. Additionally, in Munn's "Handbook of Psychological Research on the Rat" it was written that rats have very little social life and are not particularly influenced by each other's action (Munn, 1950).

The fact that rats have very complex social interactions has only been recognized 50 years ago, marked by the work of Barnett (1963, revised edition by Brain, 1976). Thereafter, and after the experiments from Harlow, other researchers became interested in social behavior of animals. In 1978, Ely and Henry were one of the first to study social relationships in laboratory colonies of mice held in semi-natural environment (Ely and Henry, 1978). Their study demonstrated the differential effects of sociality, on the endocrine response and behavior of dominant versus subordinate mice. For instance, dominant male mice are more active than subordinates. In addition, it takes about 42 days for a mice colony to become socially stable, as is seen from a decrease in aggressive behaviors. Around the same period, Poole and Fish started with the first descriptions of rat and mice play behavior (Poole and Fish, 1975; Poole and Fish, 1976). They noted that only rats show social play, while both species show solitary play behavior. Interest in this specific social behavior of rats rapidly increased and was followed by more controlled experiments on rat play behavior within laboratory during the 1980ties. For example, studies by Thor and Holloway focused on the sex differences of rat play (Thor and Holloway Jr., 1984) while early work of Pellis and Pellis investigated in detail the occurrence of specific locomotor movements (i.e. "jerks and jumps") in social or non-social situations (Pellis and Pellis, 1983). Additionally, it was quickly discovered that rat play behavior is very sensitive to periods of social deprivation and satiation (Panksepp and Beatty, 1980) and that social behavior is mediated by the endogenous opioid system (Panksepp et al., 1980).

Despite recognition of the importance of social bonding and social contact for normal development, there are regions in the world in which the historical problems regarding children's care in orphanages seems to be repeated. In Romania, there are still ongoing problems with children that are placed in institutions where they are severely neglected, a horrible humanitarian disaster. This is

mainly due to the low economic state of the country and hence, poor health care system and a lack of basic foster care (Millum and Emanuel, 2007). Luckily, efforts are made to improve the situation by both charities, foster care and science, in which the effects of this neglect and the applied interventions are studied (Bick et al., 2015; Fox et al., 2011). Unfortunately, it illustrates the necessity to address the importance of sociality, even today.

BEHAVIORAL PRECLINICAL NEUROSCIENCE 2018

At present, social behavior in preclinical research is mainly explored in the context of psychiatric diseases, such as autism, depression, schizophrenia. Parameters representative of social behavior are being used as readout parameters for recognition of these neuropsychiatric conditions. So, there is a dichotomy in studies, ranging from a more fundamental interest in the proximate and ultimate causation of social behavior, towards a more focused approach on the relation between deficiencies in social behavior and relevant neuropsychiatric conditions.

It is crucial that animals are provided with sufficient social experiences to ensure a healthy organism, free from aberrant behavior. Social contact is an indispensable ethological need for the animal; it is essential behavior for its survival. The performance, and not the consequences, of indispensable social behavior is rewarding for the animal (like other essential behaviors), to guarantee its display, irrespective of its high energy demands or potential risk. This is reflected in the brain by a release of endogenous endorphins, inducing the subjective pleasurable experience, associated with behaviors such as reproduction, play and grooming. Because of the known pleasurable subjective effects, it is also recognized that social contact is essential to maintain good animal welfare. This is reflected in the European guidelines for the accommodation and care of laboratory animals (European Directive 2010/63/EU and the Commission Recommendation 2007/526/EC) which indicate that: ‘Animals should be socially housed when and whenever possible and provided with an adequately complex environment within the animal enclosure to enable them to carry out a range of normal behavior’. Social play behavior has even been suggested as a ‘positive’ welfare indicator, since the behavior occurs only when animals are free from stress and the performance of the behavior is self-rewarding for the animal (Boissy et al., 2007).

Additionally, social behavior is a very sensitive marker, illustrating for example when an animal experiences stress or anxiety. Indeed, an increase or decrease of social behavior is observed when animals are administered or exposed with respectively, an anxiolytic or anxiogenic compound or environment (File and Seth, 2003). It is of importance that in the study of social behavior, care is taken to limit stress and possible aversive conditions to the animals, because the expression of social behavior depends on the emotional state of the animal.

RAT SOCIAL BEHAVIOR

Affiliative & play behavior

Rats express a number of different types of social behavior, including affiliative, play, aggression, maternal and sexual behavior. In this thesis, the focus will be on affiliative social behavior and play behavior. Generally, the function of play is seen as necessary for an adequate development of social skills, and also of emotional, cognitive and motor skills (Pellis et al., 1997; Pellis and Pellis, 2009). Play behavior is a kind of practice that is necessary to exhibit all of these skills required to become a successful adult (Vanderschuren and Trezza, 2013).

In rats, play behavior consists of several different elements that are usually grouped in 'rough and tumble play'. During rough and tumble play, rats frequently attack each other in the nape of the neck by gently biting or nuzzling with the snout trying to pin the other in a supine position (Pellis and Pellis, 2007). Additionally, during rough and tumble play rats rapidly approach and avoid the play partner, which can elicit a chasing bout. Similar to other self-rewarding behaviors, like grooming and reproduction, the performance of play behavior is rewarding for the animals (Pellis and McKenna, 1995). It is, therefore, not surprising that short-term isolation of 3.5-24 hours in juvenile rats leads to a rebound effect, i.e. a temporary increase of certain play behaviors such as pinning and social grooming (Niesink and Van Ree, 1989). Also, one week of solitary housing of both juvenile and adult rats leads to an increase in the frequencies of social behavior as compared to socially housed animals (Niesink and Van Ree, 1982).

Thus, short-term isolation (few hours or days) induces a non-permanent increase in social behavior. In contrast, when rats are deprived from social play for a longer period, this results in a more long lasting irreversible decrease in social behavior. Even when subsequent social housing follows after a play deprivation

period, permanent neural and behavioral changes are observed. For example, studies revealed that play deprivation during the juvenile period alters adult social behavior (Hol et al., 1999; Lukkes et al., 2009; Van Den Berg, Van Ree and Spruijt, 1999; Van Den Berg, Hol et al., 1999) and as a consequence of play deprivation, adult rats do not respond adequately during aggressive conflicts (Von Frijtag et al., 2002). Also, play deprivation caused a decrease in opioid release (Van Den Berg, Kitchen et al., 1999) and an upregulation of opioid receptors (Van Den Berg, Van Ree, Spruijt and Kitchen, 1999). Again, these observations are indicative for the rewarding value of play behavior.

Apart from play behavior, which is predominately expressed during the juvenile period, rats express other affiliative social behaviors mainly in adulthood, such as allogrooming and social investigation. These behaviors are rewarding for adult rats, as shown in place preference studies (Van Den Berg, Pijlman et al., 1999), although it is suggested that social contact is more rewarding for adolescent rats than adults (Douglas et al., 2004). However, when adult rats are deprived of social contact, also a rebound effect is observed (Niesink and Van Ree, 1982), which is again indicative for the rewarding properties of adult social behavior. Thus, two major forms of rat social behavior, i.e. play and affiliative behavior, are rewarding behaviors for rats.

Ultrasonic vocalizations

When rats display social behavior, such as affiliative, play, aggression, maternal and sexual behavior, they express ultrasonic vocalizations (USV's), that are not audible to the human ear. For an animal, such as the rat (or mouse), that is mostly active during night-time and lives in burrows, it is an important mode of communication (Brudzynski, 2013). Sensory information is mainly provided by ultrasonic sound, odor, touch (whiskers) and far less by sight. The communicative function of USV's is demonstrated by playback experiments that demonstrate that USV's can alter the behavior of receiving conspecifics. Specifically, presenting recorded 50 kHz calls to rats increases approach and investigation behaviors (Wöhr and Schwarting, 2007), while the playback of recorded 22 kHz calls increases freezing behavior and decreases locomotor activity (Brudzynski and Chiu, 1995; Wöhr and Schwarting, 2007). It was demonstrated that cooperative behavior between rats, in a task requiring simultaneous nose-pokes for a food reward, increases with the number of 50 kHz calls (Lopuch and Popik,

2011). Thus, it is suggested that USV's in rats may even be utilized to coordinate social behavior. Additionally, it is suggested that USV's at different frequencies might provide information on the emotional state of the animal. Calls around 22 kHz are produced in situations with negative associations, such as repeated experience of painful stimuli (Wöhr et al., 2005) and aversive (aggression) social situations (Burgdorf et al., 2008). Conversely, 50 kHz calls are given in situations that are perceived to be positive, such as in anticipation of play (Knutson et al., 1998) and during play bouts (Burgdorf et al., 2008). Thus, it is not merely the execution of play behavior that is rewarding for the animal, but also the expectancy of play provides rewarding value for the animal.

Within the two call categories, only within the 'positive' 50 kHz calls there is significant variation in the form of the calls. In contrast, within the 'negative' 22 kHz call category in which there are only two biological relevant variations observed. The 22 kHz calls can have a long duration, from 300 to over 3000ms, or with a short duration, of 150–300ms. The longer 22 kHz calls serve as alarm calls, emitted when the animals are confronted with external danger (Blanchard et al., 1991). The function of the short calls was for a long time unknown (Brudzynski et al., 1993). Recently, it has been suggested that the short 22 kHz calls are expressed when the animal experiences discomfort not specifically related to the presence of a stressor (Brudzynski, 2015). In the 50 kHz call category (see Figure 1) there are more distinct biological relevant subtypes recognized, and the number recognized ranges from 3 different call types (Brudzynski, 2013) to even 14 different types (Wright et al., 2010). In general, it is recognized that the 50 KHz calls are either of the same frequency (flat calls) or have a frequency that is modulated. The flat 50 kHz calls are used as contact calls that can also coordinate social behavior (Brudzynski, 2015). The frequency modulated calls, are usually subdivided in 'trills' or other forms, e.g. step calls, U-shaped, split etc. (Wright et al., 2010). In particular, trill calls, are associated with an intensive affective state (Brudzynski, 2015), such as during social play. Yet, also the other types of frequency modulated 50 kHz calls have been related to rewarding or motivated situations (Brudzynski, 2015). Several studies provide evidence for the association between rewarding value and the expression of the frequency modulated 50 kHz calls, reviewed by Burgdorf et al. (2011). For example, electric brain stimulation in regions known to be associated with reward increases the number of emitted 50 kHz calls (Burgdorf et al., 2007). Additionally, an-

imals that show significant place preference in response to an opiate agonist (DAMGO) emit more 50 kHz calls (Burgdorf et al., 2007).

Given that 50 kHz calls facilitate approach and investigative behavior (Wöhr and Schwarting, 2007), it is possible that the number of 50 kHz calls expressed by an individual may give an indication of its motivation to socially interact. Indeed, the number of 50 kHz vocalizations induced by heterospecific play (an experimenter 'tickles' the individual, attempting to model conspecific rough and tumble play) has been used to selectively breed animals for a selection line of animals that show both reduced 50 kHz calls rates and social interaction time (Webber et al., 2012). However, a lack of correlation between 50 kHz calls and social behavior during social interaction was found by (Manduca, Campolongo et al., 2014) for both the Wistar and Sprague Dawley rat strain. Thus, although it is suggested that the expression of 50 kHz calls is related to social behavior, this is not always confirmed by correlations with number of calls. Furthermore, there seems to be a high inter-individual variation in USV's (Schwarting et al., 2007).

Thus, social behavior is actually an integrated behavior of two major forms of expression, namely the display of visible behaviors and the for humans not audible acoustics. Which of the two renders most relevant information is difficult to tell. In terms of events, the number of calls and the variation can be numerous and complex. Given the evidence that a great portion of calls are associated with the emotional state of the rat, including USV's analsyis in the study of rat social behavior, is indispensable.

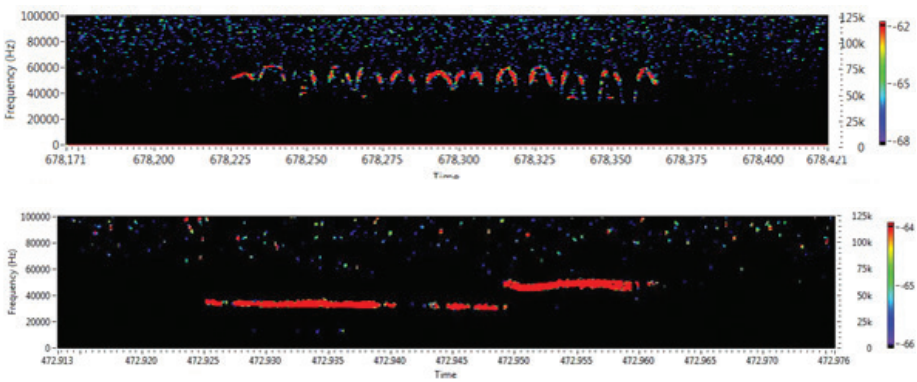

Figure 1. Two examples different types of 'positive' 50 kHz calls. Upper panel shows a frequency modulated 'trill' call, the lower panel shows a frequency modulated step up call.

Within and between strain variation

An issue not only relevant for the study of social behavior but accounting for all animal experiments, is the occurrence of natural variation. The general advice in laboratory animal science is to exclude any possible variation between animals to improve reproducibility of results. However, by trying so hard to minimize natural variation by standardization, animal science sometimes seems to have lost understanding of the real world phenomena. The problem of rigorous standardization has been termed "the standardization fallacy" (Würbel, 2002; Würbel, 2000).

In studies on social behavior this variation is an extra complicating factor, because the social stability in a cage of group-housed rats varies and this affects the phenotype of each individual animal. Indeed, Hurst and colleagues (1999) found that, in rat cages, behavior and physiology were most strongly influenced by social stability in a cage, and not so much by group densities in a cage. Behaviors related to social stability or instability in a cage, such as attempted escape behaviors and aggressive grooming, can predict differences observed in various behavioral, morphological and physiological parameters of the individual animal (Hurst et al., 1999). Additionally, when social rank per group is determined, dominant rats tend to increase the time spent in social affiliative behaviors in a social interaction test, while subordinate animals tend to express anxiety-related behaviors (Barker et al., 2017). Besides the influence of group composition, maternal care received early in life can be a source of variation. For example, natural variation in maternal care influences the levels of play fighting in rats (Parent and Meaney, 2008) whereby individuals that receive higher levels of maternal care also perform more play behaviors (van Hasselt et al., 2012). Also adult social behavior is increased when rat pups receive higher levels of maternal care (Starr-Phillips and Beery, 2014). These differences in social behavior levels due to maternal care levels are most probably in part caused by epigenetic mechanisms e.g. McGowan et al. (2011).

In rats, between-strain variation in social behavior is also very common (Himmler et al., 2016). For example, Sprague Dawley rats express higher levels of social play behavior than Wistar rats (Manduca et al., 2014). As a consequence of this baseline difference, the effects of morphine are also more pronounced in Wistar rats than in Sprague Dawley, while the effects of amphetamine are only observed in the Sprague Dawley strain (Manduca et al., 2014). Also, rats of

the Fischer-344 strain are less playful compared with rats from the Lewis strain (Siviy et al., 2003) and the Sprague Dawley strain (Siviy et al., 2011), as reflected by less play initiating behavior and less responsiveness to play solicitations. This illustrates that genetic differences are in part also accountable for differences and thus variation seen in social behavior.

Thus, a variety of sources of variation, e.g. genetic, epigenetic and inter-group stability, can cause baseline differences in social behavior. It is important to realize this in our research designs. Individual animals or strains with high levels of social behavior might be more sensitive to experimental manipulations that aim to decrease it and vice versa. Besides, it might require different statistical and methodological approaches.

SOCIAL BEHAVIOR AS PARAMETER IN ANIMAL BEHAVIOR STUDIES

Although the importance of social behavior may be acknowledged, the implementation of conditions allowing its occurrence and the study of it in both housing and experimental setups still needs attention, as will also be more extensively discussed in Chapter 2. Social behavior has been neglected for two reasons. First, it has not been recognized as a pivotal motivational system in socially living species. Second, due to its dynamic nature involving at least two individuals, it is not easy to quantify, it requires more complex test environments, behavioral observation procedures and techniques. The characteristics of social behavior as described above illustrate that social behavior is a complex behavior expressed as 'visible and invisible' behavior. The visible behavior is a highly dynamic behavior in which rats reverse roles and express for humans inaudible, but biologically highly relevant, USV's. Consequently, it is almost not possible to limit social behavior to only one behavioral parameter.

Previously, we have advocated that behavioral preclinical neuroscience should change its methodology towards a more ethologically based approach (Spruijt et al., 2014; Peters et al., 2015), because a better understanding of the mechanisms underlying behavior requires a biological relevant analysis of animal behavior. Unfortunately, there is a dangerously strong preference for 'quick, easy, low maintenance and low costs' animal experiments. This reduces behavioral analysis to the occurrence of single behaviors in short lasting tests and animals are housed in small cages with no or limited access to environmental stimuli. Especially housing conditions strongly affects animal development. Luckily,

individual housing seems to become less a standard practice and social housing of animals should not be considered an enrichment, but should be the default mode in laboratories (Bayne and Würbel, 2014). Yet despite the improvement in housing conditions, there are still many experiments in behavioral neuroscience that employ simplistic testing conditions and behavioral parameters to explain mechanisms underlying social behavior.

THE ETHOLOGICAL APPROACH

Adopting an experimental approach that allows, as much as possible, the occurrence of ethological needs, i.e. self-rewarding behaviors (Spruijt et al., 2001) of laboratory animals is certainly a challenge. Fortunately, this has been recognized and resulted in the development of systems that monitor socially interacting animals. There are, however, more methods developed to monitor social interaction between multiple mice in a group (e.g. De Chaumont et al., 2012; Giancardo et al., 2013; Kabra et al., 2013; Ohayon et al., 2013; Shemesh et al., 2013; Weissbrod et al., 2013) than there are currently developed for rats e.g. (Castelhano-Carlos et al., 2017). Such systems offer considerable advantages because they permit group-housed monitoring of animals in relatively large arenas containing for example shelters, tubes and bedding materials. The test setup should be relatively large, because the expression of social behavior requires space (see also Figure 3). Additionally, a few studies that focused on group housed rats, for example by Blanchard and co-workers, demonstrated nicely that it is possible to change housing into a rich environment that allows the display of biological relevant behavior by making use of the visible (transparent) burrow system (e.g. Blanchard et al., 1995), including the recordings of USV's in this system (e.g. Blanchard et al., 1991). Next to the optimization of these novel automated methods, there is a strong need for the development of automated systems suited for the observation of group-housed rats including USV's recordings. Current automated methods have been developed primarily for the use of mice, whereas the rat might provide a different animal model for studying social behavior.

THE HOME CAGE AS TEST ENVIRONMENT TO MEASURE SOCIAL BEHAVIOR INCLUDING USV'S

In the past, our lab has played an important role in the establishment and development of the PhenoTyper (see Figure 2) and its tracking software EthoVision; both available via Noldus Information Technology, Wageningen the Netherlands. The tracking software uses top view video images in order to recognize the animals in the cages. A rather basic feature of such software is that a contrast is made (in black and white) that allows detection of the animals in the video image, i.e. the animals are of a different color than the stationary surroundings and combined with movement of the animals the software is able to detect them. This system was originally developed for 24/7 monitoring of mice and rats in an environment that allows normal rodent behavior (exploration, sleeping, nesting, feeding) for individual animals (Spruijt and De Visser, 2006; De Visser et al., 2006). We developed, together with our partners in the SenseWell project, an

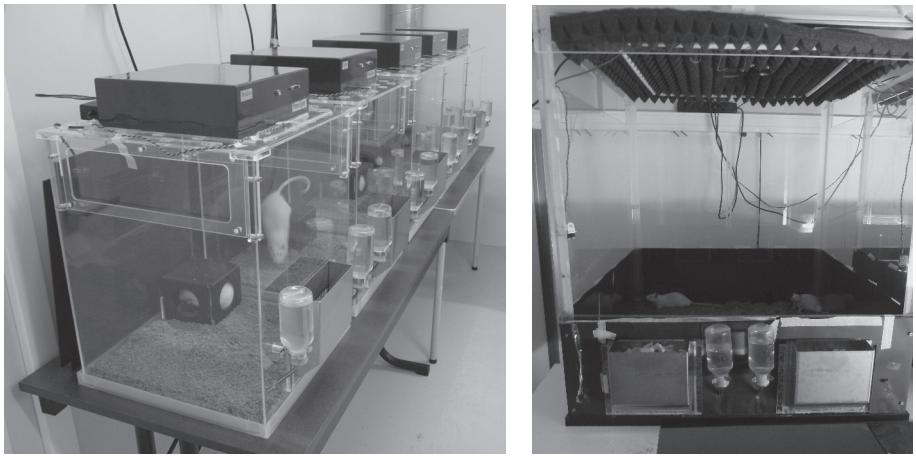

Figure 2. Examples of the PhenoTyper system as a home-cage environment. On the left a row of five PhenoTyper-4500 homecages, dimensions 45x45 cm, for individual observation of rat behavior from top view video. On the right, a PhenoTyper-9000 system, dimensions 90x90 cm, used for observation of group housed rats including ultrasonic vocalizations. Both PhenoTypers are equipped with black bedding material (Cellu-Dri Soft, Shepherd specially papers, Watertown, TN, USA) to permit video based tracking of the animals in nearly dark conditions using infrared emitting LED's. The PhenoTyper-9000 is shielded with sound attenuating pyramid foam to prevent recordings of echoes of the ultrasonic sounds.

enlarged version of the PhenoTyper (90 x 90 cm, called PhenoTyper-9000) that allows social housing of rats. Additionally, this PhenoTyper is equipped with ultrasonic microphones (Metris, the Netherlands) and first attempts were made to customize tracking software and sound recording software to automated behavioral analysis of socially interacting rats. The studies described in this thesis have utilized the PhenoTyper-9000 as a test environment for behavioral analysis of pairs of rats. During the developmental phase of the large PhenoTyper, our pilot studies confirmed the feasibility of the system to study rat social behavior (see Figure 3, adapted from Spruijt et al., 2014).

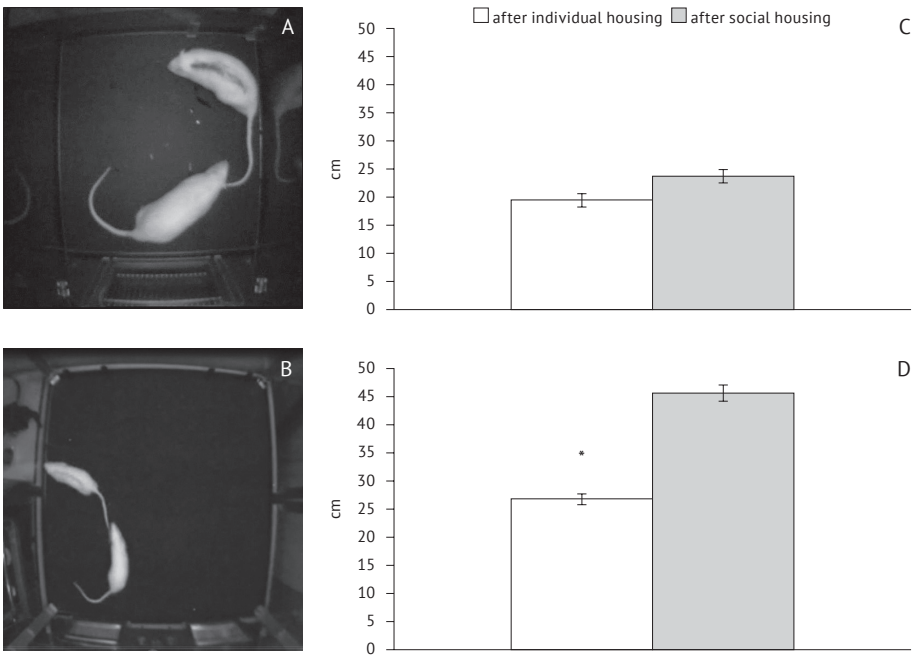

Figure 3: Measurement of social behavior and interaction requires space. Top-view of two adult male rats in the PhenoTyper-4500 (A) or the larger PhenoTyper-9000 cage (B). Panel C and D depict the effects of short individual housing (for 24 hours) on the average distance between the animals in a pair during 30 minutes sessions, measured in the PT-4500 or the PT-9000 cage, respectively. Habituated (during 2 sessions of 30 minutes) male Sprague Dawley rats were used (n = 4 pairs per group). Animals served as their own control. Only in the larger PhenoTyper cage a significant effect in distance between the animals due to short individual housing is revealed. Asterisk in D indicates a significant effect ($p < 0.005$, $t_{(3)} = 23.319$, Cohen's $d = 11.660$). Data is expressed as mean ±SEM

OBSERVATIONS OF RAT'S SOCIAL BEHAVIOR WITH TRACKING SOFTWARE AND USV SOFTWARE

The social behavior repertoire of rats consists of maternal, aggressive, sexual, play and affiliative behavior. Our studies focused on play and affiliative behavior of juvenile and adult male rats. Especially, juvenile rats exhibit high levels of social play behavior. This particular behavior, which is very expressive, is easy to recognize by a human observer as play, but presents a challenge for any automated system, relying on video images, because play behavior can occur at a high speed, especially the pursuits. Moreover, when the animals are performing a behavior referred to as rough-and-tumble play, the animals are reversing in roles in dominant and submissive roles: who is on top of whom. During these bouts tracking software needs to recognize two animals. However, since the algorithms are programmed as such that is 'looking for' a more or less cone-shaped white 'blob' (the normal body contour of a rat seen from top view video) it has great difficulties to identify the two animals. To overcome this problem, former automated studies of rat social behavior have focused on 'approach and follow behavior', which is also part of play and social behavior (Spruijt, Hol et al., 1992; Hol et al., 1999; Van Den Berg, Kitchen et al., 1999). Consequently, the automated analysis of social behavior is restricted to these behavioral parameters. Additional studies, therefore, have also utilized manual observation of other social behaviors, for example allo-grooming, social exploration and crawling under (Van Den Berg, Pijlman et al., 1999; Van Den Berg, Van Ree and Spruijt, 1999; Van Den Berg, Van Ree, Spruijt and Kitchen, 1999; Van Den Berg, Hol et al., 1999). At present, the challenge is to combine both in one system that can automatically record all or most behavioral elements of social behavior with a limited margin for errors. In the course of the development of the PhenoTyper-9000, we aimed to integrate customized tracking software from Noldus IT with additional software from Metris that records ultrasonic vocalizations.

Remarkably, only a limited number of studies in behavioral neuroscience is including the measurements of USV's in their experiments. This may partly be due to technical challenges in measuring and analyzing the high amount of USV's that are expressed during social behavior. It is not unusual to observe over 300 USV's in only a short (10 minutes) play episode of two rats. At the moment, all these USV's are manually checked by an observer and most often categorized in different types of calls. Thus, there is a strong need here for automated

methods and luckily the first steps in this process are being made (e.g. Barker and Johnson, 2017; Barker et al., 2014; Reno et al., 2013). Hopefully, such smart software tools will be able in the future to automatically classify USV's of rats, thereby increasing the knowledge on this "invisible" behavior.

RAT MODELS OF REDUCED SOCIAL BEHAVIOR

Pharmacological inhibition of the NMDA receptor

In an attempt to investigate the mechanisms underlying social behavior, animal models have been validated that potentially decrease social behavior of rats and mice. For example, to model social withdrawal, a symptom seen in for example schizophrenia, the administration of phencyclidine (PCP) is a frequently used approach (Wilson and Koenig, 2014). The compound is an ion channel blocker, N-methyl-D-aspartate (NMDA) receptor antagonist. Originally, PCP in animals was applied because in humans it was shown to have strong hallucinogenic properties. Thereafter, its administration in animals was proposed as an animal model to monitor the effects of NMDA inhibition, in order to investigate a possible causal role of the receptor in the symptoms of schizophrenia. Indeed, PCP administration effects in rats are extensively documented, see e.g. Jones et al. (2011) for a review. Thus, pharmacological inhibition of the NMDA receptor seems to be a dominant and well-established animal model for investigating schizophrenia related symptoms (Jentsch and Roth, 1999).

Play deprivation during a sensitive period

Play deprivation of juvenile rats during their sensitive period is another established model of reduced social behavior of adult rats. In this model, young rats are housed in isolation for 2 or 3 weeks directly after weaning at post-natal day 21. This is a very sensitive period during which normally high levels of social play behavior occur. A reduction in adult social behavior is observed after play deprivation (Hol et al., 1999; Lukkes et al., 2009; Van Den Berg, Van Ree and Spruijt, 1999; Van Den Berg, Hol et al., 1999). Also, play deprivation causes a decrease in opioid release (Van Den Berg, Kitchen et al., 1999) and an upregulation of opioid receptors (Van Den Berg, Van Ree, Spruijt and Kitchen, 1999). Thus, this developmental model is another model of reduced social behavior, probably with a different mechanistic background than the PCP model.

THERAPEUTIC REVERSAL OF REDUCED SOCIAL BEHAVIOR

Pharmacological modulation of the NMDA receptor

The NMDA receptor, is an ionotropic receptor which gates ion channels that are permeable to sodium, potassium and calcium. Activation of the receptor requires, next to a depolarization, the presence of glutamate and glycine (or D-serine). A recent new therapeutic approach is to modulate the NMDA receptor by inhibition of the transporter complex that normally transports glycine, i.e. glycine transporter (GlyT) inhibition. Hereby, glycine availability is elevated and this should potentiate NMDA receptor functionality. GlyT inhibition based pharmacotherapy has been suggested for numerous disorders, such as schizophrenia, pain control, alcohol and drug dependence, depression, epilepsy and obsessive compulsive disorders, as well as anxiety disorders, memory deficits, Parkinson's disease and autism (Harvey and Yee, 2013). A study in mice showed that GlyTs inhibitors have the potential to increase social exploration in the Balb/c strain, a strain that is known for its low levels of social behavior compared to other mice strains (Burket et al., 2015). So far, there are no studies that have investigated the potential of GlyTs inhibitors to reverse effects of reduced social behavior in rats.

AIMS AND OUTLINE OF THIS THESIS

The main question is how can we contribute to an improvement in laboratory animal experiments in order to increase the translational value of current animal models, with a special focus on social behavior of rodents. The general scope of this thesis is to facilitate and validate the measurements of rat social behavior. The role of social behavior in frequently used animal models and tests in behavioral neuroscience can be two fold. First, deficits in social behavior or its development may often underlie the causation of psychopathology and, second, as a sensitive read out parameter for detection of a wide group of pathological behaviors, even, when social behavior itself is not directly the cause. I study these two aspects of social behavior by using a well-known pharmacological and a developmental model. Additionally, I want to illustrate that currently available technology allows measurement of complex social behavior, namely the visible dynamic expression of it and the for humans inaudible ultrasonic calls.

I start with an explanation of the importance to change our approach when using animal models to investigate human disorders. In **Chapter 2**, I review and

promote the implementation of ethological concepts into animal models to enhance its translational value. In **Chapter 3**, I demonstrate an example of a novel approach to automatically measure and classify rat social behavior using a video tracking system. Here, I adapted an existing method to distinguish individual classes of speed and inter-individual distance when moving, into a method that classifies different social behavioral categories. The application of this method in a pharmacological model is shown in **Chapter 4**. PCP administration is a well-established animal model to study symptoms such as social withdrawal related to schizophrenia. Therefore, in **chapter 4** I investigated short and long-term effects of chronic PCP on social behavior, including USV's. **Chapter 5**, also uses the previously validated method and extends this by an elaborated set of behavioral assays. Here, I examined the behavioral effects of a frequently used model of play deprivation more closely. Additionally, I wanted to reveal if a novel and promising drug, a GlyT-1 inhibitor, could have positive effects on reduced adult social behavior. Last, **Chapter 6** provides a general discussion on the results of this thesis with focus on the validity of two frequently used animals models, pharmacological (chronic PCP) and developmental (play deprivation). I further discuss the technical challenges that are still faced when social behavior of the laboratory rat is investigated.

CHAPTER 2
Ethological concepts enhance the translational value of animal models

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ABSTRACT

The translational value of animal models is an issue of ongoing discussion, particularly in preclinical neuropsychiatric research. We argue that 'Refinement' of animal experiments is needed and this can be achieved by exploiting an ethological approach. Ethology aims to assess the functional meaning of behavioral changes, due to experimental manipulation or treatment, in animal models. In addition, an ethological approach improves the design of the animal model because more optimal testing conditions are employed that have biological relevance to the animal. Moreover, a more biological relevant analysis will contribute to clarify the functional meaning of the modeled behavior in order to determine whether it is psychopathological or adaptive in nature. More studies using animals in group-housed conditions, including the recording of their ultrasonic vocalizations, are required because, (1) social behavior is an essential feature of animal models for human 'social' psychopathologies, such as autism and schizophrenia and (2) social conditions are for social species, such as the rat, indispensable conditions for appropriate behavioral studies. Only when taking these elements into account, the validity of animal experiments and, thus, the translation value of animal models can be enhanced.

INTRODUCTION

Animal models are important sources of information for advancing our understanding of the pathophysiology of human diseases and play a critical role in the efficacy and safety screening of novel treatments for these disorders. The translational value of animal models is one of ongoing discussion, particularly in preclinical neuropsychiatric research (see e.g. Braff and Braff, 2013; Kaffman and Krystal, 2012; McGonigle and Ruggeri, 2014). Over the past years this discussion has intensified due to frequent failures in clinical trials of novel psychoactive drugs that initially had good preclinical perspectives (Belzung, 2014). As a result of these setbacks most pharmaceuticals companies are cutting back on their research activities in the field of neurology and psychiatry, while some are even abandoning them all together (McGonigle and Ruggeri, 2014). Thus, the chances for generating therapeutic advances in psychiatric drug discovery appear to be diminished. This is particularly worrying in view of the high social and economic burden psychiatric illnesses place on our society (Greenberg et al., 2003; Wittchen and Jacobi, 2005).

Especially in the areas of depression and anxiety research, it has been noted repeatedly that the majority of the animal models available for these disorders have limitations, which seriously affect their translation value (e.g. Anisman and Matheson, 2005; Belzung, 2014; McArthur and Borsini, 2006; Willner et al., 1992). For example, in the area of depression the forced swim test or the tail suspension test in rodents are frequently used. Both are short behavioral tests in which usually one readout is taken as a measure of behavioral despair, i.e. the degree of immobility shown by the animal. However, these tests only mimic an acute state and are often combined with a single treatment with an antidepressant drug, which obviously is not compatible with the chronic treatment depressed patients require (Frazer and Morilak, 2005; McGonigle and Ruggeri, 2014). In addition, these tests provide no measurements of anhedonia, which is one of the most important hallmarks of depression (Hasler et al., 2004).

Of course the complex and heterogeneous symptomatology of most psychiatric conditions complicates the modeling of these disorders in animals, specifically in rodents. The fact that most of these conditions are described by subjective and sometimes contradictory symptoms in humans clearly does not help. Nonetheless, most animal models will benefit from a required improvement

in experimental methodology. This will lead to more reliable data and consequently will increase the validity of animal experiments. This concept is also known as 'Refinement', one of the '3 Rs' (Russell and Burch, 1959). Refinement is typically seen as a tool to improve animal welfare, however, it will enhance the translational value of research as well. Ultimately, it will also help to reduce the number of animals required as the validity of each experiment is increased by improving the models and test procedures used.

Here, we discuss the limited translational value of animal models for 'social' neuropsychiatric disorders, such as autism, with a specific focus on the measurement of social behavior in these models. In order to maximize the translational value of animal studies on these human conditions, investigating behavior under social conditions is a prerequisite. However, it is striking that in most animal studies the 'social element' is minimized or even omitted. We argue that refinement of the animal experiments on social disorders is needed, because too simplistic tests are used that cannot capture the full spectrum of social behaviors.

Moreover, the test situation and housing conditions should represent a more ethological valid environment, allowing the animal to express its 'natural behavior' as much as possible. Ethology entails taking the environment (or ecological niche) in which the animal has been optimally adapted to (during evolution), into account in the design of the experimental test conditions. For instance, nocturnal animals should be tested in the dark or under dimmed light conditions. Tests involving maze learning in the water should be conducted with semi-aquatic species, such as the rat (but not the mouse), while studies investigating psychopathology that involves social behavior (such as depression, autism and schizophrenia) should be using social species. Thus, next to the classical validation criteria: predictive, construct and face validity, we would like to add the criterion of ecological validity. Finally, an important methodological improvement should be made by employing advanced automated approaches using ethological procedures. This kind of automation aids the detection of new behavioral elements by the 'computers eye', which is indispensable for assessing the quantitative aspects of movements (i.e. speed, distance, duration) or postures of animals, which cannot be analyzed quantitatively by the human eye. Furthermore, the increasing availability of new analysis algorithms enables the classification of behavior using for example machine learning.

LIMITATIONS OF CURRENT BEHAVIORAL ANIMAL MODELS AND MEASUREMENTS

An alarming preference for using easy and quick behavioral tests

There is a strong tendency in behavioral neuroscience to continue to use relatively simple, quick and seemingly easy tests, such as the open field and elevated plus maze, in which behavior of rodents is mostly captured by a single readout parameter, like time spent in a certain area. The popularity of these classical tests is rather striking as their low validity has been addressed repeatedly (e.g. Crabbe et al., 1999; Fonio et al., 2012; Haller and Alicki, 2012; Kafkafi et al., 2005; Wahlsten, 2001; Walsh and Cummins, 1976) and more advanced alternatives are available. We have recently discussed this curious 'status quo' in behavioral science and proposed several solutions to advance the measurement and analysis of rodent behavior (Spruijt et al., 2014). For instance, one critical point to keep in mind when designing a behavioral experiment is the research context in which the study is performed. Behavioral assays are used typically for two research purposes: either as a simple "one parameter" indicator with limited explanatory power, or in a more hypothesis-free context to obtain information on the functional role of behavior (Spruijt et al., 2014). In studies that assess, for example, the toxicity of a compound or its dose-response properties, the use of a simple behavioral assay can be justified. However, when the biological function of behavior is discussed in view of the results obtained with a similar simple "assay-like" behavioral test, it becomes problematic. The "assay like" test is too limited to yield conclusive answers across the full richness and complexity of the behavior. This especially applies to the investigation of social behavior, which is extremely complex.

An example of a relatively simple test typically used to investigate social behavior, such as sociability or social interest, is the three chambered test. There are several versions of this test available in mice (Moy et al., 2004), including an automated version (Nadler et al., 2004; Yang et al., 2011). In this test a familiar or unfamiliar stimulus animal is confined to a small compartment, typically an inverted wired or plastic pencil cup, within one of the three chambers. The experimental animal is all allowed to explore the three chambers freely and social interest is measured by the time the experimental animal spends sniffing the cup holding the stimulus animal and/or by preference of the experimental animal for the chamber containing the stimulus animal. It can be questioned,

whether a reduced approach time to the cup by the experimental animal indeed represents decreased sociality per se. Obviously, there is a strong confounding impact of locomotor activity in these kinds of tests, which in turn can be influenced by e.g. the species used, genetic background of the animal or the experimental manipulation. Indeed, performance in the test appears to be dependent on the background strain of the mice and the contextual novelty of the social stimuli used. Pearson and co-workers showed that C57BL/6J mice failed to exhibit preference for social novelty when the novel stimulus animal (an unfamiliar mouse of the same strain) was presented in a familiar context (Pearson et al., 2010). Moreover, the core feature of social behavior, which renders its sensitive to all kind of manipulations, namely the degrees of freedom two animals have to intertwine action-reaction patterns, is not represented in a test where the social interaction is reduced to inter-individual distance and sniffing.

Nonetheless, these tests are very popular. Most probably also because they are short (5-15 min on average), thus, relatively quick to run, and they can be used as part of a high throughput screening battery. Their short duration is at the same time another of their shortcomings. These tests typically only measure immediate reactions to novelty. We have previously shown that habituation of a mouse to a novel environment encompasses several stages, which can last up to days. Moreover, it is demonstrated that remarkable differences exist in the time course of habituation between different mouse strains (De Visser et al., 2006; Loos et al., 2014; Spruijt et al., 2014). In addition, familiarity to an environment has been demonstrated to affect sensitivity to different drugs, such as amphetamine, apomorphine, cocaine and to endogenous serotonin (Carey et al., 2005; Dunne et al., 2007; Harkin et al., 2000; Joyce and Mrosovsky, 1964). These findings were substantiated by one of our own drug studies in which we could demonstrate that a prolonged observation time (up to 3 hours) reveals more pronounced differences between drug effects compared to a 30 minute observation time (Spruijt et al., 2014).

Finally, in most of the social assays nocturnal rodents are tested during their inactive phase of the diurnal cycle, using (bright) light conditions. These test conditions do not provide an optimal challenging environment for the expression of complex social behavior. Thus, what is needed is a behavioral test that can fully captures the rich social repertoire of two or more animals concurrently under ecologically valid test conditions. Or as stated by Gerlai and Gerlai, there

is a strong need for behavioral tests investigating intra-specific social behaviors and social communication while keeping the ethological characteristics of the species in mind (Gerlai and Gerlai, 2004).

Limitations of housing and environmental conditions

Other important aspects that should be addressed when designing a study, especially when investigating social behavior, are the laboratory and experimental conditions. The importance of social conditions cannot be emphasized enough. Limitations in housing and/or developmental conditions lead to abnormal animals, which mistakenly are used in studies as baseline controls to compare, for example, treatment effects. The standard rodent cages, commonly used in most laboratories, provide very limited stimulation and can almost be better characterized as impoverished rather than standard (Bayne and Würbel, 2014). The impact of housing in an enriched environment versus a barren cage on normal neural development is widely known (e.g. Rosenzweig and Bennett, 1996; Sakhai et al., 2013).

Numerous studies have reported the negative impact of short- or long-term social isolation. For example, social isolation leads to hyperactivity in a novel environment, increased sensitivity to stressors and learning and memory deficits (Hall, 1998; Marsden et al., 2011). Moreover, chronic social stressors, like isolation, permanently alter the neuroendocrine system and increase anxiety levels (Blanchard et al., 2001). Social housing should, thus, not be considered as enrichment, but should be the default mode in laboratories (Bayne and Würbel, 2014). Of course, housing animals in captivity will always cause some restraints on the environment, but opportunities to transform the current situation in laboratories are hardly taken. Common laboratory practice, often characterized as standardization, is predominantly kept unchanged, even though its shortcomings are recognized. The encouragement for providing more enriched and social environments should not come mainly from welfare organizations, but also from scientists themselves, as good science and acceptable welfare go in hand in hand.

One important element that does not receive the required attention is the fact that the vast majority of our research using rodent models is conducted with male individuals only (see also Clayton and Collins, 2014). Most of the drug discovery results are, therefore, biased towards the male population. Females, however, are also strongly affected by neuropsychiatric disorders, albeit maybe not

in the same way, nor do they necessarily show the same sensitivity to drug treatments (Franconi et al., 2007; Soldin and Mattison, 2009). For instance, the prevalence rates for developing post-traumatic stress disorder (PTSD) and depression are higher in females compared to males (Kessler et al., 1995; Nolen-Hoeksema, 2001). Conversely, schizophrenia and autism are seen more often in males as compared to females (Baron-Cohen et al., 2011; McGrath, 2006; Newschaffer et al., 2007). The main reason for not including female rodents in animal studies is the supposedly confounding impact of their estrous cycle and fluctuating sex hormones, which is considered to affect the reliability and reproducibility of the generated data. Whether this fear is justified can be questioned as a recent meta-analysis study has shown that for most applications female mice tested throughout their hormone cycles display no more variability than males do (Prendergast et al., 2014). The procedure of excluding female animals from preclinical psychiatry studies, thus, seems to be a rather simplified one, seriously affecting translational value. Especially, as the processing of emotionality and cognition and the reactivity of the HPA axis in rodents, all show clear sex-dependent differences (Ter Horst et al., 2012). The translational value of animal studies will, thus, surely benefit from the routine inclusion of female rodents.

Variation is not a confounding or limiting factor, but a bonus

Over the years the lack of reproducibility of results between and even within studies has resulted in the notion that standardization of test environment and protocol can diminish variation and hereby enhance the reproducibility. However, so far standardization attempts have not led to a major improvement of reproducibility and it can be questioned whether this is a satisfactory strategy (Richter et al., 2009; Spruijt et al., 2014; Würbel, 2000; Würbel, 2002). Moreover, the need to standardize breeding, housing and testing have led to an impoverishment of the animal's environment (i.e. barren and densely packed cages, in brightly illuminated holding rooms), while the actual differences between animal facilities (e.g. staff workers, transportations, light and sound conditions) are virtually impossible to control between facilities. Alternatively, Würbel and colleagues have proposed a strategy of accepting variation and systematically controlling various environmental elements (Richter et al., 2009; Würbel, 2000). An inherent property of all animals, even within inbred strains, is that individuals differ. Individual differences are even seen despite the vigorous at-

tempts of standardizing everything and are impossible to completely remove.

Another strategy is the application of smart data analysis methods to circumvent the impact of variation. One of these data analysis techniques is the identification of subgroups of animals, especially with regard to their behavior. For example, subgroups of rats have been identified based on their reaction to novelty (Cools and Gingras, 1998). Rather than applying arbitrary chosen behavioral criteria, one can also identify subgroups in a population using measures that are defined based on the animal's own behavior (see e.g. Benjamini et al., 2010; Lipkind et al., 2004). Individual differences in susceptibility to treatment may also be a relevant factor in preclinical research as variability in susceptibility to disease and certain treatments is present in humans as well. In fact, it forms the foundation for a concept known as 'personalized' medicine, which receives increasing attention in today's drug discovery.

Need for a more advanced analysis of behavior

An accurate assessment of social behavior in pair- or grouped-housed rodents not only requires the right approach and experimental conditions, it also necessitates the use of advanced techniques for the automated identification and objective analysis of all social elements. Until now, social behavior is typically scored manually by a human observer quantifying only one or two endpoints (e.g. frequencies and durations of a few specific social elements), usually only of one animal at the time. Clearly the manual scoring of behavior has numerous disadvantages. It is very laborious and time consuming, subjective and prone to errors and it is limited by the properties of the human visual system. For example, the sequential organization of behavior typically remains unnoticed to the human observer, especially across a large time period. A full understanding of behavior requires not only the analysis of frequencies and durations of behavioral elements, but also the analysis of time patterns of events. This includes the sequential organization of behavior (see e.g. Spruijt and Gispen, 1984; Spruijt and Meyerson, 1987) and the temporal aspects (Casarrubea et al., 2011).

TOWARDS A MUCH REQUIRED CHANGE IN APPROACH AND METHODOLOGY WHEN MEASURING RODENT SOCIAL BEHAVIOR

A proposed solution: an ethological approach combined with automation

We have recognized several problems in the use of animal models for neuropsychiatric research and specifically the measurement of behavior in these models. In particular there is a strong negligence for two very important features of human psychopathology: the intrinsic motivation for and the expression of social behavior. We believe that improvement in the translational value of animal models can be found in the application of an ethological, more ecological valid approach when studying behavior. A minority of investigators in the behavioral neuroscience field have already adopted this strategy (see e.g. Brain et al., 1991; Chaouloff, 2013; Kršiak, 1991; Olivier et al., 1990). However, with the dominating preference for the quick and simple 'classical' behavioral assays, this alternative strategy has been slowly casted aside. Hence, what is required is a test and analysis that benefits from the information that is encoded in exploratory behaviors and dynamic social behaviors. Rather than applying a different test for distinct behavioral systems and, thus, ignoring their interaction, a behavioral test should motivate the animal to engage possibly in more than one class of motivated behaviors. Consequently, the animal will have to set priorities and make decisions. Such a set-up will allow a functional interpretation of the effect of an experimental manipulation on decisions that are made by the animals and how this translates into the expression of a behavioral reaction.

Below, we will discuss in more detail why an ethological approach is beneficial for the translation value of animal models and illustrate this with some examples. Next to that, we discuss the integration of the home-cage concept into the laboratory in order to offer a more ethological environment. We will highlight how automation of ethological procedures can enhance our understanding of animal behavior. Finally, we will review the current developments in automated social behavior observations of rodents and discuss the importance of integrating ultrasonic vocalizations recordings.

The classical ethological approach

The term 'ethology' refers to the biological study of behavior. Inspired by the work of Lorenz, Tinbergen (1963) described the approach of ethology in which he made a clear distinction between proximate (direct cause or ontogenetic) and

ultimate (evolutionary) causes of behavior. This notion formed the basis for the classical ethology. In classical ethology, behavior is observed under ecological valid conditions which allow the display of natural species-specific behaviors, as this is the biological relevant context for interpreting the function of behavior. Behavioral pharmacologist who recognized the importance of these ethological concepts introduced the term 'ethopharmocology', in which studying drug effects on the natural patterns of behavior plays a dominant role as reviewed by Kršiak (1991). This integration of ethology with pharmacology within a laboratory setting is clearly present in work of for example (Blanchard et al., 2001; Dixon, 1998; Kruk, 1997) who argue that using an ethological approach will advance behavioral pharmacology. For example, Blanchard and co-workers argue that it is important to integrate the (dys)functional role of originally adaptive behaviors into animal models for psychopathologies (Blanchard et al., 2013). We also consider it a very valuable strategy because it may help to understand the causation of maladaptive (psychopathology) and adaptive behaviors when the animal is tested under conditions in which both proximate and ultimate causation can be distinguished.

For instance, the appearance of learned helplessness, a symptom of 'depressive'-like states, can be seen as psychopathological. From an evolutionary point of view (ultimate causation of behavior), however, it appears to be an adaptive response with survival value. In an environment in which the organism has lost all control and, thus, has to limit the pointless expenditure of energy, it can be very adaptive to adopt a strategy of helplessness. Therapeutic treatment which aims to reverse this depressive-like state, should attempt to reverse the energy saving strategy into an initiating or active state. This approach has successfully been applied by Van Der Harst et al. (2005) and Kamal et al. (2010) who counteracted depressive-like behaviors by announcing and providing rewards to 'depressed' rats. The conversion from a fully passive and energy-saving strategy to an active and initiating strategy may guide the development of therapeutic strategies in depressive disorders.

Another example is the ethological perspective on the understanding of autistic disorders. It was actually Tinbergen, who devoted much of his last work to autism (Tinbergen and Tinbergen, 1972; Tinbergen, 1974). He and his wife where the first to argue that gaze avoidance behavior displayed by autistic children is an adaptive response/behavior and comparable to the response of normal chil-

dren when confronted with an aggressive stranger (Silverman, 2010). This led to the incorporation of a careful and nonintrusive approach, which currently is common practice in the interaction with autistic individuals (Silverman, 2010).

Also, the role of melanocortins on grooming behavior in rats was discovered using an ethological analysis of behavior. There are many neuropeptides, such as substance P, bombesin, oxytocin, endorphins and enkephalins, which can induce grooming behavior (Mul et al., 2013). However, only melanocortins induce grooming behavior with a sequential structure similar to naturally occurring grooming, i.e. a grooming sequence from head to tail (Spruijt and Gispen, 1984; Spruijt, Van Hooff et al., 1992). This observation directed research to the function of melanocortins as the natural ligand for inducing grooming behavior and the melanocortin-4 receptor as the 'grooming' receptor (Mul et al., 2013).

Home-cage like environments

The integration of an ethological approach into the laboratory requires changes of the test setup and the read-outs of behavior (Blanchard et al., 2013). For example, when studying social interactions the use of a semi-natural burrow system with multiple tunnels, burrows and an open area permit rodents to behave as in a natural colony setting. The advantage of such a setting is that it enables the observation of different types of social behavior, for example aggression, sexual behavior or merely affiliative social behavior (e.g. Arakawa et al., 2007; Pobbe et al., 2010). Moreover, a clear dominance hierarchy (with one dominant male and several subordinates) develops in a burrow system and this can be used as an advantage. Individual variation due to differences in rank among individuals is present in such colonies, for example subordinate animals have lower weight gain and show earlier mortality compared to the dominant animals (Blanchard and Blanchard, 1990). This setup facilitates the investigation of the coping response of subordinates animals to the social stress experienced, in order to determine whether these are adaptive or maladaptive responses. Because, social stress is more natural to the animal and differs from the artificial and acute stressors typically used in laboratories (e.g. foot shock or restraint) it has been suggested that they do not necessarily activate similar coping mechanisms (Tamashiro et al., 2004). However, current literature does not provide conclusive evidence whether this is indeed the case. Although, it has been shown that the social stress that is experienced by the subordinates alters behavior, induc-

es neural changes and effects endocrine levels (see e.g. Blanchard et al., 1993; McKittrick et al., 2000; Spencer et al., 1996). For example, subordinate animals show decreased food and water intake, decreased activity levels (Blanchard and Blanchard, 1990) and show increased ethanol consumption (Blanchard et al., 1987). Therefore, the profile of the subordinate animals in the visible burrow system has been suggested to have good comparison with the symptoms of clinical depression (e.g. Blanchard et al., 1995).

Another example of an environment that allows species-specific natural behavior is an observational or 'home/familiar' cage in which the animals are provided with sufficient room to move around freely and engage in social behavior. In the cage the animals have access to food, water and bedding material and thus they can easily remain a prolonged period of time in there. The animals are monitored continuously without the confounding effects of handling and transportation, which cause stress and anxiety. Thanks to the option of continuous and longitudinal observation novelty-induced behavior can be dissociated from baseline behavior (Tecott and Nestler, 2004).

One of the largest advantages of testing in a home-cage environment is that it can reveal novel features of behavior. For example, a recent study of Hager and co-workers (2014) revealed striking variation in long-lasting fear responses in mice when measured in a home-cage-like set-up, whereby some mice enter a shock compartment much faster than others (Hager et al., 2014). Hence, the bandwidth of responses are increased and this fosters the characterization of individual differences (Hager et al., 2014). In addition, the use of a home-cage environment with a detailed, extensive analysis of behavior (i.e. a combined analysis of kinematic parameters and sheltering behavior) has proven to be a very sensitive tool to phenotype genetically different mouse strains (Loos et al., 2014). The study by Loos and colleagues (2014) revealed some very interesting novel behavioral characteristics of mouse strains, e.g. certain strains almost never climb on top of a shelter in their home-cage (Loos et al., 2014). Furthermore, the use of a home-cage has proven to be a very sensitive tool to elucidate sensitivity to drug treatment in mice (Robinson and Riedel, 2014).

We have previously shown in our laboratory that the size of the test environment is critical for demonstrating social behavior and social interaction in rats. We could show that the effect of short individual housing, expressed by a decreased distance between pairs of rats (thus, indicative of more social interac-

tion) only becomes apparent in a home-cage with an enlarged surface (i.e. 90 x 90 cm) compared to a 'normal' cage of 45 x 45 cm (PhenoTyper model PT-4500, Noldus Information Technology, the Netherlands) (Spruijt et al., 2014). Clearly, a larger environment allows the animals to avoid each other and, thus, is pivotal for the detection of changes in social dynamics between rats.

Automation of behavioral observation

Classic ethology is based on a detailed and systematic observation of behavior and to this end ethograms are used to describe the behavior of a species in detail. However, behavioral descriptions are defined by humans, i.e. they are based on what we see. It is, therefore, limited to the human perception which does not necessarily capture a complete representation of the behavior under study. For instance, olfaction, auditory and tactile sensory information are usually ignored. It is even impossible for humans to gather most of the auditory information, as almost all sounds emitted by rodents are above the human hearing range. Furthermore, the behavioral structure (i.e. the sequential organization of behavioral elements) of species other than ourselves, cannot by analyzed in a quantitative way by the human observer. In order to have a complete understanding of behavior, an analysis of patterns is required. Despite the proven sensitivity of sequential analysis of behavioral streams (see e.g. Casarrubea et al., 2015; Spruijt and Gispen, 1984), the analysis of behavior is mostly restricted to frequencies and durations of behavioral elements.

An alternative approach is the use of automated instruments that 'replace' and complement the human eye by using computerized analyses of video camera generated images or other sources of information. Momentarily, there is an increasing amount of technological progress made in behavioral science. The behavioral biology field seems to slowly realize that it is time to change methodology towards a science that uses bioinformatics tools, e.g. automated hard- and software for the observation and analysis of animal behavior. Hereby, allowing the replacement of the human observer by smart sensors (such as cameras and microphones) that are processed by computer software. The use of automation permits a rich and detailed, thus, ethologically inspired, observation and analysis of behavior (Schaefer and Claridge-Chang, 2012).

Automation enables long-term observation. One beneficial consequence of long-term observation is that during time, a developing behavioral pattern can be followed. Typically, a relatively simple measure such as, the distance that is covered by the animal in a certain time window, is used as an indication of general activity. Yet, a much more detailed analysis of movement behavior is possible. Golani and coworkers were among the first who illustrated the application of an automated recording of the development of locomotion patterns (e.g. Benjamini et al., 2010; Drai et al., 2001; Lipkind et al., 2004). For example, when a mouse is allowed to explore a big circular open arena from an adjacent familiar cage, i.e. its home base, there is a clear developmental sequence of exploratory behavioral elements (Golani, 2012). It demonstrates that explorative behavior is not just the time spent in an open area or along the walls, but has a much more complex development of very specific behavioral sequences. Such an ethological approach shows that the analysis of temporal development of locomotion paths could yield very reliable behavioral endpoints with a high degree of discriminability (Benjamini et al., 2010; Drai et al., 2001; Kafkafi et al., 2009; Lipkind et al., 2004).

We have continued our work along these lines in the enlarged home-cage (mentioned above) by extending it with automated analysis of social behavior of rats. We have developed a method that combines velocity of movements with inter-individual distance in test pairs of juvenile rats. Our method is based on previous work of Golani and co-workers who demonstrated that locomotor behavior can be divided into distinct categories or so called 'modes' of movement (Drai et al., 2000). These categories are comparable to the use of different gears when, for instance, driving a car. For example, a rat can still move around, but hardly is leaving its location (like it is using its first gear). Alternatively, it can make a run from point a to b with a much higher velocity (using its second or third gear). We have extended this concept used to describe locomotor behavior of individual animals, to the situation when two juvenile rats are socially interacting. We found that different modes or categories of inter-individual distance are present in pairs of juvenile rats. Rats are either "in contact", "in proximity" or "not in proximity". When we combine these 'naturally present proximity categories' with velocity of movement, it yields distinct behavioral classes which are sensitive to pharmacological and environmental treatment and are consistently in line with human scored behavioral data (Peters et al., 2016).

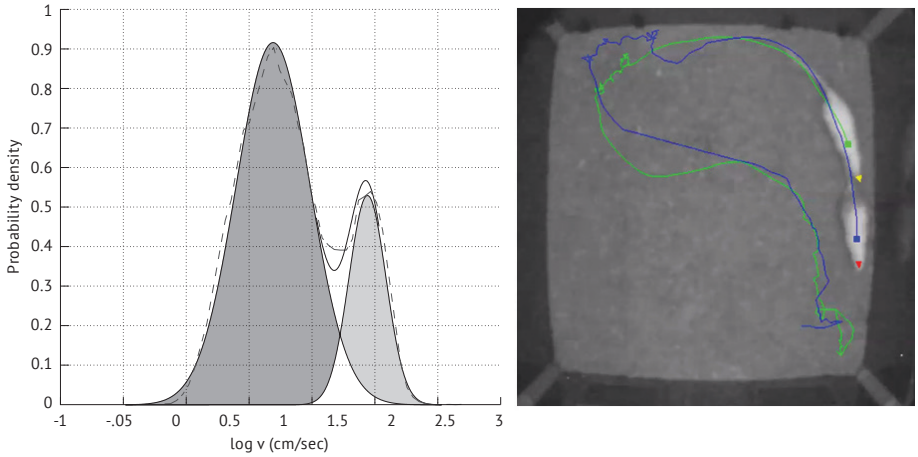

Figure 1. Example of automated data observation and analysis of socially interacting rats in a large arena. The right panel shows a top view video image of two chasing rats that are followed by tracking software. The blue and green lines show the trajectories of each animal of which various locomotion elements such as velocity can be calculated. The left panel shows an example of a frequency graph of all occurring velocities per movement bout in one test trial. Two distinct categories of movement behavior become visible: slow movements (area under the left curve) and faster movements (area under the right curve). The intersection point of the two curves is used to determine the threshold of slow and fast movements, in this example 34.35 cm/s.

Improving the measurement of rodent social behavior

Social behavior is a complex behavior because its interactive nature requires a continuous perception of the other animal's reaction to its own behavior. While interacting, animals are constantly adapting their responses to adequately react to the other animal. This dynamic behavior is difficult for human observers to follow, recognize and analyze. Especially, the sometimes very rapid movements, during for instance social play in juvenile rodents, are hard to observe with our eyes. Fortunately, considerable technological progress has been made over the last few years. Several automated methods have been introduced that all monitor social interaction between multiple mice in a group, (e.g. De Chaumont et al., 2012; Giancardo et al., 2013; Kabra et al., 2013; Ohayon et al., 2013; Shemesh et al., 2013; Weissbrod et al., 2013). Such systems do offer considerable advantages because they permit group-housed monitoring of animals in relatively large arenas with the provision of e.g. shelters, tubes and bedding materials. An elegant system has recently been described in which a three dimensional image of rats

is created from multiple depth-cameras surrounding the test environment, enabling the detection of social and sexual (e.g. copulation) behavior (Matsumoto et al., 2013). Since behavior does not only take place in a two dimensional plane, the detection of specific behaviors with the help of a three dimensional image produces a better representation of the animal's behavior. Next to the optimization of these novel automated methods, there is a strong need for the development of automated systems that are suited for the observation of group-housed rats. Since, current automated methods have been developed primarily for the use of mice, whereas, the rat might provide a better animal model for studying social behavior.

Including automated ultrasonic vocalizations recordings into behavioral analysis

A great part of the expression and interspecies communication of rodents consists of ultrasonic vocalizations which are not audible to the human ear. This was first observed in the 1960s with the help of simple bat detectors (Brudzynski, 2009). However, it has remained a substantial technological challenge to record and analyze ultrasonic calls (Brudzynski, 2009). Maybe this has caused the relatively withheld use of ultrasonic sound recordings. The number of research groups that incorporate the study of ultrasonic sound in their experiments is limited, although, the value of this type of communication is very well recognized. Evidence is growing suggesting that ultrasonic vocalizations of different frequencies might provide information on the emotional state of the animal. For example, it has been found that 22 kHz calls are emitted in situations with negative associations, such as repeated experience of painful stimuli (Wöhr et al., 2005) and aversive social situations (Burgdorf et al., 2008). Whereas rats vocalize using frequency modulated 50 kHz calls in situations that they perceive as positive, such as the anticipation of social play (Knutson et al., 1998) and during play bouts (Burgdorf et al., 2008). The communicative function of these calls is demonstrated in playback experiments where they alter the behavior of receiving conspecifics (Seffer et al., 2014; Wöhr and Schwarting, 2009). The 50 kHz calls have been shown to increase approach and investigation behaviors (Wöhr and Schwarting, 2007), while 22 kHz calls increase freezing behavior and decrease locomotor activity (Brudzynski and Chiu, 1995). More recently, it has been suggested that ultrasonic vocalizations in rats may even be utilized to coordinate behavior. It was demonstrated that cooperative behavior between rats,

in a task requiring simultaneous nose-pokes for a food reward, increased with the number of emitted 50 kHz calls (Lopuch and Popik, 2011).

Since the utilization of ultrasonic vocalizations is such a large part of the behavioral repertoire of rats, it can provide more insight into the functional meaning of social behavior. Recent studies indicated that specific types of 50 KHz calls are used just before the initiation of social play bouts in rats (Himmler et al., 2014; Kisko et al., 2015). Despite the suggestion that there are at least 14 different types of 50 kHz calls (Wright et al., 2010), it is unclear whether these different subtypes are all indicative of different behaviors. There is still a considerable challenge in the analysis of ultrasonic sound. This is partly due to the lack of automated tools to analyze the recorded sounds. After recording, the analysis of calls is carried out by human observers who inspect the spectrogram (audibly and visually) and label every recorded element. This is a very time consuming process as the number of calls can go from 500 to 1000 different elements in just one recording of 10 minutes. Recently, the first attempts to automate the analysis of ultrasonic sounds have been reported (Barker and Johnson, 2017; Barker et al., 2014; Reno et al., 2013), which hopefully will initiate the further technical development of these automated tools.

CONCLUSION AND FUTURE DIRECTION

Here, we argue that studies investigating animal models, especially those that are intended to screen potential new drugs, should exploit a more ethological environment and approach in order to permit an assessment of the functional meaning of behavior. In addition to other well-known validation criteria, such as predictive, construct and face validation, an ecological validated test setting, ethological procedure and analyses should implemented, before interpretation of behavioral results can take place. This is pivotal for an accurate evaluation of the translational value of an animal model. For example, we could recently show in our own laboratory that the application of ethological concepts is beneficial in assessing the impact of social isolation on social behavior by integrating the use of a home-cage setup (or familiar test cage) with objective, measures of social behavior and more ethologically-based analysis techniques. The computerized technical aspects both in observing and analyzing behavior are indispensable for this approach.

Another behavior of rodents, which relies even more on technical tools to record and analyze them, are ultrasonic vocalizations. These are an essential part of the behavioral social repertoire of rodents and are probably more relevant to these nocturnal animals living in enclosed environments than postures. This source of auditory information has been neglected for too long. Thus, most animal models, in particular 'social animal models' will benefit from this 'Refinement' in experimental methodology, which will increase the validity of animal experiments and ultimately the translation value of animal models. The field of behavioral neuroscience can benefit greatly from conceptual and technical progress in science when all these elements are implemented in research.

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CHAPTER 3

Novel approach to automatically classify rat social behavior using a video tracking system

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ABSTRACT

In the past, studies in behavioral neuroscience and drug development have relied on simple and quick readout parameters of animal behavior to assess treatment efficacy or to understand underlying brain mechanisms. The predominant use of classical behavioral tests has been repeatedly criticized during the last decades because of their poor reproducibility, poor translational value and the limited explanatory power in functional terms. We present a new method to monitor social behavior of rats using automated video tracking. The velocity of moving and the distance between two rats were plotted in frequency distributions. In addition, behavior was manually annotated and related to the automatically obtained parameters for a validated interpretation. Inter-individual distance in combination with velocity of movement provided specific behavioral classes, such as moving with high velocity when "in contact" or "in proximity". Human observations showed that these classes coincide with following (chasing) behavior. In addition, when animals are "in contact", but at low velocity, behaviors such as allogrooming and social investigation were observed. Also, low dose treatment with morphine and short isolation increased the time animals spent in contact or in proximity at high velocity. Current methods that involve the investigation of social rat behavior are mostly limited to short and relatively simple manual observations. A new and automated method for analyzing social behavior in a social interaction test is presented here and shows to be sensitive to drug treatment and housing conditions known to influence social behavior in rats.

INTRODUCTION

Measuring social rat behavior should be a necessity in studies using animal models for human psychopathologies, such as autism, depression and schizophrenia. However, there is a striking inconsistency between the strong relevance of social behavior and its relative minimal use in animal studies on brain disorders. Apart from methodological issues that will be addressed below, this may be due to the complex nature of social behavior. Its highly interactive character requires the continuous perception of the other animal's reaction to its own behavior and thus, continuous adaptive responses. This dynamic behavior is more difficult to recognize, analyze and interpret, than a single behavioral element that is usually manually scored. Traditional social behavioral parameters, such as time spent in playful interaction or inter-individual distance, initially seem easy to quantify and understand. However, an important shortcoming here is that behavior is simplified and degraded to a single readout parameter, thereby, losing the representation of the complex dynamic features of this behavior. Another issue is that there seems to be neither a generally adopted strategy, nor a definition, by which social behavior, especially social play, is measured and analyzed (Pellis and Pellis, 1998).

Most studies measure behavior in a relatively small and novel environment in which animals are placed for a short time period. In addition, in some cases animals are tested during their inactive phase of the diurnal cycle using bright light conditions. These test conditions do not provide an optimal challenging environment for the expression of complex social behavior. Moreover, social behavior is typically scored by a human observer quantifying only frequencies and durations of a few specific social elements. On the other hand, it is possibly to acquire more meaningful behavioral data by using detailed ethograms in combination with multivariate approaches. For example, the application of temporal pattern analysis has proven to be an effective tool to investigate the behavior of rats in an elevated plus maze (e.g. Casarrubea et al., 2013).

Whereas, in general, innovative methodologies in neuroscience are continuously becoming available and are rapidly applied, behavioral science seems hesitant in adopting new advanced hard- and software tools to measure behavior of rodents (Fonio et al., 2012; Spruijt et al., 2014). This is surprising in view of the successful use of technology in behavioral studies introduced in the 80ties and 90ties (e.g. Sams-Dodd, 1995; Spruijt and Gispen, 1983; Spruijt et al., 1992)

and the acknowledgement of numerous clear disadvantages of manually scored behavior. For instance, it is very laborious, time consuming, error prone and subjective.

The category 'social behavior' encompasses play behaviors and other affiliative social behaviors, on which we focus here, as well as agonistic, parental and sexual behaviors. Play behavior has received attention across different species because of its typical form, crucial effect on development (Cooke and Shukla, 2011; Pellis and Pellis, 2009) and because of its possible application as a welfare indicator (Boissy et al., 2007; Held and Špinka, 2011). In addition, studies have focused on the rewarding aspects of play, often in relation to reward sensitivity and abnormal brain function (for reviews see e.g. Siviy and Panksepp (2011); Trezza et al. (2011). Besides play, affiliative social behaviors comprise allogrooming, crawling over or under each other, huddling or sitting close together and following/approach behaviors. The aim of the current study is to provide an automated method that allows the detection of changes in both affiliative as well as play behaviors.

The development of advanced techniques for automated identification and objective analysis of behavior has been ongoing for a number of years now. Recently, several methods have been described allowing automated monitoring of social dyads in rodents (De Chaumont et al., 2012; Giancardo et al., 2013; Kabra et al., 2013; Ohayon et al., 2013; Shemesh et al., 2013; Weissbrod et al., 2013). When sophisticated software tools and systems are used to observe and identify behavior in full detail this automatically leads to a second issue: how to deal with the complex data. Unfortunately, little attention has been paid to the manner in which automatically acquired data, both from individual and multiple animals can be analyzed. The use of (top view) video images of behavior enables detailed analysis of these images. A few studies have tried to adopt and develop advanced statistical methods for analysis of trajectory data from automated video tracking of rats and mice (e.g. Drai and Golani, 2001; Drai et al., 2000; Kafkafi et al., 2003). The elegant approach of Golani and co-workers includes statistical methods to search for natural categories or so called 'modes' in the data itself. They could demonstrate that locomotor behavior of exploring rats can be divided into distinct categories of movement (Drai et al., 2000), comparable to the use of different gears when for instance driving a car. For example, a rat can still move around while hardly leaving its location i.e. as if it is using its first

"gear". Alternatively, it can make a run from point a to b with a much higher velocity using its second or third "gear". This method of analyzing locomotor behavior by defining different categories of velocities has been successfully applied in a few other studies using rats or mice, however, using individual animals (e.g. Grieb et al., 2014; McGinty et al., 2013). Periods of stationary movements have been called 'lingering' behavior since animals are not necessarily inactive, but can perform behaviors such as rearing and scanning of the environment. Movements with higher velocity are often called 'progression' or 'progressing'. We recently showed that drugs may differently affect lingering or progressing. For instance, morphine can enhance or inhibit progressing depending on the selected dose and time-interval after injection (Spruijt et al., 2014).

Here, we describe a novel automated method of quantifying social interactions. We take advantage of available sophisticated analysis techniques by applying automated continuous video tracking of pairs of rats. The previously noted methodological and biological shortcomings are addressed by using the proposed method as introduced above, for the distinction of different velocity categories. Yet, we extend this by applying it in a social context and on inter-individual distances to reveal possible different categories of proximity. Our method does not require a-priori defined and arbitrarily chosen criteria that define movement or being in proximity, rather, these thresholds are extracted from the frequency distribution data. In addition, we now combine velocity with inter-individual distances which leads to new behavioral classes. These behavioral classes represent for instance moving with high velocity and being in (close) proximity. We hypothesized that such classes composed of velocity of movement and inter-individual distance are sensitive to treatment with morphine and short social isolation as this has been repeatedly shown to increase social behaviors in rats (e.g. Niesink and Van Ree, 1989; Vanderschuren et al., 1995a). In addition, to provide a full validation of our method automatically obtained behavioral classes were also compared with human observer data.

MATERIAL AND METHODS

Animals

A total of 26 male Sprague Dawley (Hsd:SD) rats were weaned at Harlan, the Netherlands, at an age of 3 weeks and housed in sibling pairs. Two individuals from each mother were selected and formed a test pair. Subsequently, the pairs

arrived in this configuration at Delta Phenomics research facility (Utrecht, the Netherlands) and were housed under reversed light-dark regime (red light on at 09:00 hours, white light on at 21:00 hours.). Rats were housed in Macrolon IV-S cages with a flat lid (Techniplast, Italy). Each cage contained wood chipped bedding (Abedd® wood chips, LAB & VET Service GmbH, Vienna, Austria), a plastic tube and some tissue material. Food (CRM (E), Special Diets Services, United Kingdom) and tap water were provided ad libitum. The holding room was maintained at 21 ±1 ˚C, with relative humidity set between 45-65%. All animals were habituated on regular basis to human handling before start of the experiments. Experimental testing started when the animals were 5 weeks of age, weighing an average of 91.4 ± 6.5 gram. This age was chosen to ensure high levels of social (play) behavior. It is known that rats have a peak in play activity between 30-40 days of age (Panksepp, 1981). The experiments were performed in adherence to the legal requirements of Dutch legislation on laboratory animals (WOD/Dutch 'Experiments on Animals Act') and were reviewed and approved by an Animal Ethics Committee ('Lely-DEC').

Apparatus and software

All testing took place in an enlarged PhenoTyper® instrumented cage (Noldus Information Technology, the Netherlands) under red light conditions. The animals are provided with this large environment because the expression of social behavior requires space, see for example Spruijt et al. (2014). The cage consisted of a black floor plate (floor dimensions: 90 x 90 cm), transparent Perspex walls (height: 100 cm) and a roof equipped with infrared emitting LED's (peak range average of 950nm), on which a PhenoTyper top-unit was placed (Noldus Information Technology) containing an infrared sensitive camera (CCD 1/3" SONY SUPER HAD CCD black/white) and IR-filter (type Kodak 87C). Digital top view video recordings (25 samples per seconds) were made using a computer placed in an adjacent room. Video recordings were processed afterwards with the video tracking software EthoVision XT 8.0 (Noldus Information Technology) using the detection settings 'static subtraction'. For each sample the software stores the x- and y-coordinate of the animal's position. See Noldus et al. (2001) for more detailed information on the software. Animals were marked red or black using a permanent marker (Edding, Germany) in order for the software to individually recognize both individuals. In contrast to the black marking, the red marking is

not visible in the video because of the infrared light conditions. This way, the software recognizes a marked versus an unmarked animal, while both animals experience the same handling procedure. A similar procedure is used by (Sams-Dodd, 1995; Spruijt et al., 1992). Occasional identity swaps made by the software were corrected manually after video tracking.

Drugs

We validated our method by using a low dose of morphine that stimulates social behaviors (see e.g. Niesink and Van Ree, 1989; Vanderschuren et al., 1995a). Morphine-HCL (Centrafarm, the Netherlands) at a dose of 1 mg/kg and in a volume of 2.5 ml/kg was dissolved in NaCl (0.9%) and administered subcutaneously in the nape of the neck 30 minutes prior to testing. Control saline injections consisted of an equivalent volume of NaCl (0.9%) using the same route of administration.

Social interaction test

Before the social interaction test, animals were habituated to the experimental setup and procedure on two separate days before the start of the first test. This involved marking of the animals, a subcutaneous saline injection in the nape of the neck and habituation to the PhenoTyper cage for 30 minutes. The effect of repeated testing was considered to be minimized by these 2 days of habituation due to the fact that any initial novelty induced behavior declines after repeated exposure to the test environment (Spruijt et al., 2014). In the social interaction test animals were allowed to freely interact for 30 minutes with their familiar and similarly treated cage mate. Two social interaction tests were conducted: 1) without any isolation, thus, socially-housed (SOC) and 2) with 48 hours of short isolation of all pairs prior to the test (ISO). A repeated mixed cross-over design was used. In the first social interaction test (SOC) pairs were treated with either morphine (n=7 pairs) or saline (n=6 pairs) 30 minutes prior to testing. In the second social interaction test (ISO), the same pairs were tested again, hence the repeated design, and, again both animals of a pair received either morphine (n=7 pairs) or saline (n=6 pairs) treatment 30 minutes prior to the social interaction test.

Behavioral Category	Behavioral element	Description
Social	In proximity	Being within one tail length from the partner without actively seeking or engaging in social interaction.
	Following	Following the partner within a tail length distance.
	Social sniffing	Exploration of the partner's body by sniffing (except the anogenital region).
	Anogenital inspection	Exploration of the partner's anogenital region and tail.
	Allogrooming	Chewing and licking the fur of the partner.
	Crawling over/under	Climbing over or crawling under the partner.
	Nape attack	Snout or oral contact is directed to the neck region of the partner can be accompanied with biting and pulling fur in that region.
	Pinning/supine	The animal is standing over the partner, often using its front paws, to hold the other down (pinning), while partner is lying on its back (supine position).
	Biting/pulling	Pulling or biting the fur of the partner at any part of the body, except the neck region. Often accompanied by the receiver reacting defensively.
	Boxing/kicking	Both animals rear and box at each other with their front paws or kick with hind paws at each other defensively.
	Defense (push off/away)	Pushing away the partner, but not moving away. Includes reposition of the body during interaction. Can also be followed by an avoiding response ('avoid') if the animal pushes away and then moves away.
	Approach	Moving towards the partner. The animal moves in a straight line.
	Avoid	Moving away from the partner with at least one body length. Often the opposite of an approach, the animal moves in a straight line away.
Non-Social	Other	All individual behaviors performed when not in proximity of the partner, e.g. rearing, self-grooming, sitting.
	Mobile exploration	All individual movements through the cage.

Table 1. Ethogram. All behaviors were scored as states. Since the behavioral elements were scored from the view of one of the animals (focal animal) of a pair, behavioral elements from the category 'social' are scored either as receiver or as actor.

Manual scoring

Ten social interactions tests, were randomly selected across treatment groups and manually scored by one observer blind to the treatment. Software (The Observer XT10, Noldus Information Technology) was used to score behavior afterwards from captured video files using ½ playback speed of the video to precisely code the behavior. See table 1 for the used ethogram. Continuous focal animal sampling was used in such a way that only one animal of the pair was followed. However, when for instance following behavior displayed by the focal animal is scored , this animal can either be the one that is following or is followed (thus being actor or receiver, respectively). Individual animals of a similarly treated pair displayed. Passive or active behaviors in the same way. As a consequence, all social behaviors were listed as the behavior of a pair and information on the role of actor or receiver was not distinguished in data analysis.

Data analysis

Determining arrests and movements

After video tracking, the raw data containing the x- and y-coordinates of each animal in the pair was exported from EthoVision to MatLab® R2012b (The MathWorks, United States). Data was further analyzed with help of custom made MatLab scripts. To remove any noise, raw track data was smoothed using a robust Locally Weighted Scatter Plot Smoothing (LOWESS) filter with a 1-s time window. This connects points that are representative of the animal's trajectory and finds the most optimal fit, see (Hen et al., 2004) for a more extensive description. After smoothing other variables were calculated, such as velocity and distance between animals. Based on the statistical method introduced by Golani and coworkers, as described in Drai et al. (2000), the velocity with which a pair of animals moved was profiled.

Raw data was filtered with a repeated running median using a one-dimensional median filter with window size 13, 11, 9, 9 respectively. Data was collected with 25 Hz, producing a series of 25 x- and y-coordinates per second. This relative high sampling rate required a repeated running median approach. Basically, this means that a moving window of a few samples (13, 11, 9 and 9) is moved over the data. In this moving window the median of the consecutive samples is determined.

Tracking data was divided into movement bouts by detecting the 'segments of arrest' i.e. moments were the animal really has come to a stop without any clear visible movement of the body. Hereby, movements bouts are defined as the path between two arrests and subsequently, the maximal velocity that is reached by the animal during a movement bout can be determined. Because the tracking software always detects minor displacement of the center point, even when animals are at arrest, a threshold for arrests had to be determined. To find the optimal threshold, clear visible moments of arrests of one animal in the video were manually scored by an observer using ½ playback speed. In addition, it was scored when the animal was moving but without clear forward movement, i.e. more than two steps in the same direction, and it was scored when there was clear forward movement. Based on the distribution of arrests, a tolerance for arrest was set at 0.07 cm between 2 samples lasting at least 4 samples (0.16s). The threshold was verified by visual inspection of graphs of track visualization with velocity that were integrated with results of manually scored: 1.) arrests 2.) movements when staying in place and 3.) forward movements using this threshold.

Frequency distributions

Then, frequency distributions (histogram) of the maximal velocities of each movement bout were made. This was done for both social interaction tests (ISO and SOC) and per treatment. On these distributions the best gaussian curves that represent the data were fitted with an expectation maximization (EM) method. In short, it was first determined if different components, in this case Gaussian curves, could be recognized within the population. Thereafter, proportion, mean and standard deviation were estimated of each Gaussian curve with the EM algorithm. For more details on this method see for example Drai et al. (2000).

After visual inspection of all frequency distributions plots, two gaussian curves were plotted on the frequency distributions plots (see also Figure 1, left panel). This was verified by assuring that the mean values of the curves were at least two standard deviations apart. A positive outcome of this verification step confirmed that the two curves are indeed representing the data. Subsequently, the intersections of the curves created with the EM method were used to determine a threshold/cutoff value for the different modes or categories in which an animal moves.

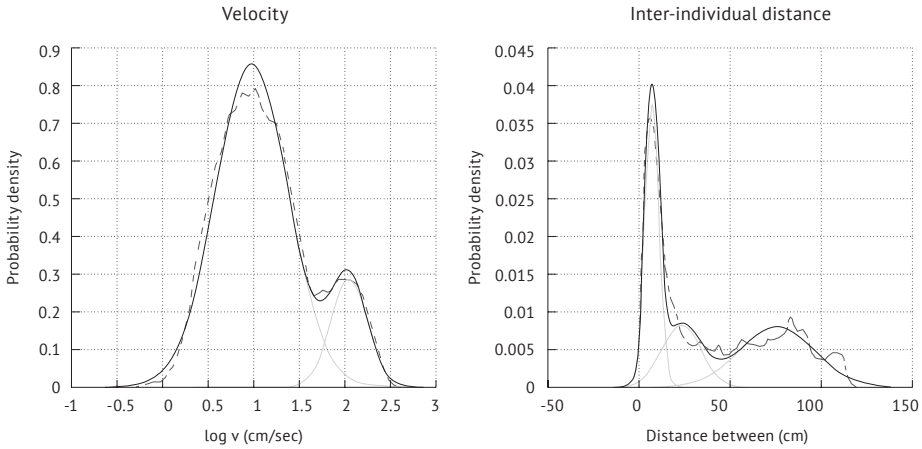

Figure 1. Two examples of the data analysis process to identify different modes or categories of velocity and social behavior (proximity) using frequency distributions of velocity (left) and distance between the two rats (right). The blue dashed line represents the empirical data, the red lines are Gaussian curves that are the result of the Gaussian expectation maximization. The cut-off values above the graphs are the intersection points of the red lines which determines the thresholds by which different modes or categories of velocity and proximity are defined.

Additionally, a similar analysis was done on the inter-individual distance between animals. The difference here is that distance between animals was calculated for each sample in the tracking data. Subsequently, all the inter-individual distances per sample were plotted in a frequency distributions. After visual inspection of all frequency distributions plots, three gaussian curves were plotted on the frequency distributions plots (see also Figure 1, right panel). The intersections of the curves created with the EM method were used to determine the different modes or categories of inter-individual distance.

Behavioral classes and statistical analysis

The identified modes or categories in velocity and inter-individual distances were combined into behavioral classes. After that, data were statistically tested using R statistical software (version 2.15.2). The pair of animals was considered as the statistical unit (thus samples size is the number of pairs) for all behaviors. Normal distribution and outlier checks were performed and after that all analyses was done using non-parametric testing. To compare the effect of morphine

with saline treatment and the effect of 48 hours short isolation, an unpaired Wilcoxon rank sum test (also known as the Mann-Whitney U Test) was executed over the full length of the social interaction test: 30 minutes.

RESULTS

Frequency distributions

Visual inspection of the inter-individual distance and the velocity frequency distributions per pair revealed no major differences between pairs of the same group and treatment. Therefore, inter-individual distance and velocity profiles were created across test condition (ISO or SOC) and across treatment (MOR or SAL). Fitting the Gaussian expectation maximization yielded two curves that divide the movements of the animals in two separate categories or modes: 1) "low velocities" and 2) "high velocities". For inter-individual distance, the application of the gaussian method resulted in three curves and thus, three different categories or modes of sociality: 1) "in contact" 2) "in proximity" and 3) "not in proximity".

Manual scoring versus automatic parameters

The percentage that each manually scored behavior occurs relative to the nine different classes of automated behavioral classes is depicted in table 2. The three distinct velocity categories are indicated with 'low' (i.e. both animals are moving with low velocity), 'high' (both are moving with low velocity) and 'low + high' (one rat is moving with high velocity, while the other is moving with low velocity). In addition, the behavior of the rat pair can fall into the "in contact", "in proximity" and not in proximity" while moving with a certain speed. The shading in table 2 highlights a high level of coincidence of the manually scored behavior (first item in each row) and the automated behavioral classes (first item of each column).

For example, it shows that all manually scored social behaviors were only occurring in the "in contact" and "in proximity" category but not in the "not in proximity". Furthermore, some behaviors were almost exclusively represented in one specific behavioral distance/velocity class. For instance allogrooming, nearly only occurred when being "in contact at low velocity". Also, all behaviors related to play were mainly found in the "in contact" category while individual

	Contact			In proximity			Not in proximity		
Category	low	high	low + high	low	high	low + high	low	high	low + high
Allogrooming	93.8	1.4	4.3	0.4	0.1	0.1	0.0	0.0	0.0
Anogenital inspection	37.9	17.0	26.6	4.1	9.5	4.8	0.0	0.1	0.0
Social sniffing	38.3	11.4	21.1	9.0	9.0	10.7	0.0	0.3	0.2
In proximity	27.8	3.0	8.3	37.5	7.2	15.0	0.2	0.5	0.5
Approaching	0.5	3.6	4.7	2.6	19.2	19.8	1.1	27.7	20.9
Avoid (moving away)	2.2	18.3	8.3	1.7	40.0	10.5	0.2	13.5	5.4
Nape attacking	24.8	38.0	24.5	0.5	9.4	2.1	0.0	0.6	0.1
Pinning/supine	65.0	16.2	18.1	0.1	0.4	0.3	0.0	0.0	0.0
Biting/pulling	33.1	34.2	23.4	2.3	5.3	1.7	0.0	0.0	0.0
Boxing/kicking	25.0	32.0	24.6	1.3	11.4	4.2	0.0	1.2	0.2
Crawling over/under	42.4	28.3	26.3	0.6	1.5	1.1	0.0	0.0	0.0
Defense	11.7	46.1	22.6	0.6	15.0	3.3	0.0	0.5	0.2
Following	1.0	26.5	4.4	0.5	56.7	3.4	0.0	7.3	0.1
Other	0.1	0.2	0.3	6.5	1.7	5.0	50.5	6.6	29.2
Mobile exploration	0.0	0.7	0.5	1.2	7.6	7.9	9.5	27.2	45.4

Table 2. Comparison of the manually scored behaviors in relation to the automatically obtained behavioral parameters. The percentage that each manually scored behavior occurs relative to the nine different classes of automated behavioral classes is depicted. The three distinct velocity categories are indicated with 'low' (i.e. both animals are moving with low velocity), 'high' (both are moving with low velocity) and 'low + high' (one rat is moving with high velocity, while the other is moving with low velocity). The shading highlights a high level of coincidence of the manually scored behavior (first item in each row) and the automated behavioral classes (first item of each column).

behaviors (other and mobile exploration) were almost only seen in the "not in proximity" category. Also, following (chasing) behavior is performed at high velocity being both "in contact" and "in proximity".

Effects of short isolation and compound validation

Total distance moved

An overall effect of morphine on the total distance moved per animal pair during the 30 minutes social interaction test was detected (Figure 2). Morphine significantly increased the total distance moved as compared to saline ($U = 37$, $p = 0.022$) in the social test condition. In the short isolation test condition, morphine also increased the total distance moved as compared to saline, however, this was only a near significant trend ($U = 35$, $p = 0.051$). Furthermore, an effect of short isolation (48 hours) on total distance moved per pair was also observed in the saline ($U = 36$, $p = 0.002$) and morphine ($U = 47$, $p = 0.002$) treated groups.

Movement with low or high velocities

Both high velocity (Figure 3) and low velocity movements (data not shown) were affected by drug treatment when given after a short isolation. In the social interaction test after short isolation, morphine significantly increased the time spent moving with high velocity as compared to saline ($U = 38$, $p = 0.014$). Also, morphine treated animals spent significantly more time moving with high velocity when tested after short isolation ($U = 43$, $p = 0.017$).

Inter-individual distance combined with velocity of movement

The classification of the velocity modes combined with that of the inter-individual distance resulted in nine different behavioral classes that represent sociability. These nine classifications are defined by a specific combination of a pair that can either be "in contact", "in proximity" or "not in proximity" (the "social modes") and move with high velocity or low velocity, or one is moving with high velocity and the other is moving with low velocity ('the velocity modes'). In the latter mode, both animals moving at different velocity, there were no differences seen between the treatments in both conditions (social and short isolation).

Morphine compared to saline, significantly increased moving with "high velocity when in contact" ($U = 42$, $p = 0.001$) after the short isolation condition, (Figure 4A) but not after the social condition. In the interaction test after social

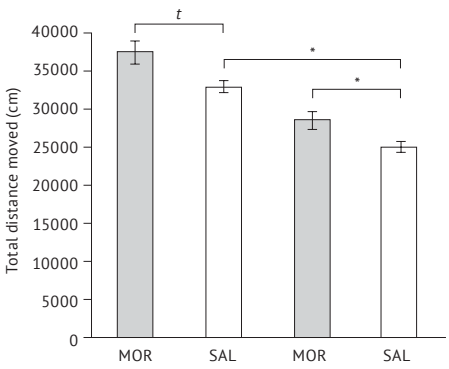

Figure 2. Average (±SEM) total distance moved in centimeters during the 30 min-social interaction tests for the two treatment groups after short isolation housing (48 hours) and social housing. Morphine (MOR, n = 7) 1 mg/kg and saline control (SAL, n = 6). Asterisks indicate significant differences; *p<0.05 and t indicates a trend p<0.1

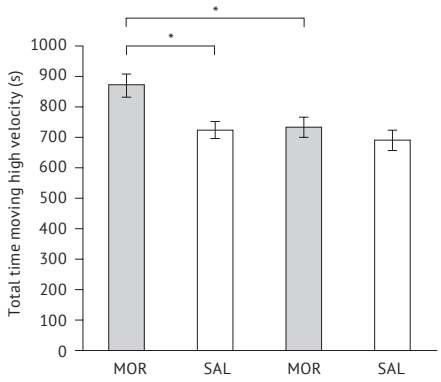

Figure 3. Average (±SEM) total time moving with high velocity during the 30 min-social interaction tests for the two treatment groups after short isolation housing (48 hours) and social housing. Morphine (MOR, n = 7) 1 mg/kg and saline control (SAL, n = 6). Asterisks indicate significant differences; *p<0.05

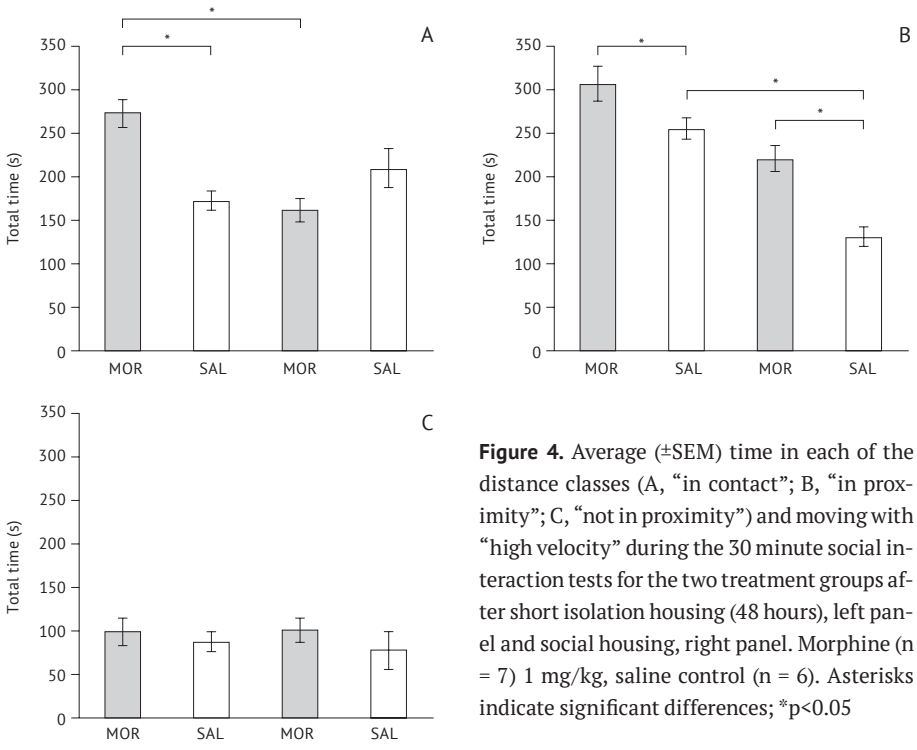

Figure 4. Average (±SEM) time in each of the distance classes (A, "in contact"; B, "in proximity"; C, "not in proximity") and moving with "high velocity" during the 30 minute social interaction tests for the two treatment groups after short isolation housing (48 hours), left panel and social housing, right panel. Morphine (n = 7) 1 mg/kg, saline control (n = 6). Asterisks indicate significant differences; *p<0.05

housing morphine significantly increased moving "with high velocity when in proximity" compared to saline ($U = 41$, $p = 0.002$) but not in the isolation condition (Figure 4B). When animals were moving "with high velocity when not in proximity" no effects of morphine or short isolation were observed (Figure 4C). In addition, the effects of short isolation housing were observed in both morphine and saline treated animals. For morphine treated animals, short isolation significantly enhanced the time moving with "high velocity in contact" (Figure 4A, $U = 49$, $p < 0.005$) and "high velocity in proximity" (Figure 4B, $U = 44$, $p = 0.011$) compared to social housing. Saline treated animals also spent significantly more time in moving with "high velocity in proximity" in the social interaction test after short isolation as compared to social housing (Figure 4B, $U = 36$, $p = 0.002$).

DISCUSSION

Our method revealed that juvenile rats show two distinct modes or categories of movement: movements with low or high velocity. This is in line with observations of others in mice (e.g. Kafkafi et al., 2003) and in rats (Drai et al., 2001; Drai et al., 2000). In addition, by applying the same method -now for the first time - on inter-individual distance we detected three distinct modes or categories of inter-individual distances: "in contact", "in proximity" and "not in proximity". Interestingly, the combination of velocity with inter-individual distance yielded distinct behavioral classes which are sensitive to pharmacological and environmental treatment and which are consistently in line with human hand scored behavioral data.

In the "in proximity category", hand scored behaviors mainly observed are: in proximity (without being actively engaging in social interaction), approach or avoidance and following (socially active). While, in the "in contact" category (very close: one whisker-length away) most observed behaviors are the play behaviors, social investigation or social grooming. This suggests that our automated method is capable of distinguishing real physical contact behaviors from social behaviors that do not necessarily involve physical contact (touch) such as following behavior (chasing) or approach/avoidance. In addition, by combining inter-individual distance with velocity of movement the automated behavioral classes also identifies following behavior which takes place at high velocity. Moreover, it characterizes behaviors such as allogrooming, social sniffing and

play contact behaviors that are mostly performed when animals are both moving with a lower velocity. The automated behavioral classes are in line with manual scoring at a compatible level as two human observers, which usually achieve 80% reliability. In the present study this is sufficient to detect the effects of morphine treatment and environmental manipulation i.e. isolation.

Short isolation from peers before the test is a well-known manipulation to increase social behavior of rats (e.g. Niesink and Van Ree, 1982). The here defined (automated) behavioral classes demonstrate a clear effect of short isolation on social behavior. Both morphine and saline treated animals move more with high velocity when "in contact" or "in proximity" in the social interaction test after short isolation. In addition, our study shows that morphine intensifies social behavior by increasing moving with high velocity, while simultaneously decreasing the inter-individual distance from "in proximity" to "in contact". Probably, morphine strengthens the intrinsic motivation to engage in social behavior with the two aforementioned effects as a consequence. This is in line with previous studies showing that morphine increases levels of social (play) behavior (Niesink and Van Ree, 1989; Vanderschuren et al., 1995b) after a short period of individual housing before treatment. The effects of morphine are most clearly seen in the high velocity category which is comparable with the earlier finding that morphine increases moving with high velocity in individual animals (Spruijt et al., 2014).

The automatically determined "in proximity" matches relatively poor with the manually scored "in proximity". In proximity is defined in the manual soring as: "Being within one tail length from the partner without actively seeking or engaging in social interaction". A human observer is biased towards scoring changes in behavior in contrast to the system, which scores at a fixed frequency (every sample) the behavior again. Thus, our current automated observation tool determines per sample in which category the sample belongs which could have resulted in a slight overestimation of frequencies as compared to human scoring. In addition, human observers tend to interpret behavior after a behavior or behavioral sequence has occurred and, therefore, short events are tend to be neglected by human observers. This way of observing may escape human awareness. On the other hand, also non intended proximity behaviors may have occurred which is not recognized by the human observer as in proximity, for example when the animal is highly active and runs around it can cross the oth-

er individual's proximity area. When humans do not see the intention to be in proximity they also do not score it, whereas when objectively measured they are. The factor intention as easily and unaware used by humans to identify behaviors is not part of automatic system and may, thus result, into differences. This could have caused the relatively poor matching of these categories.

In the social interaction test animals perform a mixture of social behavior and otherwise motivated behaviors. The present method provides an objective tool to focus on the efficacy of a treatment on the "real" social episodes in the test. Those episodes are characterized by a decrease in the inter-individual distance which occurs in the "in contact" or "in proximity" distance categories. When trial duration increases to hours and even days, our method could extract the specific episodes of behavior in which social interaction takes place. This is important when possible effects of a treatment are expected to preferentially occur in the social domain alone. Hypothetically, when animals which show aberrant social behavior are treated with an antidepressant agent or anxiolytics this should restore baseline social behavior. In our test setup, which has the benefits of a home-cage approach, this could easily be measured by automatically selecting the social episodes and subject those episodes to further analysis

The use of automated (social) behavioral parameters based on coordinates of the animals is not new. For example, Sams-Dodd (1995) used an automated parameter for social interaction in rats. In his study, a fixed threshold was used: animals are in close proximity when their center of gravity points are within 20 centimeters from each other. It was mentioned that (p.161): "selection of a criterion value of 20 cm is based on systematic variation of this parameter from 0 to 50 cm" and "the value of 20 cm resulted in the least variation in the data". It is exactly this variation that we now use to obtain the different categories of proximity by using the frequency distributions of inter-individual distances. An important benefit of our approach is that the threshold is not assessed artificially (arbitrarily) by limiting variation but based on the animal's own behavior. When the size of animals changes (due to age or gender) or different setups are used, the arbitrarily chosen cutoff values have to be assessed again, whereas in our approach this is deduced from the actual data. What is regarded as high velocity or being in proximity is defined by the variation in the occurrence of different velocities or inter-individual distances and not by an arbitrarily chosen value.

In the future, nose and tail point recognition or even 3 dimensional image

building of the animals, see for example Matsumoto et al. (2013), could have an added value, because, it might be possible then to capture the orientation of animals towards each other. However, the current tracking of body contour is still a challenge for the software when the animals are close together. Here, the overlapping pixels cause merging of body contours and that makes it difficult for algorithms to recognize the individual rats and, thus, also to correctly identify the nose or head area. Still, in future, ideally, tracking software should be able to recognize full body contour and movement of individuals even when they are close together. Many behavioral elements have such specific characteristics such as the "pinning" behavior and nape attacks, that these characteristic might be useful in "learning" the software to recognize these behaviors. A similar approach was recently applied in mice, where 2 relatively simple behaviors 'walk and follow' could be recognized by the system (Kabra et al., 2013). Most computers systems are not a hundred percent error free. It is especially difficult for a computer system relying on only top view camera images to identify different animals properly when they are close together or overlap partially. In our setup, using commercially available tracking software, we needed to manually correct identity swops before our analysis could take place. Also, other software systems have dealt with these issues. For example, some provide tracking supervision in which the tracks can be corrected frame-by-frame when two blobs of animals are overlapping in such a way the software does not recognize two individuals (De Chaumont et al., 2012).

Due to all the recently developed new systems it seems that long-term monitoring of laboratory rodents in a group should be readily adopted in future research. Application of such methodology though, requires a vast understanding of the basic principles behind the software and the subsequent statistical methods to analyze the data. There is still a huge gap between the extraction of biological relevant information from complex data sets derived from long-term group housed animals and the current state of the techniques (Branson, 2014). Regardless of the system used, our approach of data analysis could potentially also be applied on data output from other systems, as long as it allows an accurate identification, determination of the velocity of movements and the inter-individual distance. In the future, we plan to integrate the behavior with ultrasonic vocalizations of the rat pairs which will further increase the sensitivity of our system and leads to a better understanding of the social behavioral

profile of rats. The ultimate goal is to continuously monitor group housed rats in a home-cage environment to study their behavior in a relatively fast, automated and objective way without interference of human handling.

ACKNOWLEDGMENTS

We kindly appreciate the help of Esteban Portal in creating the custom R scripts for data processing, exploration and (statistical) analysis. We would also like to thank Jan van der Kieft and Niek van Stipdonk for their help with the manual corrections of the tracking data. The PhenoTyper-9000 cage was developed as part of the SenseWell project. We acknowledge our partners in this consortium (Noldus Information Technology BV, Metris BV, Telemetronics Biomedical BV, BioMetris Wageningen University, Faculty of Veterinary Medicine Utrecht University and EEMCS TU Delft) for their support. This research was partially financed by a grant from Agentschap NL (NeuroBasic-PharmaPhenomics) and ICTRegie (SenseWell).

CHAPTER 4

Short- and long-term behavioral analysis of social interaction, ultrasonic vocalizations and social motivation in a chronic phencyclidine model

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ABSTRACT

Phencyclidine (PCP) has been suggested to induce symptoms of schizophrenia. However, animal models using PCP administration have produced ambiguous results thus far. It seems that acute effects are similar to symptoms of schizophrenia, however, it is not clear if PCP can induce permanent behavioral changes that reflect schizophrenic-like symptoms. Therefore, we assessed the ability of chronic PCP administration (3 mg/kg, 14 days) to induce short or long lasting behavioral changes in rats. Social behavior, including ultrasonic vocalizations and motivation for social contact were investigated at different time points, up to 29-36 days, after cessation of PCP treatment. During a social separation test, performed at 5 and 36 days, PCP treated rats spent less time near the divider that separates them from their familiar cage mate compared with saline (SAL) treated rats. Further, at short-term, PCP was able to induce a decrease in social behavior. In contrast, at long-term, PCP treated animals spent more time in contact when exposed to an unfamiliar partner as compared to SAL treated rats. But, this difference was not observed when exposed to a familiar partner. We did not find any difference in ultrasonic vocalizations production at all time points. The results of our study indicate that PCP is unable to induce overt long-term deficits in social interaction behavior. Rather, it seems that PCP diminishes motivation for social contact. The long-term consequences of chronic PCP administration on social behavior in rodent models remain complex, and future studies addressing this are still needed.

INTRODUCTION

Phencyclidine (PCP) is a non-competitive ion channel blocker, n-methyl-D-aspartate (NMDA) receptor antagonist with strong psychotomimetic effects. Originally, it was used as an anesthetic, however, after patients reporting the full spectrum symptoms of schizophrenia its use in clinical settings ended (Javitt and Zukin, 1991). Also, chronic PCP abusers were misdiagnosed as being schizophrenic patients (Morris et al., 2005). PCP is also known as the party drug 'angel dust' because of its hallucinogenic capacities (Javitt and Zukin, 1991). Sub-chronic PCP administration has been suggested as an animal model that mimics schizophrenia. Schizophrenia is a neurological disorder with a variety of symptoms, including auditory and visual hallucinations, delusions and thought disorder. In addition, negative symptoms manifest as the absence of or deficits in functions that are normally present, such as alogia (speech poverty), avolition (reduced motivation), anhedonia (decreased sensitivity to rewarding stimuli), social withdrawal and depression. Social interaction and social withdrawal are important measures for investigating the negative symptoms of schizophrenia (Wilson and Koenig, 2014).

The effectiveness of NMDA antagonists such as ketamine, dizoclipine (MK-801) and PCP to induce schizophrenia symptoms has led to many studies investigating these compounds in laboratory animals. Administration of PCP in rats has demonstrated that NMDA receptor blockade is able to induce behavioral and neurochemical changes relating to the positive (Sturgeon et al., 1982), negative (Sams-Dodd, 1996) and cognitive symptoms (Jentsch et al., 1997) seen in schizophrenia patients. However, similar to their differences in potency, the three NMDA receptor antagonists appear to differ in their ability to model the core symptoms of schizophrenia. It seems that the majority of attempts to pharmacologically model schizophrenia have utilized PCP over other NMDA receptor antagonists. In particular, PCP administration has been applied to model social withdrawal in animals.

Indeed, previous studies on the acute effects of PCP in rats (Audet et al., 2009; Sams-Dodd, 1996; Sams-Dodd, 1998a; Koros et al., 2007; Tanaka et al., 2003) are largely in agreement that the time spent in social interaction is reduced after sub-chronic PCP administration, or after single PCP administration (Corbett et al., 1995). Compared to the large amount of research on merely the acute effects of PCP (typically within 24 hours after last administration) there

have been far less clear results on whether PCP can induce enduring deficits on social behavior. Sub-chronic administration of PCP has been found to cause social deficits in male rats that can last anywhere from 72 hours (Lee et al., 2005) to 1 week (Matricon et al., 2016; Seillier and Giuffrida, 2009; Seillier et al., 2010; Seillier et al., 2013). However, other research has not been able to replicate these findings: both sub-chronic and chronic PCP treatment regimens fail to produce enduring changes in social behavior, 72 hours (Egerton et al., 2008), 1 week (Li et al., 2012), 2 weeks (Metaxas et al., 2014), or 7-21 days (Sams-Dodd, 2004) after last injection. In contrast, one study even found increased social behavior of male rats after PCP administration, lasting even 6 weeks post-treatment (Jenkins et al., 2008). Interestingly, one research group has consistently shown social deficits even lasting up to 6 weeks in female Long-Evans rats (Snigdha and Neill, 2008a; Snigdha and Neill, 2008b). It is possible that these contrasting findings are due to gender differences in compound uptake and metabolism, since, there is evidence that female rats have higher blood plasma levels of PCP than males after being administered the same dose (Nabeshima et al., 1984).

Currently, there are limited studies on PCP that include a full spectrum behavioral analysis including ultrasonic vocalizations. It is known that rats express ultrasonic vocalizations (USV) between 32-90 kHz, usually referred to as '50 kHz calls', during social behavior (e.g. Knutson et al., 1998; Burgdorf et al., 2008; Willey et al., 2009). Diminished social behavior typically implies that a lower amount of 50 kHz calls is expressed (Lukas and Wöhr, 2015). Despite their potential to provide an additional measure of social behavior, in the chronic PCP model, ultrasonic vocalizations during social interaction or after separation have hardly been investigated. One study, investigating the effects of prenatal PCP exposure in combination with isolation housing found an increase in 50 kHz USV levels during social interaction, but no difference in total social interaction behaviors (Watson et al., 2016). In contrast, Boulay and colleagues (Boulay et al., 2013) observed a decrease in 50 kHz USV in rats exposed to human tickling, after a single PCP injection of 1 mg/kg but not of 0.5 mg/kg.

Thus, there is no clear view on whether repeated PCP administration can induce enduring social deficits in male rats and this prompted us to conduct a thorough study on PCP and social behavior. Especially, the lack of information on the effects of chronic PCP on USV production warrants further investigation. We aimed to observe and analyze the behavior of the rats in an etholog-

ical relevant manner with use of automated tracking software and recordings of ultrasonic vocalizations. As we (Peters et al., 2015; Spruijt et al., 2014) and others (Benjamini et al., 2010; Fonio et al., 2012; Zilkha et al., 2016) have previously argued, it is necessary that behavioral measurements are obtained and analyzed in a way that addresses the behavior from the animals point of view. We try to accomplish this by using a relatively big arena, testing rats that are socially housed throughout the experiment and by testing during the dark, and thus active, phase of the diurnal cycle using dim-red lighting conditions. In addition, behaviors are analyzed with automated tracking and objective behavioral parameters using a method we have previously validated (Peters et al., 2016).

This experiment was designed to test the effects of PCP on social behavior shortly after the cessation of a 14 day treatment period as well as testing any potential long-lasting effects of PCP 29-36 days after the treatment period ended. At both time points we monitored; 1) free social interaction behavior in pairs and, 2) the acute response to separation from a familiar cage mate, to investigate the effect of chronic PCP on motivation for social contact. Additionally, after 32 days, it was tested if a single clozapine injection was able to reverse any behavioral changes possibly induced by the repeated PCP injections. As our protocol closely resembles the study of Lee et al. with a positive result (Lee et al., 2005), it seems plausible to predict that we will find diminished social behavior at least 72 hours after the final PCP injection. Similarly we would expect our additional assays, USV's and separation tests, to show reduced pro-social 50 kHz vocalizations and a reduced motivation to stay in contact with the cage mate. The long-term effects are harder to predict since there is limited data on the effects of PCP after more than 5 weeks.

MATERIALS & METHODS

Rats and housing

Male Sprague-Dawley rats (Hsd: Sprague Dawley® SD®, n=30) were purchased from Harlan (Horst, the Netherlands) and arrived at PND 25 ±3 (weighing 49.5g ± 6.5). The choice of strain was based on the general notion that Sprague Dawley rats tend to be more expressive. In addition, it has been demonstrated that when compared to the Wistar (Manduca et al., 2014) and Long Evans (Ku et al., 2016) strains, Sprague Dawley rats exhibits higher levels of play behavior. The high levels of social (play) behavior of the Sprague Dawley rats potentially

increased the likelihood to discovery a reduction in social behavior.

Rats were weaned at Harlan, the Netherlands, at an age of 3 weeks and housed in sibling pairs. Two individuals from each mother were selected and formed a test pair. Subsequently, the pairs arrived in this configuration at Delta Phenomics research facility (Utrecht, the Netherlands). At arrival, rats were housed in sibling pairs in their homecages; Macrolon type IV-S cages with standard flat lids (Techniplast, Italy). After 4 weeks, the standard flat lids of the homecages were changed with raised wire lids with drop-in food hopper (series -119/-224, Techniplast, Italy) to allow the rats to completely stand up in their homecages, in line with EU-guidelines for housing laboratory rats. During the first 12 days after arrival, the rats were allowed to acclimatize to their new environment and habituated to human handling. Both rats in the cage received the same treatment. This set-up was adopted from Sams-Dodd (1995); Sams-Dodd (1996).

During the first 4 weeks Cellu-Dri Soft (Shepherd specialty papers, Watertown, TN, USA) was used in the homecages. This bedding was stored and mixed with fresh, clean bedding to provide bedding material for the social interaction test (see section experimental apparatus for an explanation). After this period, wooden chips (Abedd® wood chips. LAB & VET Service GmbH, Vienna, Austria) were used for bedding. In each cage a cylindrical semi-transparent amber-colored tube and two pieces of tissue paper were provided. Animals were kept on a reversed 12h light/dark schedule with white lights on 21:00 hrs, off at 09:00 hrs and dim-red lighting on during dark period. Rats had ad libitum access to food (Teklad Global 2018, Harlan, Madison, WI, USA) and tap water throughout the experiment. The housing room was maintained at a temperature of 20-22 ˚C and a humidity of 40-50% with constant background noise provided by radio. The experiments have been performed in adherence to EU-guidelines and national legal requirements concerning animal research (Wod/Dutch 'Experiments on Animals Act') and have been approved by an Animal Ethics Committee ('Lely-DEC').

Drugs and drug administration

PCP hydrochloride and clozapine were purchased from Lipomed AG (Arlesheim, Switzerland). PCP was dissolved in 0.9% sodium chloride solution (NaCl), and was administered at a dose of 3mg/kg (similar to Lee et al. (2005)) and at a volume of 1ml/kg using a sub-cutaneous (s.c.) injection in the nape of the neck. An s.c injection was chosen over an intraperitoneal (i.p) injection as it has been

found that injections of PCP via the s.c. route yield higher concentrations of the compound in the blood and brain of rats than via the i.p route (Kalinichev et al., 2008). Animals (n = 16) received one daily PCP injection between 09:00 and 11:00 hours for 14 consecutive days (similar to Lee et al., (2005). The animals in the control group (n = 14) received one daily saline (SAL) injection (0.9% NaCl) at an volume of 1 ml/kg, instead of a PCP injection. At the beginning of the PCP treatment period the animals were at PND 37±3 (weighing 130g ±16.4). Clozapine was dissolved in 0.9% NaCl and 1M lactic acid. The solution was then brought back to neutral pH using 10M sodium hydroxide (NaOH). Clozapine 2.5 mg/kg (similar to Corbett et al. (1995)) was administered via an i.p. injection at a volume of 5ml/kg 40 minutes before the social interaction test started. To control for the effect of the injection, a control injection consisting of 0.9% NaCL, 1M lactic acid and brought back to neutral pH using 10M NaOH, further named vehicle, was given to all the animals.

Experimental apparatus

All behavioral testing was carried out in an enlarged PhenoTyper® instrumented cage (PhenoTyper 9000, Noldus Information Technology, the Netherlands) under red light conditions (Figure 1). The animals are tested in this large environment because the expression of social behavior requires space, see for example Spruijt et al. (2014). The cage consisted of a black floor plate (floor dimensions: 90 x 90 cm), transparent Perspex walls (height: 100 cm) and a roof equipped with infrared emitting LED's (peak range average of 950nm), on which a PhenoTyper® top-unit was placed containing an infrared sensitive camera (CCD 1/3" SONY SUPER HAD CCD black/white) and IR-filter (type Kodak 87C). Video recordings were made on computers placed outside the experimental room and were processed afterwards using EthoVision XT 9.0 research edition (Noldus Information Technology, the Netherlands). A total of 4 PhenoTyper 9000 cages were connected to 1 computer and used simultaneously. The floor of the arena was covered with Cellu-Dri Soft (Shepherd specialty papers, Watertown, TN, USA) which had previously been exposed to all the subjects, by mixing the bedding that had been used in their home cages, thereby providing a baseline level of odor. In this way we attempted to reduce the novelty of the arena while not encouraging territorial behavior. In addition, it has been demonstrated that a novel arena with clean bedding can suppress the number of USV (Wöhr et al., 2008).

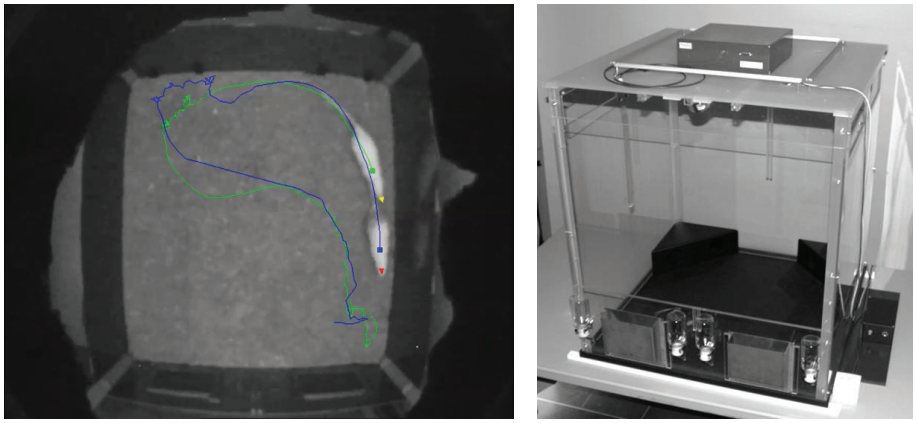


Figure 1. Example of an enlarged PhenoTyper 9000 (90x90x100 cm) and top view video tracking image of two rats using EthoVision XT 9.0 (Noldus, the Netherlands).

To record the USV a gold foil electrostatic transducer microphone (Metris, the Netherlands) with a frequency range of 15-125 kHz, minimum receiving sensitivity of -42 dB at 50 kHz and with a beam angle of 12 degree at 3 dB down (at 90 kHz) was integrated into the PhenoTyper-9000. The microphone was connected to a control unit, which was connected to a data acquisition board (National Instrument, M-series PCI-6250). Sonotrack software (Version 2.2.4, Metris, the Netherlands) was used to continuously record ultrasonic sound. The microphone was placed at the roof of the PhenoTyper cage at a height of 93 cm. Additionally, the roof of the PhenoTyper was shielded with sound attenuating pyramid foam to prevent recordings of echo's of the ultrasonic sounds.

Behavioral tests

This experiment was designed to test the effects of PCP on social behavior directly after the cessation of a 14 days treatment period as well as testing any potential long-lasting effects of PCP 29-36 days after the treatment period. In each of these two testing periods social interaction tests as well as one separation test were conducted to assess social motivation (Figure 2). All behavioral tests were conducted in the dark (i.e. active) phase of the animals' diurnal cycle, under infrared and red-light conditions, and were carried out in an experimental room with the same conditions as their housing environment.

Treatment PCP/SAL 14 days	Behavioral test:	Social interaction		Seperation	Social interaction			Seperation
	Pairing:	Familiar	Unfamliar	Familiar	Familiar	Unfamiliar + SAL	Unfamiliar + clozapine	
	Days after treatment	2&3	4*	5*	29 & 30	31	32	36

Figure 2. Schematic outline of the time schedule of the two behavioral testing periods carried out with the two treatment groups. After the animals arrived at PND 25±3, they were allowed to habituate to their new housing and handling for the first 12 days. After this period the animals were injected (s.c.) once daily with either PCP or SAL for 14 consecutive days. The behavioral testing consisted of two testing periods, the first commenced 2 days after the end of treatment and the second started 29 days after the end of treatment. *from social interaction test 4 days after treatment & from the separation test 5 days after treatment the USV's measurements were analyzed

Social interaction test

A social interaction test consisted always of two similarly treated rats being simultaneously placed into the PhenoTyper 9000, for 20 minutes, during which time top view video (see Figure 1) and ultrasonic sound were recorded. For the purposes of subject identification a color was randomly assigned to each individual within a pair, either black, or red (for control-purposes of the effect of the marking, i.e. red is not visible under infrared lighting conditions in the cage). Then the appropriate color was applied to the rear half of the animal using a marker pen (Edding, Germany) 30 minutes prior to the test, see also Figure 1, left panel for an example. After marking the animals were returned to their home cage until the start of the test. After the two initial social interaction tests on day 2 and 3 after treatment, the animals were tested in the social interaction test with the same protocol but with the exception that the subjects were paired with an unfamiliar, similarly treated and weight-matched partner (within 10% of the weight of the larger animal). Twenty-nine days after their final injection the animals were tested in the same test battery with the following exceptions: a) in the trials at 29 and 30 days after treatment the animals were fixated as well as marked 30 minutes prior to the test, to habituate them to the injection procedure, b) the social interaction tests at day 31 and 32 were preceded by a control i.p. injection, (vehicle) on day 31 and the atypical antipsychotic clozapine on day 32, c) for logistic reasons, the animals were injected and marked 40 minutes

prior to the test and then returned to their home cage. In all other respects the protocol for these two tests were identical to the ones carried out in the first week after treatment.

Separation test

Five and 36 days after treatment, the animals were also placed in a separation testing paradigm. The arena in which they were tested, the PhenoTyper 9000 (Figure 1), was the same as the one used in the social interaction test. Cage mates were placed into the arena simultaneously prior to the test and left for 10 minutes to acclimatize. At the end of the acclimatization period a solid Perspex divider, 60 cm high, was placed down the middle of the area separating the pair from each other. Since the divider did not reach the roof of the arena, we assumed that the animals were still able to hear each other's USV's and therefore, would be aware of the other's presence. In addition, in the corners animals could be aware of each other by some odor outflow trough tiny spaces between the walls and the divider. The separation test lasted for 20 minutes, during which both video and ultrasonic sound were recorded. Animals were not marked or injected during the separation test days.

Automated analysis of social behavior

Software

All behavioral tests were analyzed from video recordings using the automatic tracking software package EthoVision (EthoVision XT 9.0 Research Edition, Noldus Information Technology, Wageningen, the Netherlands). This tracking software, with the aid of the markings on the backs of the animals, is able to track the movements of both subjects within the arena. Comparable software algorithms have been successfully used in the past to assess social interactions in rats (Sams-Dodd, 1995; Spruijt et al., 1992). In short the software detects contrasts, recognizing objects through their differences in shade. The software takes 25 samples per second, and within each sample the location of each animal is detected by comparing the video frame to a reference image of the empty arena. The software is further able to distinguish the two animals using size based identification criteria, whereby, the black marking on the back of one of the rats makes its apparent size smaller relative to the other rat. The tracks of the subjects' movements during a trial allow us to measure several parameters, such as

the intra-individual distance and speed of moving. Occasional identity swops made by the software were corrected manually after video tracking.

Data analysis

Tracking data of the social interaction tests was analyzed with a previously validated method described in Peters et al. (2016). After video tracking, the raw data containing the x- and y-coordinates of each animal in the pair was exported from EthoVision to MatLab® R2012b (The MathWorks, United States). Data was further analyzed with help of customized MatLab scripts. First, raw data was smoothed using a robust Locally Weighted Scatter Plot Smoothing (LOWESS) with a 1-s time window and filtered with a repeated running median using a one-dimensional median filter with window size 13, 11, 9, 9 respectively. Tracking data was then divided into movement bouts by detecting the 'segments of arrest' i.e. moments were the animal really has come to a stop without any clear visible movement of the body anymore. Hereby, movements bouts are defined as the path between two arrests and subsequently, the maximal velocity that is reached by the animal during a movement bout can be determined.

Then, frequency distributions (histograms) were generated of 1.) the maximal velocities of each movement bout and 2.) the inter-individual distance between animals per sample. On these distributions the best Gaussian curves that represent the data were fitted with an expectation maximization (EM) method. It was first determined if different components, in this case Gaussian curves, could be recognized within the population. Thereafter, proportion, mean and standard deviation were estimated of each Gaussian curve with the EM algorithm. After visual inspection of all frequency distributions plots, 2 Gaussian curves were plotted on the frequency distributions plots of the velocities, while 3 Gaussian curves were plotted for the inter-individual distances. Subsequently, the intersections of the curves created with the EM method were used to determine a threshold/cutoff value for the different modes or categories. Resulting in animals that move either with "low velocity" or "high velocity" and are "in contact", "in proximity" or "not in proximity". The identified categories in velocity and inter-individual distances were combined into behavioral classes, see Figure 3. Similar to our former study (Peters et al., 2016), it was again found that based on the frequency distributions of the rat pairs

the velocity of the animals can separated into low or high velocity, with an average threshold of 30.5 cm/s ± 5.8 for the social interaction tests at day 2-4 after treatment and an average threshold of 23.7 cm/s ±5.0 at the social interaction tests at days 29-32. For the inter individual distances, we found an average threshold of 17.1 cm ±1.2 for in contact and 43.8 cm ±6.2 for the threshold between in proximity and not in proximity at day 2-4 after treatment. For the social interaction tests at days 29-32, the thresholds were: in contact, 19.5 cm ±2.9 and in proximity, 51.5 cm ±8.4. These thresholds are consistent with findings of other rat pairs monitored in our lab and also fit with the rats growing older and thus bigger during the course of this study.

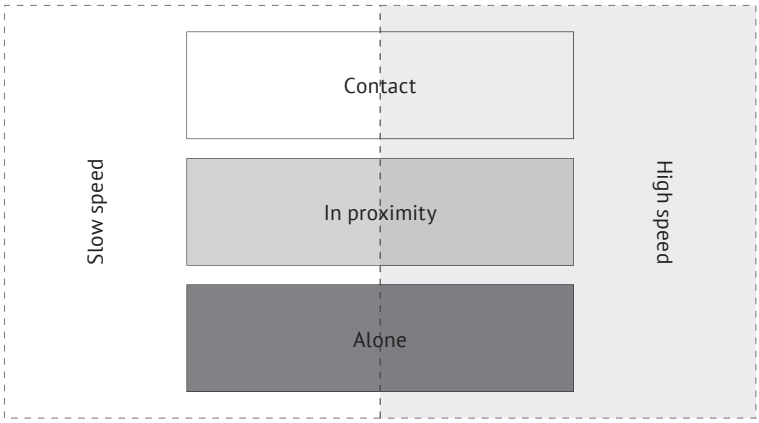


Figure 3. Behavioral classes that were generated after frequency distributions and the threshold between the distributions of velocity and inter-individual distances were obtained.

Ultrasonic vocalizations

Sound was recorded throughout all the behavioral tests, using the microphone placed at the top and center of the testing arena. From the social interaction test 4 days after cessation of PCP or SAL treatment and the separation test 5 days after cessation the USV recordings were analyzed and presented here. The sound data was processed by the software package Sonotrack (Version 2.2.4, Metris, the Netherlands) which creates the visualization (spectrogram) of the sound file as well as making the ultrasonic sounds audible to the human ear, using a technique based on the time expansion method. This method essentially works by

slowing down the sound recording, bringing the frequency down to a level that is within an audible frequency range. USV were first detected by using the automatic detection settings in the program. These settings marked any sound that was longer than 5ms, fell between the frequency 18 and 90 kHz and was above the threshold amplitude of 15.0 as an USV, any sounds that did not meet these criteria were not analyzed. This initial automated analysis was followed by the manual checking of the trials to correct for noise. Any noises that could not be categorized as an USV, after both a visual and auditory inspection, were discarded, e.g. noise from the environment such as nails of the animals on the transparent acrylic walls. The position of the microphone relative to the position of the animal during a call occasionally meant that the USV might not be fully recorded. Therefore, an extra criterion was implemented whereby elements that were separated by a gap of less than 10 ms were considered to be part of the same call, whereas elements that were more than 10 ms apart were considered separate calls, similar as in Reno et al. (2013). After detection calls were further split into categories based on their characteristics see Table 1. USV were categorized as 22 kHz calls, fixed frequency (FF) 50 kHz calls or frequency modulated (FM) 50 kHz calls.

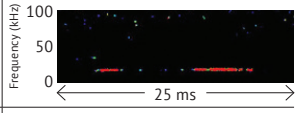
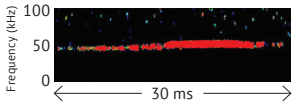
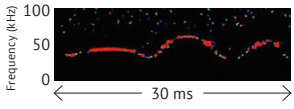
Category	Criteria	Example sonograms
22 kHz call	Call must fall between the frequencies of 18 and 32 kHz	
50 kHz call	Call must fall between the frequencies of 32 and 90 kHz	
Fixed frequency (FF) call	Max and Min frequency of the call must not be more than 7 kHz apart (i.e. bandwidth of 7 kHz)	
Frequency modulated (FM) call	Does not meet criteria for "F", can contain more than one or more FM components	

Table 1. The USV categories used to analyze the sound data along with the criteria used to categorize the calls. On the right there are example sonograms of a 22 kHz call, and a FF and FM 50 kHz call.

Statistical analysis

Data were statistically tested using R statistical software (version 2.15.2) and SPSS statistics package (Version 21.0). The pair of animals was considered as the statistical unit (thus samples size is the number of pairs; n = 8 for PCP and n = 7 for SAL) for all behaviors in the social interaction and separation tests. For comparison of the behavioral parameters of the PCP treated animals with the SAL treated animals a Welch unequal variances Two Sample t-test was used. And for within group comparisons a paired student t-test was used. For analysis of the USV, the non-parametric Mann-Whitney U (between group comparison) or the Wilcoxon signed rank test (within group comparison) was used. All tests were two-tailed and significance was set at $p < 0.05$.

RESULTS

General activity in social interaction tests

The automated analysis of the social interaction tests, revealed no differences in overall activity between the treatment groups: no significant differences were present in the average total distance moved by the PCP and SAL treatment groups (Figure 4). However, the administration of clozapine caused a significant reduction in overall activity (Figure 4) due to a decrease in total distance moved by both the PCP treatment group (SI(31) + vehicle: 15225 ±8546 compared with SI(32) + clozapine: 8546 ±3361, $t_{(7)} = -8.21$, $p < 0.0001$) and the SAL treatment group (SI(31) + vehicle: 15075 ±1937 compared with SI(32) + clozapine: 7538 ±3537, $t_{(6)} = -5.06$, $p < 0.004$).

In all social interactions tests, no significant differences were present between the PCP and SAL treated animals in time spent moving at high velocity or low velocity (data not shown). Also, no significant differences were observed in mean velocity between PCP and SAL group (data not shown).

Social Interaction

Duration in contact

The automated analysis of the social interaction tests, revealed that at 31 days after the last PCP injection, PCP treated animals spent more time in contact with each other when paired with another unfamiliar similarly treated rat (Figure 5, SI(31) + vehicle: PCP 420 ±47; SAL 325 ±53, $t_{(10.24)} = 3.50$, $p < 0.005$). At all other time points, no significant treatment-effects were present.

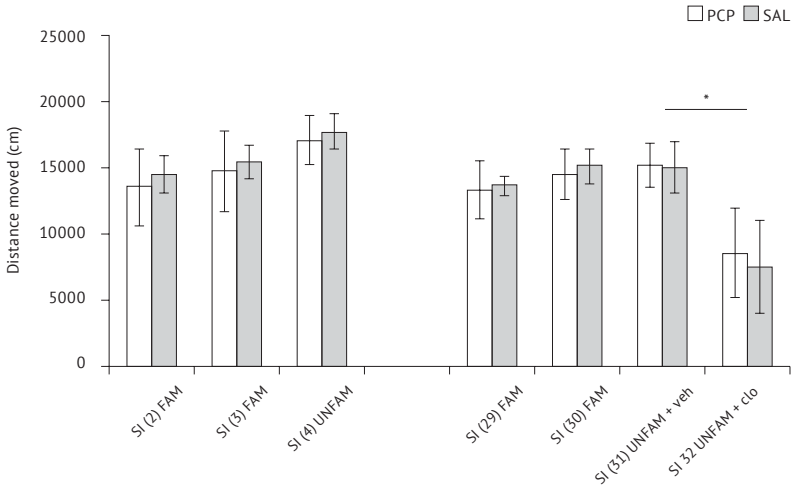


Figure 4. Average activity (±SD) of each group per social interaction test session of 20 minutes after chronic PCP or SAL treatment of 14 days. * = $p < 0.004$; SI = Social Interaction test, (#) = number of days after treatment, FAM = animals were familiar to each other, UNFAM = animals were unfamiliar, veh = vehicle, control group.

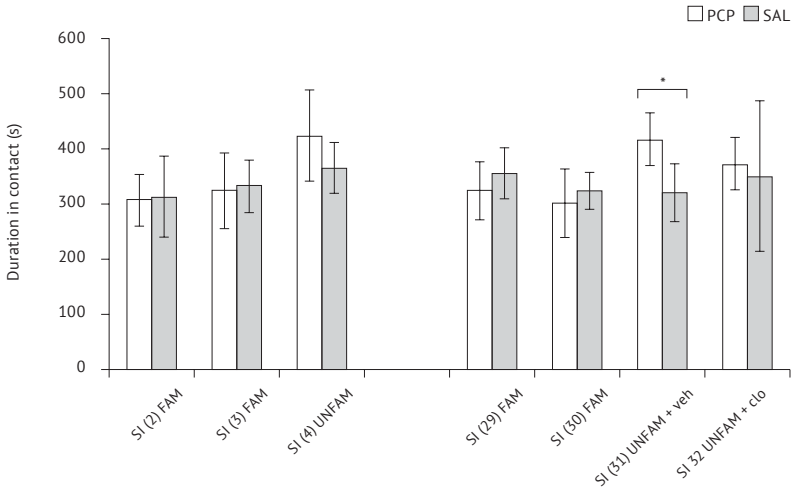


Figure 5. Average duration in contact (±SD) of each group per social interaction test session of 20 minutes after chronic PCP or SAL treatment of 14 days. * = $p < 0.005$; SI = Social Interaction test, (#) = number of days after treatment, FAM = animals were familiar to each other, UNFAM = animals were unfamiliar to each other, veh = vehicle, control group.

Duration in proximity

The automated analysis of the social interaction tests, revealed that in the social interaction test at 3 days after last PCP injection, SAL treated animals spent more time in proximity to each other when paired with their familiar and similarly treated cage mate (Figure 6, SI(3): PCP 420 ±47 sec.; SAL 325 ±53, $t_{(10.24)}$ = 3.50, p < 0.005). In addition, SAL treated pairs (88 ±21) spent more time in proximity when moving with high velocity than PCP treated pairs (67 ±12) at 4 days after the last PCP injection when paired with a similarly treated, but unfamiliar rat ($t_{(9.41)}$ = -2.40, p = 0.039) data not shown). At all other time points, no significant treatment effects were present.

Separation test

In both the first (5 days after final injection) and the second (36 days after final injection) separation test the PCP treatment group spent significantly less time close (< 15cm) to the divider separating them from their cage partner than the SAL treatment group (Mann-Whitney U; first separation: U = 44.0; p = 0.004; d = 3.44; second separation: U = 55.0; p = 0.017; d = 2.89; Figure 7).

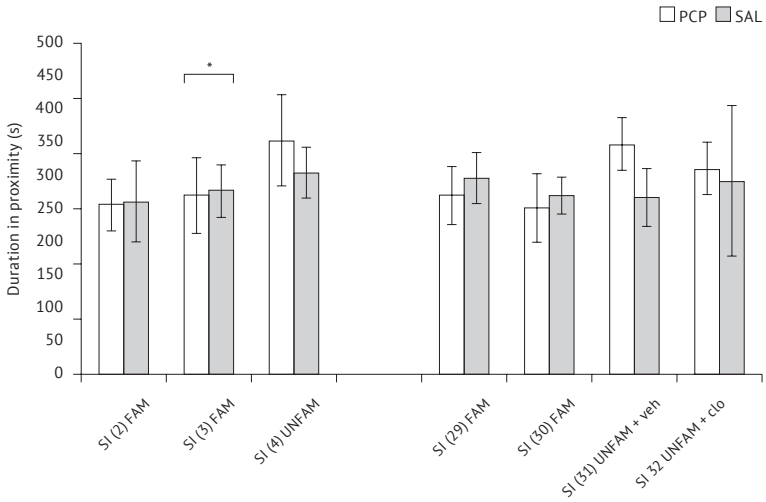


Figure 6. Average duration in proximity (±SD) of each group per social interaction test session of 20 minutes after chronic PCP or SAL treatment of 14 days. * = p < 0.01; SI = Social Interaction test, (#) = number of days after treatment, FAM = animals were familiar to each other, UNFAM = animals were unfamiliar to each other, veh = vehicle, control group.

This difference was not accompanied by differences in activity levels as within the first and second separation test there was no significant difference between the total distance moved by the PCP (1: 6110 cm ± 270; 2: 5776cm ± 265) and the SAL (1: 6435cm ± 207; 2: 6075cm ± 248) treatment groups (Mann-Whitney U; $p > 0.3$; data not shown).

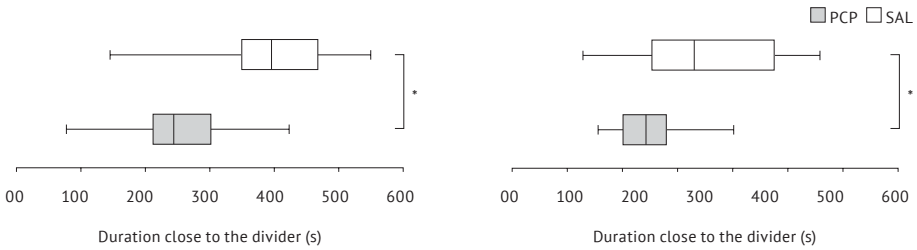


Figure 7. Time spent within 15 cm of the divider that separated the cage partners during each of the two separation tests after treatment with chronic PCP or SAL treatment of 14 days. left: Separation test 1 at 5 days after treatment, right: Separation test 2 at 36 days after treatment. * $p < 0.05$

Ultrasonic Vocalizations

Ultrasonic vocalizations during the social interaction test

The ultrasonic vocalizations produced by the rat pairs in the social interaction test after 4 days of chronic PCP or SAL treatment were analyzed. Due to the high number of calls in the trials, only every other minute was analyzed. No significant differences were observed between both treatment groups in the total amount of vocalizations or the different types of vocalizations (Table 2).

Ultrasonic vocalizations	PCP median (Q1, Q3)	SAL median (Q1, Q3)	W	p
Total	323 (233, 467)	283 (210, 435)	29.0	0.62
50 kHz Frequency modulated	114 (81, 151)	121 (81, 144)	25.0	1.00
50 kHz Flat frequency	188 (148, 303)	172 (125, 267)	31.0	0.46
22 kHz	2 (1, 4)	0 (0, 11)	27.5	0.74

Table 2. Amount of ultrasonic vocalizations produced by the pair of rats during the social interaction test after 4 days of treatment with PCP or SAL. Unpaired Wilcoxon signed rank test revealed no difference between the treatment groups in total number of calls or in the different categories.

Ultrasonic vocalizations during the separation test, 5 days after treatment

Due to the high number of calls in the acclimatization trials, in which cage mate are together in the arena for 10 minutes, only every other minute was analyzed. While, the whole 20 minutes of the period after the separation was analyzed. The analysis of the first separation test at 5 days after treatment with PCP or SAL revealed that separation significantly decreased the total number of USVs compared to the acclimatization for both PCP (Z = -2.52, p= 0.012) and SAL treated animals (Z = -2.37, p= 0.018, Figure 8). However, no significant difference between the total number of USV's produced by PCP (n= 8) and SAL (n= 7) treated pairs of rats during the acclimatization period were observed (Figure 8). During the separation test, the SAL group (median=15.0, Q1=7.0, Q3=34.5) did not produce more USVs than the PCP group (median=6.5, Q1=4.5, Q3=7.8), (U = 16.5, p = 0.19). Within the acclimatization trial no significant difference was present between the number of FF or FM 50 kHz calls produced by the PCP (FF: median=179.5, Q1=138.3, Q3=243.0, FM: median=53.0, Q1=42.0, Q3=73.8) and the SAL (FF: median=200.0, Q1=131.5, Q3=257.5, FM: median=60.0, Q1=30.0, Q3=257.5) treated pairs. Throughout all the acclimatization trials a total of eight 22 kHz calls were found, split evenly between the two treatment groups. Too few calls were recorded in the separation tests to make any meaningful comparisons between the different categories of calls displayed.

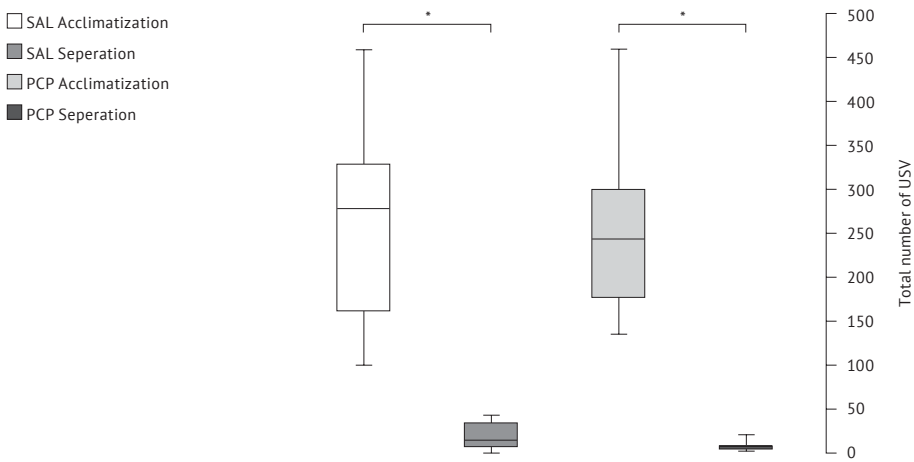


Figure 8. Total number of USV given by pairs of rats treated with PCP and SAL during the acclimatization (physical contact) and separation components of the first separation test, performed 5 days after cessation of treatment. Separation reduced the number of USV for both treatment groups, * $p < 0.05$.

DISCUSSION

We assessed the ability of chronic PCP administration (3 mg/kg, for 14 days) to induce short- and long-lasting behavioral changes in male Sprague Dawley rats using an ethologically relevant assessment of behavior. During the social separation test performed at 5 and 36 days after chronic treatment, PCP treated rats spent less time near the divider that separates them from their familiar cage mate compared with SAL treated rats. The results of our study further showed that PCP treated animals spent less time in each other's proximity after 3 or 4 days of chronic treatment, regardless of being exposed to a familiar (cage) partner or unfamiliar partner. Particularly, time in proximity when moving with high velocity was reduced for PCP treated pairs. This behavioral category mostly reflects approach, avoidance and following behavior (Peters et al., 2016). In contrast, at 31 days after treatment, PCP treated animals spent more time in contact when exposed to an unfamiliar partner as compared to SAL treated rats. But, this difference was not observed at 29 or 30 days after treatment when exposed to a familiar (cage) partner. In contrast to the in proximity category, the in contact category mostly represents (passive) behaviors such as allogrooming, social sniffing and crawling under/over or being in supine or pinning position (Peters et al., 2016).

Taken together, these results suggest that PCP, at short-term, is able to induce diminished (active) social interaction behavior. But, probably it is not able to induce overt permanent long-term decrease in (active) social interaction behavior. Furthermore, it seems that chronic PCP affects the motivation for engaging in social behavior. From our results of the separation test, we conclude that diminished social motivation is a permanent effect of the chronic PCP treatment. Thus, its use in an animal model should be aimed at assessing motivation for social contact, instead of the previous focus in the literature on PCP as a model for social withdrawal.

In all social interaction tests the results were not accompanied by a difference in ultrasonic vocalizations between PCP and SAL treated animals. This is in contrast to previous studies which demonstrated that a single administration with PCP (Boulay et al., 2013) and MK-801 (Panksepp and Burgdorf, 2000) reduced the number of tickle induced 50 kHz calls. However, both tests were carried out within 30 minutes after the injection with the NMDA receptor antagonist and therefore, it is possible that the decrease was caused by acute ef-

fects of the drugs. On the contrary, Watson et al. combined postnatal PCP administration with long-term social isolation housing, and showed an increased number of 50 kHz calls during social interaction (Watson et al., 2016). However, no difference in social interaction behavior was observed in this study. A lack of correlation between 50 kHz calls and social behavior during social interaction, was also found by Manduca et al. (2014) for both the Wistar and Sprague Dawley rat strain. Thus, although it is suggested that the expression of 50 kHz calls is related to social behavior, this is not always confirmed by correlations with number of calls. Furthermore, the lack of finding in our study might also be due to a high inter individual variation in USV, which is commonly observed in rats (Schwarting et al., 2007). Indeed, in our study, we also observed animal pairs with highly variable amounts of calls. This stresses the need for increasing numbers of animals or different statistical approaches when investigating USV.

Our results are in line with other sub chronic studies (Audet et al., 2009; Bruins Slot et al., 2005; Sams-Dodd, 1996; Sams-Dodd, 1998a; Koros et al., 2007; Tanaka et al., 2003) investigating short-term effects of PCP and demonstrated that repeated PCP treatment (minimum: 3 injections; maximum: 15 injections) induces social deficits in rats measured within 24 hours after last treatment. Also, we found comparable results to the study of Lee and colleagues (2015), from which we adopted the scheme of 14 daily injections of 3 mg/kg, who also found sub-chronic administration of PCP to cause social deficits in male rats after 72 hours (Lee et al., 2005). Similar to the studies of Li et al. (2012; Metaxas et al. (2014); Sams-Dodd (2004), we could not find evidence that chronic PCP treatment induces permanent social interaction deficits. Rather, on the long-term our results suggest that PCP treated animals seek more (passive) social contact when paired with an unfamiliar, but similarly treated, partner. In contrast, Matricon et al. (2016); Seillier and Giuffrida (2009); Seillier et al. (2010); Seillier et al. (2013) have consistently confirmed diminished social behavior after chronic PCP exposure using a 7 days washout period. This is however, a shorter washout period compared to ours (29-39 days), which leads to the suggestion that the effects of PCP are more transient and not permanent (Metaxas et al., 2014; Sams-Dodd, 2004). Only one study confirmed decreased initial social interaction (during the first 8 minutes of the test) lasting 28 days after PCP treatment (2 daily injections for 2 consecutive days) (White et al., 2009). In this latter study, PCP was ad-

ministered during later development (PND50-51) which is somewhat later than our study (start treatment at PND 37±3). Though, the study of Metaxas et al. (2014) is comparable to our study, using 35-42 day old animals, here there was no enduring effect of PCP administration. Although, age during PCP treatment can be an interesting factor to consider, currently, there is no clear view on whether this is indeed causing the different outcomes in studies regarding sub-chronic PCP administration. Obviously, a study on age dependent effects of PCP administration would be welcome (similar for strain).

There are currently only limited investigations into the specific effect on motivation for social contact in rats after chronic PCP treatment. Similar to our diminished social motivation in the separation test, Schwabe and colleagues found in a conditioned place preference paradigm, that sub chronic PCP reduced the preference for the social chamber, lasting 7 weeks after last PCP treatment (Schwabe et al., 2006). Acute MK-801 administration, is also able to reduce social investigation behavior of rats that are physically separated from each other (Gururajan et al., 2012). However, recently, others have investigated the effects of sub chronic PCP (7 days) on social cognition using an adapted version for rats of three chambered social apparatus (Nadler et al., 2004; Moy et al., 2004). While McKibben et al. (2014) found no effect of chronic PCP on sociability or preference for social novelty, Seillier and Giuffrida (2016) found diminished interest for social novelty, but no abnormal sociability (i.e. normal interest in the initial social chamber compared to the empty chamber). This rather suggests a social cognition deficit rather than a motivation deficit and could thus not help to explain the diminished social motivation observed in our study. It has been suggested that chronic PCP induces anhedonic effects (i.e. lowered sucrose consumption). Indeed, both acute and sub chronic PCP has been shown to decrease voluntary sucrose consumption on short-term, (e.g. Baird et al., 2008; Turgeon and Hoge, 2003). However, Lydall et al. (2010), using a similar dosing regime of 7 days but a longer washout of 7 days, could not replicate these results on sucrose- consumption after sub chronic PCP. Additionally, the hypothesis that PCP administration has anhedonic effects has been put into doubt, since the evidence is not fully supportive (see Neill et al., 2014 for a review). Thus, although our finding of decreased motivational levels for social contact fits the anhedonia hypothesis it is too preliminary to draw

hard conclusions. Future studies that included measurements of motivation after sub chronic PCP treatment can help to understand this.

It has previously been shown that clozapine has a sedative effect that suppresses the activity of rats (Sams-Dodd, 1998b; Wiley, 1994). It is therefore, not surprising that acute treatment with clozapine significantly reduced the activity levels of both treatment groups compared to vehicle, along with a concomitant reduction in the levels of active social interaction displayed by both groups. Clozapine was not able to reveal any differences on long-term in the social interaction test. However, it is suggested that in the PCP model, repeated treatment with clozapine is necessary before onset of therapeutic effect (Sams-Dodd, 1998b), whereas we only gave a single injection.

In addition to executing the social interaction test in a more ethologically relevant manner, assessment of the outcome of this model could be strengthened through utilizing additional behavioral assays, like the inclusion of USV or tests that measure the motivational aspects of social behavior. Similar to the test set up utilized by Gururajan et al. (2012) our separation test set-up may represent another way to detect differences in social motivation. However, one must be careful in employing 'standard' and seemingly easy behavioral assays for social behavior. For example, in assays were physical contact is prevented, different outcomes than in a social interaction test can be observed (Ku et al., 2016). Thus, a combination of behavioral assays that aim to assess several aspects of behavior, e.g. motivational and rewarding properties, could highly benefit future research. Furthermore, our automated method and objective behavioral parameters could, as previously argued (Spruijt et al., 2014; Peters et al., 2015) help to increase replicability and validity. This is important since the results on social behavior using the sub-chronic PCP model still vary between studies conducted in different laboratories (see also Gururajan et al. 2010).

Social interaction and social withdrawal are important measures for investigating the negative symptoms of schizophrenia (Wilson and Koenig, 2014). NMDA antagonism is a widely used approach to mimic social withdrawal, however, further investigation reveals that results on sub chronic PCP administration are not consistent. Our study adds to the evidence that indeed short-term effects of chronic PCP can be used to mimic social deficits, however, we found no evidence that chronic PCP induces long-term social withdrawal. More attention for USV during social behavior is needed to reveal whether this aspect of social

behavior can also be a read out parameter in animal models using PCP. The long-term consequences of chronic PCP administration on social behavior in rodent models remain complex, and future studies addressing this are still needed.

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CHAPTER 5

Effects of play deprivation in male Wistar rats on activity, social interest & recognition, separation, social interaction and ultrasonic vocalizations

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ABSTRACT

Play deprivation during the juvenile period is a paradigm used to model social withdrawal or social behavioral deficits related to critical early life events. Two or 3 weeks of play deprivation in early life can reduce social contact behavior in the social interaction test, when tested as adults. The possible role of the NMDA (Nmethyl-daspartate) receptor was investigated by applying a glycine transporter (GlyT)-1 inhibitor. In line with our earlier work which attempts to incorporate an ethological approach we analyzed the behavior of rats using different behavioral assays including ultrasonic vocalizations as well as previously validated automated measurements of social interaction.

Male Wistar rats were divided into 2 groups: 1. Play deprived from postnatal day (PND) 23 till 35 and, 2. socially housed in groups of 4 from PND 23 till day 35. After the play deprivation period, at PND 36, all animals were rehoused in pairs for a subsequent period of 5 weeks. Then, at 10 and 11 weeks of age, all rats were subjected to: 1. Individual test in the home cage for general behavioral activity, 2. an social-sexual interest and recognition test using female odor, 3. An automated social interaction test and 4. a separation test. In the social interaction test animals were given one acute injection of 1 mg/kg (R)-(N-[3-(4'-phenylphenoxy)propyl]) sarcosine, NFPS, a GlyT-1 inhibitor.

We found that play deprivation induced higher individual locomotor activity in a novel home cage. Interest in a social-sexual scent over a neutral scent was present, regardless of rearing condition. Additionally, in our protocol, both socially reared and play deprived rats showed no increased interest in a novel social-sexual scent over a familiar social-sexual scent. In the separation paradigm, results showed that play deprived rats spent more time close to the divider that separated them from the cage mate. It appears as if, play deprived animals were more motivated to seek social stimuli. Last, in the social interaction tests no differences between play deprived or socially housed rats were found in activity, social behavior or ultrasonic calls. Thus, the difference between play deprived and non-play deprived is limited to the appetitive aspects rather than the consummatory aspects of social behavior.

Despite our efforts we could unfortunately not replicate results of earlier studies showing reduced sociability after play deprivation during the juvenile period. Results from our study show the sensitivity of the model to methodological differences. Further, it stresses the need to carefully consider experimental protocols and report details concerning housing or other experimental conditions.

INTRODUCTION

Animal models are important sources of information for advancing our understanding of the pathophysiology of human diseases. Despite continuous research efforts, it remains essential to obtain better insight into the neurobiological mechanisms underlying symptoms of psychiatric disorders. Psychiatric symptoms can be regarded as difficult to model in mice or rats. But, when care is taken to measure and analyze animal behavior in a biological relevant setting, it can provide useful insights (Spruijt et al., 2014; Peters et al., 2015). Rats are, as humans, social animals that display high level of social behavior. Therefore, they are potentially good candidate animals to model social behavior deficits. It has been recognized that social deprivation in a critical early life period, is a biologically relevant method to study abnormal neurodevelopment and behavior (Robbins, 2016).

Early life social experiences are critical for development of normal social behavior. Continuous post weaning social isolation housing of rodents is a frequently used manipulation because of its potential to induce permanent changes in various behavioral domains, e.g. anxiety (Fone and Porkess, 2008), stress behavior (Bartolomucci, 2007), depression like behavior (Heidbreder et al., 2000), and, social behavior. Conflicting findings, however, are present in the literature regarding the effects of social isolation on social behavior. In part, this is likely due to the differences in isolation protocols, in particular the timing and duration of isolation or the use of different strains (Ferdman et al., 2007). Here, we focus on a model that deprives rats temporarily from social play behavior during the juvenile period and after which social re-housing is followed. Uniqueness of this model is that it specifically focusses on the effects of play deprivation. Juvenile rats are highly motivated to frequently play with their conspecifics during a few weeks following weaning. The highest levels of social play behavior occur between days 40-50 of age (Panksepp, 1981). Social play behavior has rewarding properties and the opioid and dopaminergic system are involved (Niesink and Van Ree, 1989; Panksepp et al., 1984; Trezza et al., 2011; Vanderschuren et al., 1997; van Ree and Niesink, 1983). Post weaning isolation deprives animals from social play behavior and this had led to the hypothesis that reward related systems involved in regulating social behavior are not adequately developed in adulthood. Studies revealed that play deprivation during the juvenile period alters adult social behavior (Hol et al., 1999; Lukkes et al., 2009; Van Den Berg, Van

Ree and Spruijt, 1999; Van Den Berg, Hol et al., 1999) and as a consequence of play deprivation, adult rats do not respond adequately during aggressive conflicts (Von Frijtag et al., 2002). Furthermore, play deprivation decreases motivational properties of sucrose and preference for social contact (Van Den Berg, Pijlman et al., 1999) and morphine treatment during the play deprivation period can counteract the effects of play deprivation (Van Den Berg et al., 2000). Also, it decreases opioid release (Van Den Berg, Kitchen et al., 1999) and causes upregulation of opioid receptors in distinct brain regions like the amygdala, hippocampus and substantia nigra (Van Den Berg, Van Ree, Spruijt and Kitchen, 1999). Thus, 2 or 3 weeks isolation in early social development leads to persistent neurobiological changes such as diminished sociability, even when social rehousing is applied after the play deprivation period. Moreover, together the set of studies from Van den Berg provide good evidence that the absence of opioid release during the play deprivation period is most likely responsible for an altered development of the opioid system.

There is no doubt that the dopamine system is implicated in reward behavior and motivation and, thus, also is involved in social play behavior. For example, blockade of dopamine receptors in the nucleus accumbens decreases social play behavior in highly motivated animals by applying short-term play deprivation (Manduca et al., 2016) and, rats exposed to long-term isolation from weaning (PND 28-77) have greater dopamine release and uptake in nucleus accumbens and the dorsalmedial striatum (Yorgason et al., 2016). Thus, play deprivation during the juvenile period is expected to have an effect on normal development of the reward system involved in regulating motivational aspects of social behavior.

Recent studies also implicate a role for the NMDA (n-methyl-D-aspartate) receptor in social behavior in mice (e.g. Jacobs and Tsien, 2017; Deutsch et al., 2011). It is not unlikely that the dopaminergic system, in part, regulates NMDA receptor activity. At different levels, the interaction between the dopamine and glutamate system is being investigated (De Bartolomeis et al., 2014). Additionally, dopamine is suggested to be a regulator of the NMDA receptor, though, the precise mechanisms are rather complex, see Fabrizio and Camilla (2015) for an review. Other studies have indicated that NMDA receptor knock-out in mice models yields aberrant social behavior including; increased physical distance between cage mates and social withdrawal in the resident-intruder paradigm

(Mohn et al., 1999) and reduced preference for a social stimulus mice and reduced ultrasonic vocalizations during sexual behavior (Saunders et al., 2013). We hypothesize that play deprivation during the early juvenile period may also reduce NMDA receptor functioning, possibly due to a complex interplay between the development of the reward and glutamatergic systems.

The NMDA receptor is an ionotropic glutamate receptor that requires co-activation of glutamate with either glycine or d-serine. Recently, the glycine transporters (GlyTs) have become new targets for pharmaceutical development. Inhibitors of the GlyTs are of interest, because, they will elevate glycine availability and therefore, potentiate NMDA receptor functionality. GlyT based pharmacotherapy has been suggested for many disorders such as schizophrenia, pain control, alcohol and drug dependence, depression, epilepsy and obsessive compulsive disorders, as well as anxiety disorders, memory deficits, Parkinson's disease and autism (Harvey and Yee, 2013). A study in mice showed that GlyTs inhibitors have the potential to increase social exploration in the Balb/c strain, a strain that is known for its low levels of social behavior compared to other mice strains (Burket et al., 2015). So far there are no studies that have investigated the potential of GlyTs inhibitors to reverse effects of reduced social behavior in rats. Our question was if the play deprivation model, which has repeatedly led to reduced sociability in rats, is sensitive to treatment with a GlyT inhibitor which has previously successfully increased social exploration of mice.

In this study we aimed to: 1. Test if our previously validated method to automatically classify social behavior (in contact, in proximity, not in proximity) could reveal effects of play deprivation 2. use our additional behavioral assays; USVs recordings, social-sexual interest & recognition, separation test for assessing motivation for social contact, to provide a more extensive analysis of the play deprivation effects and 3. to examine the role of the NMDA receptor in this model by using a single treatment of a GlyT-1 inhibitor, NFPS ((R)-(N-[3-(4'-phenylphenoxy)propyl]) sarcosine). To this end, Male Wistar rats were divided into 2 groups: 1. Play deprived from postnatal day (PND) 23 till 35 and, 2. socially housed in groups of 4 from PND 23 till day 35. After the play deprivation period, at PND 36, all animals were rehoused in pairs for a subsequent period of 5 weeks. Then, at 10 and 11 weeks of age, all rats were subjected to: 1. Individual test in the home cage for general behavioral activity, 2. a social-

sexual interest and recognition test using female odor, 3. An automated social interaction test and 4. a separation test. In the social interaction test animals were given one acute injection of 1 mg/kg NFPS, a GlyT-1 inhibitor.

MATERIALS AND METHODS

Rats and housing conditions

Male Wistar rats (HsdCpb:WU, n = 32) were purchased from Harlan (Horst, the Netherlands). Rats were weaned at Harlan, at an age of 21 days. There, all pups were divided into two groups before transported to the research facility. One group was individually housed (n = 16) the other group was socially housed in 4 groups of 4 animals (n = 16). At Harlan, two male individuals from each litter were selected and assigned to: 1. socially housed group which we will term the play group or 2. Isolation housing which we will term the play deprived group.

Play deprived animals were physically isolated from day 21 till day 35 (weeks 4 and 5) in Macrolon type III standard lid cages (Techniplast, Italy; l x w x h: 42 x 26.5 x 5.5 cm). Animals from the play group remained in groups of 4 during these two weeks in Macrolon type IV low lid cages (Techniplast, Italy; l x w x h: 60 x 38 x 20 cm). All cages were placed in the same animal room and allowing visual, auditory and olfactory contact. Besides handling during weekly weighing and cleaning of the cages, there was no physical contact. After the play deprivation period (postnatal day 35) all 32 animals were rehoused in pairs with an unfamiliar rat from the same housing condition with similar body weight in Macrolon type IV cages (Techniplast, Italy; l x w x h: 60 x 38 x 20 cm) with raised wire lids. The standard flat lids of the homecages were changed with these raised wire lids with drop-in food hopper (series -119/-224, Techniplast, Italy) to allow the rats to completely stand up (rearing) in their homecages, in line with EU-guidelines for housing laboratory rats. During the pair housing period after the play deprivation, one animal was unexpectedly found dead in its cage, leading to exclusion of this animal and his cage partner from all experiments. Resulting in n = 16 rats that were play deprived during the juvenile period and n = 14 rats that were socially housed throughout the study.

All cages contained bedding (Abedd® wood chips, LAB & VET Service GmbH, Vienna, Austria). In each cage a cylindrical semi-transparent amber-colored tube and two pieces of tissue paper were provided, as form of enrichment. Animals were kept on a reversed 12h light/dark schedule with white lights on 21:00 hrs,

off at 09:00 hrs and dim-red lighting on during dark period. Rats had ad libitum access to food (Teklad Global 2018, Harlan, Madison, WI, USA) and tap water throughout the experiment. The housing room was maintained at a temperature of 20-22 ˚C and a humidity of 40-50% with constant background noise provided by radio. The experiments have been performed in adherence to EU-guidelines and national legal requirements concerning animal research (Wod/Dutch 'Experiments on Animals Act') and have been approved by an Animal Ethics Committee ('Lely-DEC').

Drugs and treatment protocol

(R)-(N-[3-(4'-phenylphenoxy)propyl]) sarcosine (NFPS) was purchased from Tocris Bioscience, Bristol, UK and dissolved in 5% 2-hydroxypropyl-β-cyclodextrin. NFPS was injected intraperitoneally 90 minutes prior to the test session at a dose of 1 mg/kg. The no-drug controls received an injection with an equivalent volume of a saline (0.9% sodium chloride). Dose and treatment time were determined based on previous studies (Karasawa et al., 2008; Shimazaki et al., 2010). Rats were tested in a repeated design. Prior the social interaction test 3, saline was given and a NFPS injection prior to the fourth and last social interaction test. Drugs were prepared fresh daily. In all other tests animals were left untreated.

Experimental apparatus

All behavioral testing was carried out in an enlarged PhenoTyper® instrumented cage (PhenoTyper 9000, Noldus Information Technology, the Netherlands) under red light conditions (Figure 1). The animals were tested in this large environment because the expression of social behavior requires space, see for an example Spruijt et al. (2014). The cage consisted of a black floor plate (floor dimensions: 90 x 90 cm), transparent Perspex walls (height: 100 cm) and a roof equipped with infrared emitting LED's (peak range average of 155 950nm), on which a PhenoTyper® top-unit was placed containing an infrared sensitive camera (CCD 1/3" 156 SONY SUPER HAD CCD black/white) and IR-filter (type Kodak 87C). Video recordings were made on computers placed outside the experimental room and were processed afterwards using EthoVision XT 9.0 research edition (Noldus Information Technology, the Netherlands). A total of 4 PhenoTyper 9000 cages were connected to 1 computer and used simultaneously.

To record the USV a gold foil electrostatic transducer microphone (Metris, the Netherlands) with a frequency range of 15-125 kHz, minimum receiving sensitivity of -42 dB at 50 kHz and with a beam angle of 12 degree at 3 dB down (at 90 kHz) was integrated into the PhenoTyper-9000. The microphone was connected to a control unit, which was connected to a data acquisition board (National Instrument, M-series PCI-6250). Sonotrack software (Version 2.2.4, Metris, the Netherlands) was used to continuously record ultrasonic sound. The microphone was placed at the roof of the PhenoTyper cage at a height of 93 cm. Additionally, the roof of the PhenoTyper was shielded with sound attenuating pyramid foam to prevent recordings of echoes of the ultrasonic sounds.

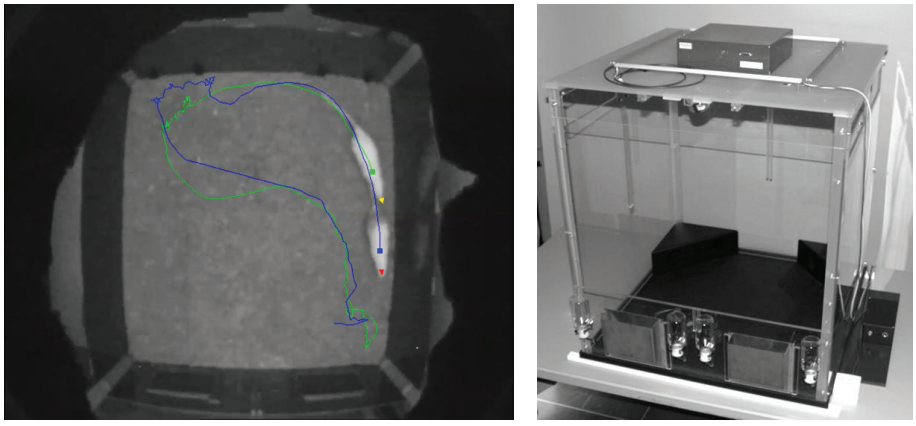


Figure 1. Example of an enlarged PhenoTyper 9000 (90x90x100 cm) and top view video tracking image of two rats using EthoVision XT 9.0 (Noldus, the Netherlands).

Behavioral tests

This experiment was designed to test the effects of 2 weeks of play deprivation during the juvenile period on social behavior in adulthood. In a 2 week testing period at week 10 and 11 of age, general activity, social interest and social recognition, social interaction and a separation test were conducted to assess social motivation (Figure 2). All behavioral tests were conducted in the dark (i.e. active) phase of the animals' diurnal cycle, under infrared and red-light conditions, and were carried out in an experimental room with the same conditions as their housing environment.

<table>
<tr><td rowspan="4">Play deprivation or group housing

PND 21-35</td><td rowspan="4">Housing in (same treated) pairs

5 weeks</td><td>Behavioral test:</td><td>General Activity</td><td>Social interest & recognition</td><td>Social interaction</td><td>Social Sepera-tion</td><td>Social interaction</td></tr>
<tr><td>Pairing:</td><td colspan="2">individual</td><td>Familiar & same treated</td><td>-</td><td>unfamiliar & same treated</td></tr>
<tr><td>Treatment:</td><td colspan="2">-</td><td>-</td><td>-</td><td>NFPS or vehicle</td></tr>
<tr><td>Age:</td><td colspan="2">10 weeks</td><td colspan="3">11 weeks</td></tr>
</table>

Figure 2. Schematic outline of the time schedule of the behavioral testing periods carried out with the two groups, the play deprived group (isolation housing during PND21-35) or the play group (social housing in groups of 4 during PND 21-35). There were 4 social interaction test in total, performed on 2 consecutive days before the social separation and on 2 consecutive days after the social separation test. All animals were subjected to the same behavioral tests in a repeated design.

Monitoring general activity

At 10 weeks of age all rats were tested in the social interest and social recognition test and before onset of these tests, all animals were housed individually for 24 hours in the PhenoTyper 9000, which was used as a home cage. Each PhenoTyper was split into half with a Plexiglas partition to decrease the testing area (90 x44cm). The PhenoTyper contained black bedding (Cellu-Dri Soft, Shepherd specially papers, Watertown, TN, USA), a shelter was provided for shelter and tap water and food (Teklad Global, Harlan, Madison, WI, USA) were available ad libitum. Animals were given 22 hours (12 p.m.-10a.m.) to habituate and were free to move, drink eat and sleep during their stay. By habituating them for a day, and leaving the animals in this temporary home cage during testing, stress levels and novelty induced exploration during the test can be reduced (Spruijt et al., 2014). Additionally, during habituation animals were monitored using EthoVision to measure general activity, home cage behavior and circadian rhythm prior to testing.

General overview of the social interest and recognition test

After 22 hours of habituation in the experimental home cage testing began at 10 a.m. The test consisted of two sessions: a social interest and a social recognition session. In the first session general interest in social scent over a non-social scent is measured for each individual. After a 30 minute retention interval, the second

session took place. Here, the individual's recognition memory for social scent in the first session was tested (see Figure 3). Scents were presented in stainless steel scent cups, as previously used in (van den Bos et al., 2002), measuring 4 x 4 x 4 cm with a lid that contained twelve 3mm diameter holes. The lid could be secured by placing a screw in a drilled hole in the center of the lid. The cup could be placed in a stainless steel holder which was applied to the bottom of the cage and was already present during habituation. Cups were placed at one third of the cage length, between the feeding zone and the outer part of the shelter. Social scents were collected from animals from different home cages in the animal facility. From these home cages, bedding (Abedd® wood chips, LAB & VET Service GmbH, Vienna, Austria), scented with urine and feces for 5 days, was collected from 2 unfamiliar adult female Sprague Dawley rats who were both housed separately and had a synchronized estrus cycle. Bedding was always handled with clean gloves and stored in different air sealed, labelled plastic tubes for a maximum of 1 week. For the non-social scent clean wood chip bedding was used. At the moment of testing, bedding was placed in the scent cups and the cups were placed in the cup holders in the experimental home cage, always in the same order (first in the left holder, then right). Locations of the different scent-filled cups were randomized between experiments to prevent side bias between the left and right side of the cage. The test started immediately after the cups were placed in the holder.

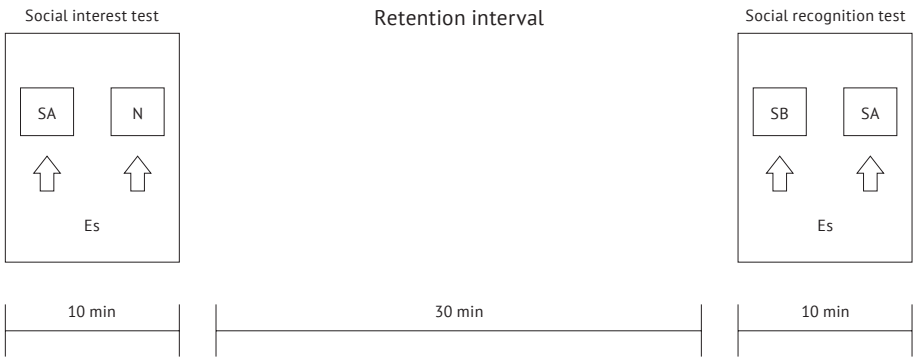


Figure 3. Scheme of social interest and social recognition session. During the social interest session the experimental subject (Es) can explore cups containing either social scent (SA) or neutral, non-social scent (N). After a 30 minute retention interval, the experimental subject can explore either the same social scent (SA) or a new unfamiliar social scent (SB) (recognition session).

Social interest session

During the social interest test session, general interest in social scent was measured during the acquisition phase of the learning of the social scent for the recognition test. Animals were given the choice between spending time with the cup containing clean bedding (non-social, N) or with the cup with bedding from an unfamiliar adult female rat (social scent, SA). The rats were allowed to sniff and explore the cups for 10 minutes before they were removed.

Social recognition session

The second session, during the retrieval phase of the social recognition test, after a retention interval of 30 minutes time spent with the now familiar, social scent as used in the first session (SA) or with a cup containing bedding from a second social scent from a second unfamiliar adult female Sprague Dawley rat (SB) is measured. All cups removed from the first session were emptied and cleaned using warm water, and dried using tissues. Then, clean cups were refilled in preparation for the social recognition test. After the 30 minute retention interval new cups were again randomly introduced into the experimental home cage. The rats were again allowed to sniff the cups for 10 minutes before they were removed.

During both sessions all animals were observed using a fully automated observing system (EthoVison XT, Noldus Information Technology, The Netherlands). From each animal a nose-, tail- and center point was identified by the software and coordinates were stored (25 samples per second). During the tests different behaviors were measured, such as locomotor activity and time spent with each scent cup. Using EthoVision, a 2 cm square zones was created around the cups. When an animal's nose point was within the 2 cm zone of a scent cup (an area defined as 'small interest zone') EthoVision recorded and stored this information, hereby mimicking human observation of animals sniffing the cup. Occasional identity swops of nose and tail point of the animal, made by the software, were corrected manually after video tracking. Preference for a social scent was said to be present when the time spent in proximity (nose-point within 2cm) with one scent cup was significantly higher than the other.

During the social interest test, there were 3 animals from the play group and 1 animal from the play deprived group that were not responding, i.e. not leaving their shelter during the full 10 minutes of testing. In addition, 1 play deprived

animal managed to remove the cup containing the scented sawdust out of the cup holder during the social recognition test. These animals were all excluded from data analysis, resulting in analysis of n = 11 in the play group and n = 14 in the play deprived group.

Social interaction test procedure

At the age of 11 weeks social behavior was tested using an automated social interaction test. The test arena was again the PhenoTyper-9000 placed in a test room, with the same light and climate conditions as the animal housing room. The experimental cage was covered with used bedding (Cellu-Dri Soft, Shepherd specially papers, Watertown, TN, USA) with mixed scent from all experimental animals, since it is known that absence of scents might reduce or even inhibit the amount of ultrasonic vocalizations made by the rats when socially interacting (Wöhr et al., 2008). Animals were habituated in pairs, with their familiar cage partner, to the test set-up and procedure for 30 minutes on two consecutive days (social interaction test 1 and 2). Then, in social interaction test 3 and 4, two unfamiliar rats and same treated rats (e.g. play group or play deprived) were placed together. Pairs were given the similar treatment (NFPS or semi-vehicle) via i.p. injection 90 minutes prior to the social interaction test. Testing order of pairs was randomized on all days. Body weights of the rats were matched as closely as possible, with a maximal difference in bodyweight of 25%. All rats were socially housed at the time of testing. Observation of behavior started immediately after placing the animals in the experimental cage. A social interaction test consisted always of two rats being simultaneously placed into the PhenoTyper, for 30 minutes, during which time top view video (see Figure 1) and ultrasonic sound were recorded. For the purposes of subject identification a color was randomly assigned to each individual within a pair, either black, or red (for control-purposes of the effect of the marking, i.e. red is not visible under infrared lighting conditions in the cage). Then the appropriate color was applied to the rear half of the animal using a marker pen (Edding, Germany) 30 minutes prior to the test, see also Figure 1, left panel for an example. The animals were observed, again using the fully automated observing system (EthoVison XT, Noldus, Wageningen, The Netherlands).

Automated analysis of social behavior in interaction test

Software

All social interaction tests were analyzed from video recordings using the automatic tracking software package EthoVision (EthoVision XT 9.0, Noldus, the Netherlands). This tracking software, with the aid of the markings on the backs of the animals making the animals differ in size in terms of identified pixels, is able to track the movements of both subjects within the arena. Comparable software algorithms have been successfully used in the past to assess social interactions in rats (Spruijt, 1992; Sams-Dodd, 1995). The software takes 25 samples per second, and within each sample the location of each animal is detected by comparing the video frame to a reference image of the empty arena. The software is further able to distinguish the two animals using size based identification criteria (number of white pixels detected by the computer), whereby, the black marking on the back of one of the rats makes its apparent size smaller relative to the other rat. The tracks of the subjects' movements during a trial allow us to measure several parameters, such as the intra-individual distance and speed of moving. Occasional identity swops made by the software were corrected manually after video tracking.

Data analysis

Tracking data of the social interaction tests was analyzed with a previously validated method described in (Peters et al., 2016). After video tracking, the raw data containing the x- and y-coordinates of each animal in the pair was exported from EthoVision to MatLab® R2012b (The MathWorks, United States). Data was further analyzed with help of customized MatLab scripts. First, raw data was smoothed using a robust Locally Weighted Scatter Plot Smoothing (LOWESS) with a 1-s time window and filtered with a repeated running median using a one-dimensional median filter with window size 13, 11, 9, 9 respectively. Tracking data was then divided into movement bouts by detecting the 'segments of arrest' i.e. moments were the animal really has come to a stop without any clear visible movement of the body anymore. Hereby, movements bouts are defined as the path between two arrests and subsequently, the maximal velocity that is reached by the animal during a movement bout can be determined.

Then, frequency distributions (histograms) were generated of 1.) the maximal velocities of each movement bout and 2.) the inter-individual distance be-

tween animals per sample. On these distributions the best Gaussian curves that represent the data were fitted with an expectation maximization (EM) method. It was first determined if different components, in this case Gaussian curves, could be recognized within the population. Thereafter, proportion, mean and standard deviation were estimated of each Gaussian curve with the EM algorithm. After visual inspection of all frequency distributions plots, 2 Gaussian curves were plotted on the frequency distributions plots of the velocities, while 3 Gaussian curves were plotted for the inter-individual distances. Subsequently, the intersections of the curves created with the EM method were used to determine a threshold/cutoff value for the different modes or categories. This resulted in animals that move either with "low velocity" or "high velocity" and are "in contact", "in proximity" or "not in proximity". The identified categories in velocity and inter-individual distances were combined into behavioral classes.

Similar to our former studies (Peters et al., 2017; Peters et al., 2016), it was again found that based on the frequency distributions of the rat pairs, the velocity of the animals can be separated into low or high velocity, with average thresholds of: 36.75 cm/s ± 9.72 (SI1), 35.75 cm/s ± 7.91 (SI2), 41.28 cm/s ± 12.45 (SI3) and 46.91 cm/s ± 14.19 (SI4). For the inter individual distances, we found an average threshold of 26.32 cm ±3.42 for in contact and 58.20 cm ±3.95 for the threshold between in proximity and not in proximity, averaged overall 4 social interaction tests. We did not observe any statistical differences in these thresholds, between ISO or SOC animals or between different social interaction tests. These thresholds are consistent with findings of other rat pairs monitored in our lab and also fit with the rats age (11 weeks) during the social interaction tests.

Social separation test

Between social interaction test 1-2 and 3-4, animals were subjected to a social separation test, similar as done in (Peters et al., 2017). Cage mates were placed into the arena simultaneously prior to the test and left for 10 minutes to acclimatize. At the end of the acclimatization period a solid Perspex divider, 60 cm high, was placed down the middle of the area separating the pair members from each other. Since the divider did not reach the roof of the arena, we assumed that the animals were still able to hear each other's USV's and therefore, would be aware of the other's presence. In addition, in the corners animals could be aware of each other by some odor outflow trough tiny spaces between the walls and the

divider. The separation test lasted for 20 minutes. Animals were not marked or injected during the separation test days.

Ultrasonic vocalizations

Sound was recorded using the microphone placed at the top and center of the testing arena. From the social interaction tests 3 and 4 USV recordings were analyzed and presented here. The sound data was processed by the software package Sonotrack (Version 2.2.4, Metris, the Netherlands) which creates the visualization (spectrogram) of the sound file as well as making the ultrasonic sounds audible to the human ear, using a technique based on the time expansion method. This method essentially works by slowing down the sound recording, bringing the frequency down to a level that is within an audible frequency range. USV were first detected by using the automatic detection settings in the program. These settings marked any sound that was longer than 5ms, fell between the frequency 18 and 90 kHz and was above the threshold amplitude of 15.0 as a USV, any sounds that did not meet these criteria were not analyzed. This initial automated analysis was followed by the manual checking of the trials to correct for noise. Any noises that could not be categorized as an USV, after both a visual and auditory inspection, were discarded, e.g. noise from the environment such as nails of the animals on the transparent acrylic walls. The position of the microphone relative to the position of the animal during a call occasionally meant that the USV might not be fully recorded. Therefore, an extra criterion was implemented whereby elements that were separated by a gap of less than 10 ms were considered to be part of the same call, whereas elements that were more than 10 ms apart were considered separate calls, similar as in Reno et al. (2013). After detection calls were further split into categories based on their characteristics see Table 1. USV were categorized as 22 kHz calls, fixed frequency (FF) 50 kHz calls or frequency modulated (FM) 50 kHz calls.

Statistical analysis

Data were analyzed using IBM SPSS statistics 20, R Studio Version 0.97.551 and MatLab® R2012b. Data was checked for normal distribution using Shapiro Wilk's test and the equality of variances was checked using Levene's test. For comparing data between groups in the social interest and recognition test and the separation test, the independent samples t-test or the non-parametric Mann Whit-

ney U were used. For comparing data within a group, the paired samples t-test or non-parametric Wilcoxon test were used. For the social interaction test, a two way repeated measures ANOVA was used for comparing data between and within groups over different time points. In addition, it could reveal an interaction effect between time and rearing condition. Significance was set at $p<0.05$, and all tests were 2-tailed.

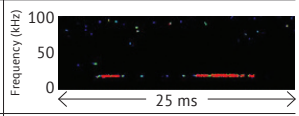
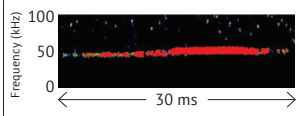
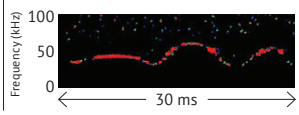
Category	Criteria	Example sonograms
22 kHz call	Call must fall between the frequencies of 18 and 32 kHz	
50 kHz call	Call must fall between the frequencies of 32 and 90 kHz	
Fixed frequency (FF) call	Max and Min frequency of the call must not be more than 7 kHz apart (i.e. bandwidth of 7 kHz)	
Frequency modulated (FM) call	Does not meet criteria for "F", can contain more than one or more FM components	

Table 1. The USV categories used to analyze the sound data along with the criteria used to categorize the calls. On the right there are example sonograms of a 22 kHz call, and a FF and FM 50 kHz call.

RESULTS

Body weight and locomotor activity

All rats gained weight over the course of their housing period at the animal facility (two way repeated measures ANOVA, $F_{(6,168)}=3411.54$, $p < 0.001$; data not shown). There were no significant weight differences found between animals in the play group compared to the play deprived group (Two way repeated measures ANOVA, $F_{(1,28)}= 1.079$, $p = 0.308$ (data not shown).

Regarding locomotor activity in the first hour of habituation to a novel environment (Figure 4), both rearing conditions showed a significant decrease in

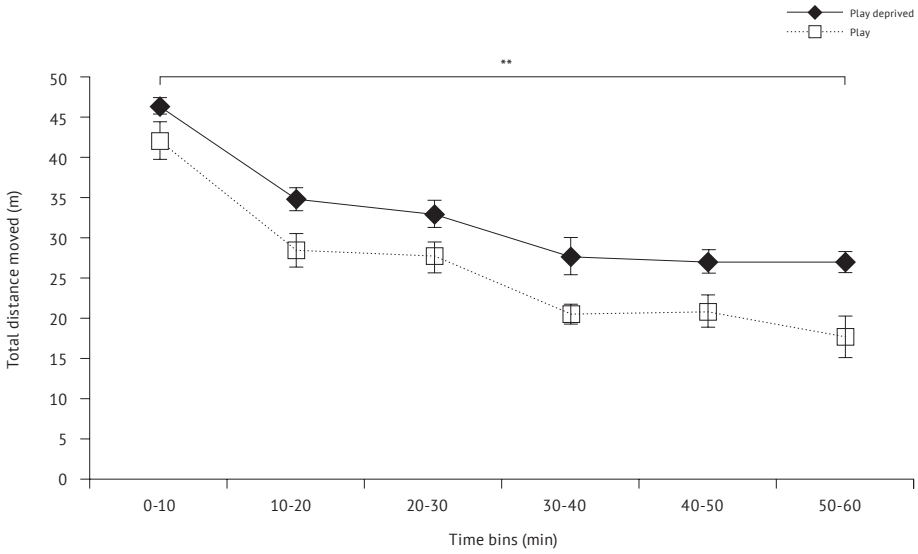


Figure 4. Individual locomotor activity (mean ± SEM) in rats from the play deprived group (n =16) and the play group (n =14) during the first hour of habituation to a novel environment. ** $p < 0.01$, effect of rearing condition

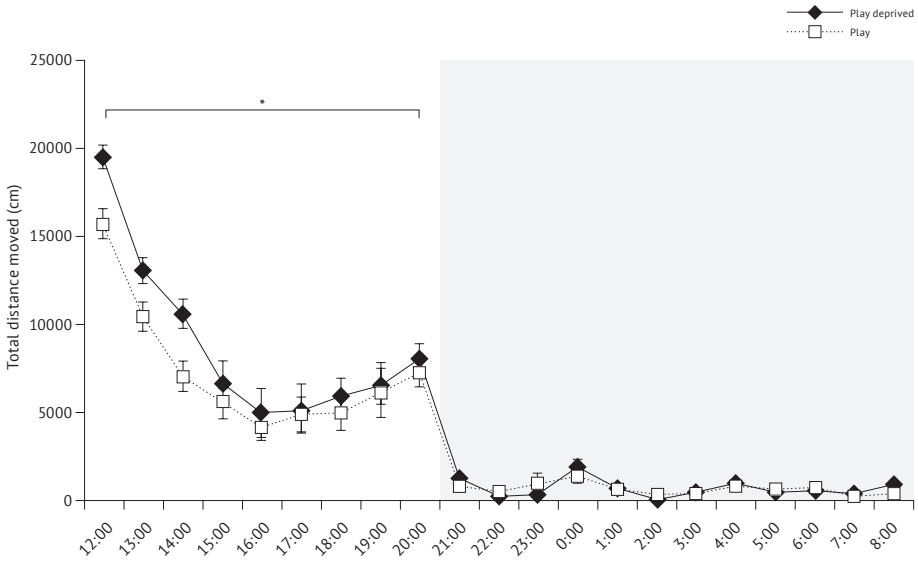


Figure 5. Individual locomotor activity (mean ± SEM) during the first day in a novel home cage of rats from the play deprived group (n =16) and the play group (n =14). Gray area represents period of inactive behavior during the period of white lights on. * $p < 0.05$, effect of rearing condition

locomotor activity over time during the first hour of habituation of the social recognition test (Mixed ANOVA, $F_{(5,140)}$ = 63.917, p <0.001). Also, there was a significant effect of rearing condition found (Mixed ANOVA, $F_{(1,28)}$=12.979, p = 0.001). Additionally, this effect was also observed during the complete 22 hours of habituation to the novel environment (PhenoTyper 9000). Play deprived animals showed significantly higher locomotor activity as compared to animals from the play group (Figure 5) during the active period of the diurnal cycle (Mixed ANOVA, $F_{(1,28)}$ = 4.831, p = 0.036)

Social interest and recognition test using scent cups

During the social interest session, both the play group and play deprived group showed a significant preference for the unfamiliar social scent (SA) over the non-social scent (N) ($t_{(10)}$ = 2.405, p = 0.037 (play); $t_{(13)}$ = 8.943, p < 0.001 (play deprived)). There was no significant difference in scent preference during the social recognition session (Figure 6) between play ($t_{(10)}$ = -0.424, p = 0.681) and play deprived animals ($t_{(13)}$ = -1.155, p =0 .269). In addition, the total time spent with both cups during each session was used as a measure for general interest in the cup. In both groups animals showed a significant decrease in interest in the cups during the social recognition session (data not shown; $t_{(10)}$ = 3.451, p = 0.006 (play); $t_{(13)}$ = 2.455, p = 0.029 (play deprived)).

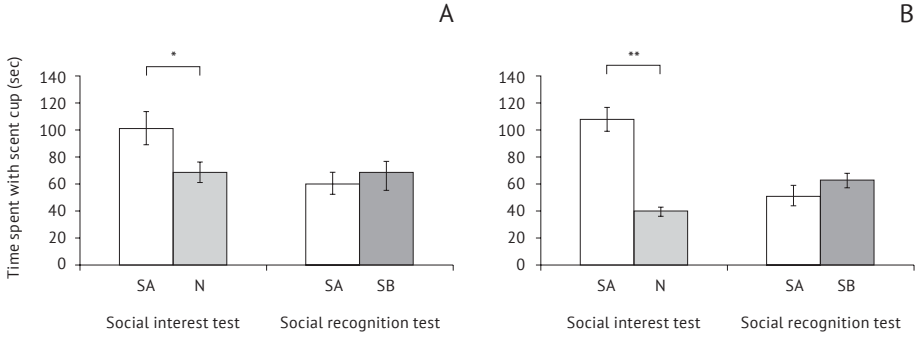


Figure 6. Time spent (mean ± SEM) with each cup during the social interest and recognition test session for the play group (A, n=11) and play deprived group (B, n=14). Time spent with the unfamiliar social scent (SA) versus non-social scent (N) during the social interest test and now familiar social scent (SA) versus unfamiliar social scent (SB) during the social recognition trial was measured. * p < 0.05, ** p < 0.001

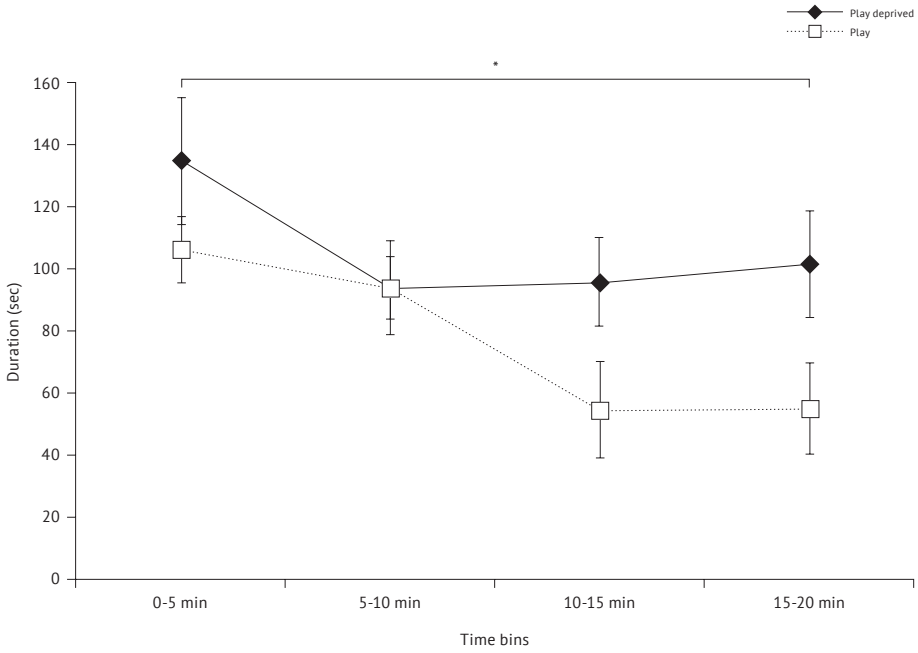


Figure 7. Time spent (mean ± SEM) near the divider of rats from the play deprived group (n =16) and the play group (n =14) during the social separation test. * p <0.05

Social separation test

Regarding the time spent near the divider, there was a significant decrease found over time (Mixed ANOVA, $F_{(3,84)} = 12.496$, $p < 0.001$). There was a significant interaction effect of time x rearing condition ($F_{(3,84)} = 3.132$, $p = 0.046$), with play deprived animals spending more time near to the divider (Figure 7).

Social interaction tests

During the first social interaction test, one trial (data of 2 animals) was lost due to an unexpected shut down of one of the computers. As a result, there was data from n = 14 for the play deprived group and n = 14 for the play group during this first social interaction test. During the other social interaction tests data was available from all animals. Between the play and play deprived group, there was no difference in activity found, calculated as average total distance moved, during all social interaction tests (data not shown). Additionally, there were no

difference between both groups, in speed of moving, either with high or low velocity, observed during all social interaction test (data not shown).

Regarding social behavior, analysis of 'in contact', 'in proximity' or 'out proximity' behavior of the pairs during all 4 social interactions tests yielded no significant differences between rat pairs from the play deprived and play group. Only, in social interaction test 4, animals from the play group showed a trend towards spending more time in contact (Figure 8) as compared to the play deprived animals after being treated with NFPS ($t_{(8.3)}$ = -2.085, p = 0.069).

Ultrasonic vocalizations during the social interactions tests 3 and 4

Play deprived or socially housed rats displayed no differences in USVs during the social interaction test 3 (vehicle injection) and 4 (NFPS injection) when paired with a similarly treated, unfamiliar partner (Figure 9). Additional analysis of frequency flat and frequency modulated 50 kHz calls revealed no statistical differences between or within social and play deprived rats. 22 kHz calls were sporadically observed and for that reason not included in any analysis.

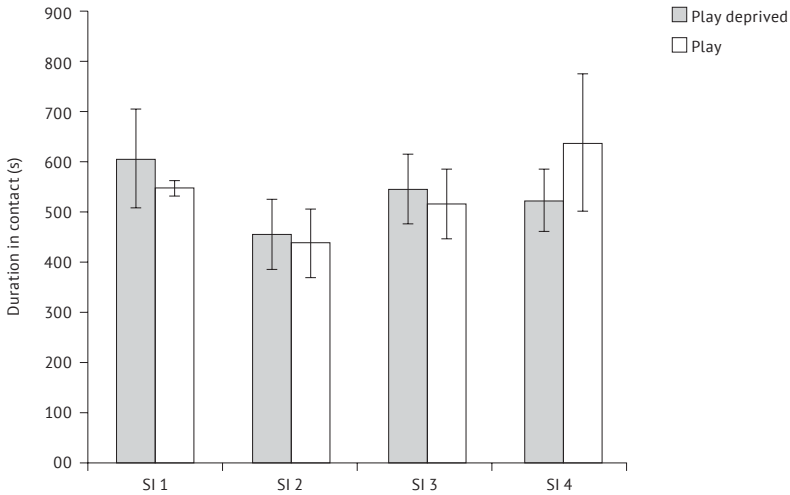


Figure 8. Time spent (mean ± SD) in contact in rat pairs from the play deprived group (no-play, n =16) and the play group (play, n = 14), during the social interaction (SI) tests. During SI 1 and 2 animals were tested as familiar and similarly treated pairs without any additional treatment. Before SI 3, animals received a vehicle injection, before SI 4 they received NFPS 1 mg/kg and in both test animals were unfamiliar and similarly treated. No significant differences between the play or play deprived group were observed, except for a trend towards significance in SI 4.

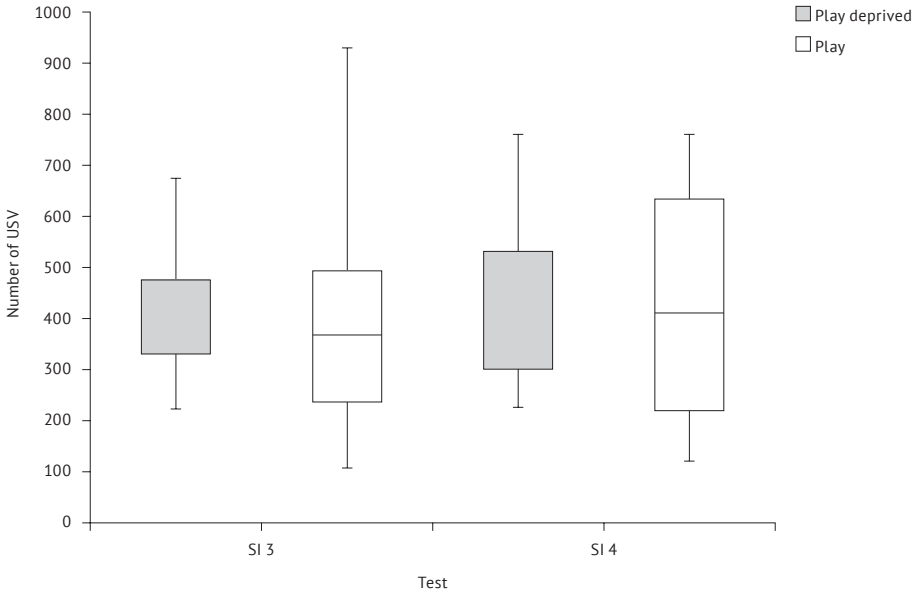


Figure 9. USV's in rat pairs from the play deprived group (no-play, n =16) and the play group (play, n = 14) during the social interaction (SI) tests 3 and 4. Before SI 3, animals received a vehicle injection, before SI 4 they received NFPS 1 mg/kg, and in both test animals were unfamiliar and same treated. No significant difference between the play or play deprived group were observed.

DISCUSSION

We investigated a previously used play deprivation model using an elaborate set of behavioral assays: individual activity, social-sexual interest and social-sexual recognition, reaction to social separation, social interaction and, USVs. Additionally, we examined a possible role for the NMDA receptor in this model by applying a single treatment of a GlyT-1 inhibitor (NFPS) prior to the social interaction test.

Results indicate that play deprivation during the juvenile period followed by subsequent social rehousing, induced persistent higher individual locomotor activity in a novel home cage when animals are 10 weeks of age. This effect was not only observed in the first hour of habituation, but was persistent over at least the first 12 hours in the novel environment. This latter effect is of particular interest because, although, it has been well recognized that post weaning early short-term isolation induces spontaneous higher locomotor activity in a novel environment (for review see e.g. Jones et al., 2011), this is mostly observed in short lasting tests and using a protocol where animals are kept in long-term isolation throughout the study, (e.g. Fone et al., 1996; Silva-Gómez et al., 2003; Dalley et al., 2002). Here, we show that this effect is likely to be more persistent and is occurring even after rehousing animals socially for 5 weeks after the 2 weeks isolation procedure, i.e. the deprivation of play. This is of importance when studying behavioral effects of play deprivation.

In the separation paradigm, our results showed that play deprived rats spent more time close to the divider that separated them from the cage mate. This suggests an increased motivation for seeking social contact rather than a general diminished sociability, which, is not in line with results obtained in other play deprivation studies also applying a subsequent social rehousing period (Van Den Berg, Van Ree, Spruijt and Kitchen, 1999; Van Den Berg, Pijlman et al., 1999; Van Den Berg et al., 2000; Hol et al., 1999; Von Frijtag et al., 2002). These latter studies showed a decline in social interactions and measured interactions representing the consummatory phase, whereas our separation paradigm is more focused on the appetitive phase of social behavior.

In all 4 social interaction tests performed at 11 weeks of age no differences in activity, social behavior or USV's between rats from the play group and play deprived group were found. In addition, a single acute treatment with NFPS, a GlyT-1 inhibitor, did not induce any changes in social behavior or USVs between

play deprived and socially housed rats. Thus, despite the observed persistent outcome on individual locomotor activity and increased social interest, we could not replicate results of earlier studies showing reduced sociability in adulthood after play deprivation during the juvenile period.

This may have been caused by two methodological differences: 1.) similarly reared rats (play deprived or non play deprived) were tested in all of our social interaction dyads and 2.) provision of enrichment in the cages (cylindrical tube, tissue paper and an increased height of the cage to allow rearing) during isolation and the rehousing period. The latter protocol difference with previous studies has been confirmed (Spruijt, personal communication), although, there was no specific reports on details of any cage materials in the earlier studies (Van Den Berg, Van Ree, Spruijt and Kitchen, 1999; Van Den Berg, Pijlman et al., 1999; Van Den Berg et al., 2000; Hol et al., 1999; Von Frijtag et al., 2002). As numerous studies have shown the major impact of enriched housing on behavior and development (for reviews see Simpson and Kelly (2011); Bayne and Würbel (2014)) this must be taken into account as possibly relevant. For example, enriched housed animals show reduced reward sensitivity compared to standard housed rat (Van Der Harst et al., 2003). Additionally, environmental enrichment stimulates social play behavior in rats that are exposed to prenatal stress, which normally decreases social behavior (Morley-Fletcher et al., 2003). It is likely that, the enrichment provided to the rats in our study may have counteracted the effects of play deprivation during the juvenile period. Perhaps enrichment facilitated solitary (object) play. An alternative explanation is that a relatively deprived housing condition in combination with play deprivation was in the former studies a too poor environment for proper development, which is reflected then as decreased social interaction behavior.

In this study we paired similarly reared rats, play deprived or socially housed, in each of the social interaction tests. This is different to the social interaction tests in the studies of (Hol et al., 1999; Van Den Berg, Van Ree, Spruijt and Kitchen, 1999; Van Den Berg et al., 1999) in which play deprived rats were paired with a socially housed individual. But, another study of Van den Berg also paired similarly treated rats (isolated or socially housed) in the social interaction test (Van Den Berg et al., 2000). Additionally, in Van Den Berg, Van Ree, Spruijt and Kitchen (1999) an overnight isolation prior to testing was applied to increase social behavior during testing, but this was not done in the other studies. Thus, it is not

completely clear whether these differences are indeed influencing the results. Conversely, it could also be argued that pairing play deprived rats in a social interaction test would strengthen diminished sociability because both rats would not be inclined to initiate social behavior, while a normal socially housed rat is likely to elicit social behavior of a play deprived interaction partner. Thus, in our setup this would then be reflected in less in contact or in proximity behavior.

In the social interest and recognition test, normal interest in a social scent over a neutral scent was present, regardless of rearing condition. Additionally, in our protocol, both play and play deprived rats showed no increased interest in a novel social-sexual scent over a familiar social scent. First, this indicates that play deprivation did not induce deficits in social-sexual interest. Second, results also indicate that from both rearing groups, animals could not distinguish between the novel social-sexual scent and the familiar social-sexual scent. This is remarkably because the test we used was previously successfully validated (van den Bos et al., 2002) and we also first ran a successful pilot experiment (not reported) using the exact same setup. It is, however, recognized that novel object recognition is sensitive to many experimental factors including strain, housing conditions, gender and age (Akkerman et al., 2012). It remains thus unclear what is accounting for the absence of novel scent recognition.

To conclude, in this study, play deprived animals are, when tested individually, more sensitive to a novel environment and this effect persists at least for 12 hours, and, motivation for social contact seems increased compared to socially housed animals. Thus, rather than diminishing sociability, it seems that play deprived rats are actually more sensitive for social contact. Results from our study illustrate the sensitivity of the play deprivation model to methodological differences. Further, it emphasizes the need to carefully consider experimental protocols and report details concerning housing or other experimental conditions. This would help to increase reproducibility and thereby improves validity of animal models.

The GlyT-1 inhibitor has been shown to stimulate social exploration in balb/c mice (Burket et al., 2015). Here, we hypothesized that due to play deprivation the reward systems is less responsive to social stimuli. The lack of any effect of GlyT-1 does not support this. Also, the enhanced social interest of the play deprived animals suggests rather an increased sensitive towards social stimuli than an insensitivity. Perhaps the play deprivation has sensitized the animals

for social rewards, whereas in previous studies the relation between social stimuli and reward has not been developed due to a lack of stimuli in general: poor environment and no social stimuli. More research on the specificity of absence of social stimuli during a critical period is required. Additionally, it remains of interest to investigate if GlyT-1 inhibitors could also have a positive effects in rat models of diminished sociability.

ACKNOWLEDGMENTS

We kindly appreciate the help of Esteban Portal in creating the custom R scripts for data processing, exploration and (statistical) analysis. We would also like to thank Raymond de Heer for his technical support and initial development of the MatLab scripts. The PhenoTyper-9000 cage was developed as part of the SenseWell project. We acknowledge our partners in this consortium (Noldus Information Technology BV, Metris BV, Telemetronics Biomedical BV, BioMetris Wageningen University, Faculty of Veterinary Medicine Utrecht University and EEMCS TU Delft) for their support. This research was partially financed by a grant from Agentschap NL (NeuroBasic-PharmaPhenomics) and ICTRegie (SenseWell).

CHAPTER 6
General discussion

Suzanne M. Peters

BACKGROUND AND AIMS

At present, the preclinical research interest in rodent social behavior is focused on its use as readout parameter in animal models for neuropsychiatric disorders ('translational research'). These animal models are used to understand the mechanism by which disease symptoms manifest, with the hope that this will ultimately result in possible treatments. However, in the area of translational research there are some major limitations that hamper progress (Belzung, 2014). Pivotal is the limited translational value of the animal models (Barrett, 2015; Braff and Braff, 2013; McGonigle and Ruggeri, 2014). This becomes apparent by the lack of reproducibility of research results (Fonio et al., 2012) and an overestimation of treatment effects (Reichlin et al., 2016). We argue here that this can be partly attributed to a currently common practice in behavioral neuroscience: i.e. a limited and too simplistic analysis of animal behavior (see Figure 1). In general, animal behavior is reduced to a few single behavioral parameters instead to covering the full complexity of all behavioral elements displayed by the animal. This simplistic view becomes particularly problematic when the behavioral outcomes of such experiments are used to interpret the functional meaning of observed behavioral differences. We have identified several elements, which are fundamental in this process (Figure 1). First, inadequate housing and developmental conditions. It is crucial that animals are provided with sufficient social experiences to ensure a healthy organism, free from aberrant behavior. This is also reflected in the European guidelines for the accommodation and care of laboratory animals (European Directive 2010/63/EU and the Commission Recommendation 2007/526/EC) which indicate that: 'Animals should be socially housed when and whenever possible and provided with an adequately complex environment within the animal enclosure to enable them to carry out a range of normal behavior'. Secondly, there is a reduction in natural variation of behavior, through the process of inbreeding and by selection relatively tame, easy to handle, animals. Finally, the performed behavioral tests tend to be too simplistic and too short (to capture the full repertoire of behaviors demonstrated by the animal), while the analysis of the behavioral readouts is too limited (even if animal demonstrates its full behavioral repertoire, the current analysis methods are not able to qualify/quantify it completely).

This thesis aims to contribute to an improvement in animal behavioral experiments in order to increase the translational value of current animal mod-

els, with a special focus on social behavior of rodents. This issue is addressed by showing that a different approach can yield more appropriate measures of behavior. Two main characteristics of this approach are: 1) ethology and 2) automation. Ethology is a discipline in which behavior of animals in studied in the context of proximate and ultimate causation. The functional meaning of behavior depends on these two distinct causes. Thus, also the biological relevance of observed behavioral changes is taken in account. The automated analysis of behavior offers great advantages as it allows the measurement of complex behaviors, that usually escape the human eye; it is objective and less prone to errors, plus it can record behavioral changes faster than a human observer (Schaefer and Claridge-Chang, 2012; Spruijt et al., 2014; Robie et al., 2017).

In this thesis, I have adopted an ethological approach combined with automation of behavioral analysis to facilitate measurements of rat social behavior. To this end, I have developed a methodology allowing continuous monitoring of social interactions in an experimental setup, including species-specific characteristics. Next, this approach has been applied and tested on two established animal models of impaired social behavior: a chronic phencyclidine (PCP) model and the play deprivation model. Both models were chosen because of their known efficacy to reduce social behavior.

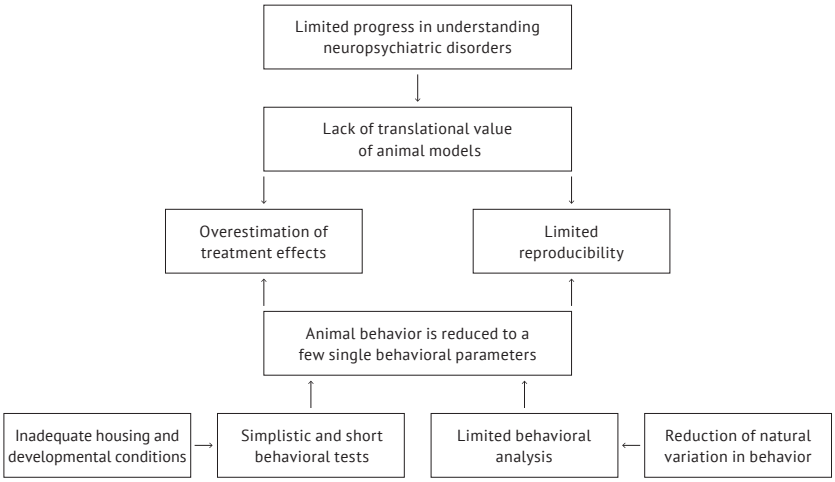


Figure 1 Schematic overview of the current status of behavioral neuroscience in preclinical research.

SUMMARY OF MAIN FINDINGS

This thesis starts with highlighting the importance of social behavior and the necessary change in approach that is needed in order to measure rodent social behavior accurately (Chapter 2). I hope to have (re)fueled the discussion in behavioral animal science with the argument that social behavior is an essential feature of several human psychopathologies and various known social conditions are necessary for appropriate neurobehavioral studies. Additionally, I would like to increase awareness of the problems arising from too simplistic behavioral tests and promote an ethological approach allowing assessment of the functional meaning of changes or differences in behavior within and between groups.

In Chapter 3, I described our experimental setup, i.e. the adjusted large home-cage enclosure permitting observations of group-housed rats. Further, I validated our approach of automated analysis, based on the original idea of Prof. Ilan Golani and co-workers (e.g. Drai et al., 2000). It consists of the analysis of frequency distributions of automatically tracked parameters and subsequent classification of behavioral modes according to Gaussian curves that best represent the data. I show that rat social behavior can be categorized in three distinct modes of inter-individual distances, 'in contact', 'in proximity' and 'not in proximity'. A combination of the social modes with the velocity modes, 'moving with low or high speed' yields specific categories of behavior. These are susceptible to pharmacological treatments and environmental manipulations. Furthermore, the behavioral categories I defined with my automated analysis approach are consistent with the categories obtained with human scoring of these behaviors (hand scoring of the same experiments). Therefore, automated recognition of patterns in social behavior is a powerful tool for data analysis.

In Chapter 4, I continue with the application of methodology to analyze short- and long-term effects of chronic PCP administration. It revealed that at short-term, PCP is able to induce diminished (active) social interaction behavior. However, it is not able, probably, to induce overt permanent long-term decrease in (active) social interaction behavior. Results from additional behavioral screening, i.e. a separation test, suggest that chronic PCP affects the motivation to engage in social behavior. Remarkably, behavioral ultrasonic vocalizations (USV's) analysis, now also incorporated for the first time, did not reveal significant changes in social behavior. It remains unclear, unfortunately, whether this

is due to a real absence of differences in social behavior or reflects a current limitation of the USV analysis. Taken together, the validity of chronic PCP administration as a model for diminished social behavior is limited to short term effects, because, it only results in a temporarily reduction of social behavior. Moreover, the consummatory phase, i.e. the phase of active social interaction behavior, is not permanently affected, yet, the appetitive phase of social behavior seems permanently effected by chronic PCP, as reflected by a reduction in motivation to engage in social behavior.

In the play deprivation model (Chapter 5), play deprivation during the sensitive period leads to diminished adult social behavior, according to current literature. However, my behavioral analysis revealed a different effect of play deprivation on adult social behavior. First, motivation for social contact seemed increased in play-deprived animals as compared to socially housed animals. Thus, rather than diminishing sociability, it seems as if play-deprived rats were actually more sensitive to social contact, i.e. effect of play deprivation merely on the appetitive phase. Second, play-deprived animals were more sensitive to a novel environment, when tested individually for 24 hours and this effect persisted at least for 12 hours. Thus, play deprivation clearly had an effect, although it was not completely the effect I had expected based on the current literature concerning this model. The contrast in results illustrate the sensitivity of the play deprivation model to methodological differences. Further, it emphasizes the need to carefully consider experimental protocols and report details concerning housing or other experimental conditions.

MAIN CONCLUSION

Based on the findings presented in this thesis, my main conclusion is that the ethological approach and novel automated methodology that I introduced here can significantly contribute to the understanding of current animal models for social behavior and, thus, increase the translational value of them. The setup and automated behavioral analysis provides us with various social categories of behavior, which are sensitive markers for changes induced by pharmacological treatment or environmental manipulation. Furthermore, subtle differences can be revealed in rat social behavior as I demonstrated that an absence of decreased/increased social contact behavior does not necessarily imply an absence of other (appetitive) aspects of social behavior. In both models I investigated,

I have seen that although behavioral changes occur that reflect the appetitive phase of social behavior, a clear effect of the mere performance of the behavior (consummatory phase) is not always apparent. This suggests that current behavioral tests for monitoring rat social behaviors might be focused on limited information, i.e. only the consummatory phase, by investigating social behavior in a social interaction test only.

ADVANTAGES OF THE ETHOLOGICAL AND AUTOMATED APPROACH

The benefits of adopting an ethological approach in behavioral neuroscience have been recognized (Brain et al., 1991; Chaouloff, 2013; Fonio et al., 2012; Kršiak, 1991; Olivier et al., 1990; Zilkha et al., 2016). However, till present it is still not as promptly implemented as one would expect based on those benefits. One of the main features of an ethological approach is the simultaneous activation of more than one motivational system. This is important, because behavior does not consist of independent behavioral systems, rather it is the intertwined result of several behavioral systems. Thus, the classical approach of applying batteries of tests, each to analyze a single parameter (relating to a single behavioral system), ignores this interaction.

The advantages of automated analysis of social interaction behavior have been widely recognized. Moreover, automation might reveal potentially new features (Robie et al., 2017). For example, sequential and temporal structures of behaviors, such as quantitative aspects of movement (speed), usually remain unnoticed by a human observer. In our approach, automation and subsequent behavioral analysis adds an extra benefit. The behavioral classes are based on the distribution of the data and, therefore, no arbitrary thresholds have to be predetermined and set per experiment. Each pair of animals reveals its own thresholds, separating one category from another, as shown by the distribution curves. These categories, defined by the animal's own variation, have been termed 'animal centered measures' (Benjamini et al., 2010). Thus, variation in data and between experiments, for example due to age or strain differences, does not impose a conflicting factor in our behavioral analysis. Additionally, our experimental setup and automated behavioral analysis method is a first step towards continuous recordings of pair-housed rats and eventually group-housed rats.

ETHOLOGICAL CONSIDERATIONS

Limited reproducibility of animal models

My research and that of others clearly indicated that the validity of two frequently used animal models to mimic persistent impaired social behavior (chronic PCP treatment and play deprivation) can be questioned. Social interaction and social withdrawal are important measures for investigating the negative symptoms of schizophrenia (Wilson and Koenig, 2014). NMDA antagonism is a widely used approach to mimic social withdrawal, however, a close inspection of the current literature already revealed inconsistent results of repeated PCP administration on social rat behavior. Our results complement this, because, no persistent impaired social behavior was observed. Decreased social behavior was only observed, within a short time window after ending repeated PCP administration.

This automatically brings the following question in mind: are studies applying this model investigating symptoms associated with withdrawal of PCP, instead of PCP-induced symptoms? It is beyond the scope of this thesis to provide a full discussion on the validity of PCP administration, however, a critical review on the use of PCP to mimic social impairments is certainly needed. In view of the behavioral results presented in my thesis in combination with evidence from current literature, we have to conclude that chronic PCP administration has only limited translational value when applied with a purpose to permanently impair social behavior.

Similar inconsistent finding have been observed with regard to the play deprivation model. Literature on the play deprivation model is consistently revealing reduced adult social behavior. However, it should be noted that these findings are almost completely limited to one laboratory, with the exception of the work of Lukkes and co-workers (Lukkes et al., 2009). The fact that these observed effects seem to be confined to 1-2 laboratories only, would indicate that they can most likely be attributed to different husbandry or housing conditions. For example, the use of enrichment material in the cages of the animals during deprivation. This illustrates the sensitivity of the play deprivation model to environmental changes (e.g. housing conditions) and hence, it limits its translational value.

Influence of strain differences and selective breeding

Variation in social behavior levels between strains and within a strain has not been recognized yet and should not be underestimated in causing reproducibility problems between experiments. Strain differences and selective breeding may also have influenced our results. Laboratory rodents have been maintained for generations in a laboratory environment and, throughout that period they may have been selected on their docile character and tameness. Hence, selection pressure might not necessarily have favored high levels of social interaction behavior. Indeed, in rats clear differences between strains have been observed in the levels and occurrence of social behavior in rats (Ferguson and Cada, 2004; Ku et al., 2016; Manduca, Servadio et al., 2014; Siviy et al., 2003) and mice (Pobbe et al., 2010; Moy et al., 2004). Additionally, comparing wild rats with commonly used laboratory strains indicates a modified expression of play behavior, which can be attributed most likely to the process of domestication (Himmler et al., 2016). For example, wild rats tend to initiate less play and more often use an evasive defense tactic during play, compared to laboratory strains (Himmler et al., 2016).

In our studies we have used rats from two outbred strains: the Sprague Dawley, Hsd: Sprague Dawley® SD® (Chapters 3 and 4) and Wistar strain, HsdCpb:WU (Chapter 5), obtained from a commercial breeder (Harlan, the Netherlands). In Chapter 5, we employed the play deprivation model similar to earlier studies, for example (Hol et al., 1999; Van Den Berg, Pijlman et al., 1999; Van Den Berg et al., 2000), wherein also rats of the Wistar strain were used. However, initially our preference was the use of rats from the Sprague Dawley strain, because, assuming that strain differences are conserved across commercial breeders, they show higher levels of social behavior compared to other strains; Wistar (Manduca et al., 2014) and Long Evans (Ku et al., 2016). Since both models are aimed at diminishing social behavior, it seemed favorable to start with initial high levels of social behavior.

Dyads of social behavior – differences between individuals or not

When a pair of rats is the statistical unit, as is the case in our methodology, two factors need to be considered. First, pairing configuration (i.e. same treatment, familiarity of individuals) can influence results due to initial differences between

the animals. Second, individual differences between animals in a pair are not analyzed. Below these considerations are discussed in somewhat more detail.

We paired same treated, unfamiliar weight-matched rats in our experiments, to ensure equality of the partners in the dyad. When, unfamiliar rats are paired together for the first time, it is not yet established who of the two is dominant. To ensure weight could not play a factor in this process, we have weight-matched the individuals which would form a pair. Dominance hierarchy within a group of rats has an effect on individual social interaction behavior (Barker et al., 2017). Thus, pairing familiar cage mates that differ in dominance rank, in a social interaction test most likely introduces initial individual differences in social behavior. However, possibly it reduces novelty-induced activity and thus, reflects a more stabilized situation. We implemented a strategy to first habituate animals to the experimental setup and procedures. In order to prevent introducing an additional factor of novelty, familiar cage mates were paired during these habituation tests.

In addition the pairing configuration, i.e. exposing normal or experimental animals together, has an influence on the behavioral outcome of a dyad. For example, it was observed that play-deprived animals were attacked more frequently by other animals (Van Den Berg, Van Ree and Spruijt, 1999). Furthermore, solitary housed adolescent rats were less appealing to normal socially-housed adolescent rats, as was reflected by avoidance behavior from the social rat towards an isolated rat (Douglas et al., 2004). In our studies we paired equal partners, i.e. both normal control animals or both experimentally treated animals, together in a social interaction test. This was expected to amplify the effects of treatment. Analyzing pairs of animals that are differently treated could, however, potentially reveal differences in social interaction behavior remaining unnoticed in a same treated configuration. Therefore, in future studies we recommend to include analysis of individual behavior as well.

To listen or to see?

Social behavior in rodents is an integrated behavior of two major forms of expression, display of visible behaviors and the for humans not audible acoustics (USV's). Given the evidence that a great portion of USV's are associated with the emotional state of the rat, it is impossible to study social behavior of rats with-

out listening to them. Hence, we should listen more to what rats communicate when socially interacting and efforts to understand their language should complement the analysis of social behavior. Unfortunately, measurements of USV's production by rats in social situations were hardly implemented in laboratory studies on rodents, even though its relation with the positive (50 kHz calls) and negative (22 kHz calls) emotional states is widely recognized. Careful analysis of these calls can provide more insight into the emotional states driving social behavior. Unfortunately, its analysis at the moment is an exhaustive task because of the abundant number of all kind of calls and the manual labelling it requires, see also technical challenges further in this discussion. Despite a lack of significant effects in our studies (Chapter 4 and 5), we strongly believe that meaningful information can be deduced from these readouts.

Pilot studies of synchronized behavioral categories with USV's revealed that 50 kHz USV's are indeed expressed during states in which the rats are in contact and moving with high speed (indicative of intense social interaction behavior) and far less when rats are not in proximity of each other. These preliminary results are line with observations of 50 kHz calls expression during playful interactions (Brunelli et al., 2006; Burgdorf et al., 2008; Knutson et al., 1998). High or low levels of play behaviors do, however, not necessarily correlate with high or low levels of 50 kHz calls (Manduca et al., 2014) and in our studies we observed a similar effect.

Standard practice is to analyze at least two categories of different 50 kHz calls; the frequency flat (FF) and the frequency modulated (FM). In some studies these two categories are even subdivided into 14 different call types. However, information on the occurrence of specific 50 kHz USV's during specific social behaviors is largely incomplete and whether they have a specific meaning is unclear. Qualitative analysis of USV production during social interaction has shown that the frequency at which 50 kHz calls are emitted increases just before playful attacks (Himmler et al., 2014; Kisko et al., 2015). Additionally, devocalization of one partner, by surgically removing the recurrent laryngeal nerves, leads to more playful encounters turning into aggressive behavior in a pair of adult rats. This suggest a critical role of the 50 kHz calls during play behavior (Kisko et al., 2015; Kisko et al., 2016). In individual rats anticipating a playful session, certain call types (split, composite and multi-step) were expressed during running and

jumping, whereas, trill calls were mainly expressed during slower movements, such as walking or shaking, during a 2-minute period of anticipation (Burke et al., 2017). However, it should be noted that the animals were alone and not yet socially interacting.

Evidence for the use of specific 50 kHz subtypes as play signals is currently limited. The most common observed subtype, the frequency modulated 'thrills', could not be related to specific behavioral elements of social interaction. The short 50 kHz calls, however, are more likely to occur after an 'evasion', i.e. moving away from the attacker, and are therefore suggested to be used as tactical signals during social interaction (Himmler et al., 2014). Thus, despite the presence of many subtypes of USV's, their communicative function during social interaction is still not completely clear. Our understanding of the language of rats, at least in social interaction behavior, thus remains limited. This urges the need for more qualitative assessment of rat USV production, comparable to the very thoroughly inspection of calls underlying behavioral elements done in a recent study of Burke et al. (2017).

METHODOLOGICAL CONSIDERATIONS

Continuous monitoring – large data set issues

Our experimental setup and behavioral analysis allows continuous recordings of pair-housed rats. There are some practical limitations for which a solution is yet not readily available. For example, continuous monitoring demands excessive data storage capacity when both video and data samples of tracking will have to be stored. A 24 hours video of a single PhenoTyper set-up is 20 Gigabytes in size, thus running 4 PhenoTypers on one computer will demands a storing capacity of at least 80 GB per day. A possible solution could be not to store the video and to run the tracking software real-time during the observation period. However, as a manual check and subsequent correction of the tracking data is required this is not an option. In fact, applying 'offline' tracking of the animals from stored video files is common practice in all currently available automated systems. The demand of tracking 2 animals simultaneously adds even more pressure on the storage and computing capacity. First, it requires considerable processor power during tracking of live video streams and second, the current tracking software has difficulties with correctly identifying the individual animals separately. For

both issues, the solution is to store video files and perform tracking and correction afterwards. Thus, depending on the experimental setup a choice between two not ideal situations has to be made. Once reliable tracking data has been obtained, it has to be analyzed using MatLab. Here, large data sets do not necessarily limit analysis, but slows down analysis.

Difficulties in automated social behavioral tracking

To accurately track two or more animals in a group-housed setting using red lights conditions to ensure meaningful analysis of behavior during the active phase of the animals, is a major challenge for software engineers. See also, Robie et al. (2017) for some practical advices on experimental and technical setup. Especially, occlusions occurring when two individuals are above and under each other, are a major reason for unreliably data acquisition of socially interacting rats. When rats are in close contact most automated systems, developed thus far, have significant problems recognizing specific contact behaviors. As a consequence, specific contact behaviors such as allogrooming, nape attacking, pinning, are often clustered into a behavioral class of "social contact" (Lorbach et al., 2017). It is suggested that, thereafter, the observer could have the option to manual annotate the behavioral sequences that consists of 'contact behavior' into a more detailed ethogram.

Technical challenges in recording of USV's

Manual labelling of thousands of recorded USV's is a very exhaustive task. It is common practice in behavioral neuroscience to limit, therefore, analysis to only a few minutes per pair of socially interacting rats. We also have experienced that analysis of USV's is very time consuming, even with a time reducing strategy, and this has significantly slowed down our analysis. Some recent advances, further discussed below in 'future directions', might in future help to limit analysis time of USV recordings.

Before recording can start, some technical issues need to be considered and if required evaluated on forehand. USV's recording requires the following set-up: high-frequency microphone(s), sound amplifier, sound processor and a software program to analyze the recording. Several systems are available: Sonotrack (Metris, the Netherlands), SASLab Pro (Avisoft Bioacoustics, Germany), RavenPro

system (The Cornell Lab of Ornithology Ithaca, NY) and currently in use by different laboratories. What is actually unknown is whether technical differences in components of these systems can lead to different outcomes. Therefore, at the start of our studies we performed a methodological evaluation. We simultaneously recorded rat USV's using the Sonotrack system and the Avisoft system. Initial results of these recordings showed that we recorded comparable sounds with comparable features.

In all experimental setups employing USV recordings care must be taken to ensure the recordings have sufficient quality. While using one overhead microphone is most probably sufficient to record most USV's of the animals, it can also be beneficial to apply multiple microphones in one setup. Application of a single microphone is often the choice because it is less complicated and only a minor risk of missing calls is assumed (Himmler et al., 2014). The second setup, is more complicated, has a risk of overestimation of calls, and requires post-hoc filtering but ensures that no calls are missed. In our studies, we utilized a single microphone placed at the top of the cage. The microphone was placed at the maximally possible height, according to the specifications of the manufacturer. The used height was somewhat higher than in most studies. Therefore, we first performed a methodological check, using both a single and a microphone array of 4, to ensure an underestimation of total USV's was not present. Another technological difficulty is that recordings can be filled with 'ghost' sounds, caused either by reflection of the ultrasonic frequencies (echo's) or by other sounds made by animals (for example nails that scratch surface). The 'ghost' sounds are picked up due to the high sensitivity of the recording system. Echoes can be partially prevented by shielding part of the experimental apparatus with sound absorbing pyramid foam, but other ghost sounds need to be manually excluded from analysis. Surprisingly, very limited discussion on these technical challenges is currently reported. To minimize the risk of within laboratory bias, a comparative investigation conducted by independent technical experts is urgently warranted.

Recommendations for future studies

Based on all that we have learnt from our studies, I infer five recommendations for future studies to analyze social rat behavior:

1) Selection of strain and individuals has to be done with understanding of the baseline differences and variation, which are there due to epigenetic and/or genetic mechanisms and developmental conditions.
2) Test conditions are chosen such that the animals can express the full spectrum of social behavior and the impact of possible confounding factors, such as novelty or familiarity of the test setup or testing partner(s), is known.
3) Investigation of social behavior should be aimed at addressing both the motivational properties and the execution of it. Besides social interaction behavior, the motivation to engage in social contact should also be investigated.
4) The behavioral parameters are analyzed such that they implement all variables that are relevant for the biological function of the behavior.
5) Ultrasonic calls are recorded and analyzed in a relevant manner.

FUTURE DIRECTIONS

Ethological assessment of animal behavior

The problems occurring from a simplistic view on laboratory animal behavior has been repeatedly addressed and a change towards an ethological approach is advocated (Anderson and Perona, 2014; Blanchard et al., 2013; Fonio et al., 2012; Gerlai, 2002; Spruijt et al., 2014; Zilkha et al., 2016). Unfortunately, progress towards a different mindset regarding the measurements of rodent behavior is still slow. Hopefully, the current improvements in technology (see below) will help to advance the implementation of an ethological approach, with the ultimate goal to monitor socially housed laboratory rodents continuously. In a social setting, it will also be possible to investigate the development of social behavioral in socially housed animals and this is of importance because: 1) neuropsychiatric disorders also have a certain course of development; 2) developmental periods are characterized by their difference in type of social behavior (play behavior during adolescence and affiliative behavior during adulthood); and 3) effects of experimental manipulation or treatment can be observed for longer time period.

Ideally, a long-term monitoring system should also enable analyses of individual behaviors, apart from social behavior. Moreover, although very challenging, USV's expression should be included.

Automated monitoring

It is 2018 and tremendous improvements have been made in various technologies in science (e.g. in the fields of molecular and cellular science). Yet, for some mysterious reason the field of behavioral neuroscience is still lagging behind. Automated behavioral assessment systems are in development, but their implementation to the level that is required to capture complex behaviors is minimal. Only a few papers have been published over the past 20 years that describe innovative tools for the use of for example automated analysis of socially interacting rodents. Yet, very limited methodology is developed for rats, see Table 1. Systems developed for social interaction of rats will hopefully soon follow. What than awaits is the routine implementation of automated methodology in behavioral neuroscience.

A frequent occurring issue in automated analysis of socially interacting animals is that of correct animal identification. Manual checking of the track data afterwards by a human observer is still necessary to correct 'identity swops', this has been reported for all systems depicted in Table 1, and we also suffered from this technical limitation of the tracking software. A suggested solution is the use of RFID assisted tracking (Weissbrod et al., 2013). However, this has a few important drawbacks. First, each animal has to be equipped with a transponder and this requires sedation of the animal. Second, the transponder and antenna's used to receive the signal from the transponder emit radio frequencies that are audible to rodents and might therefore influence their behavior. Third, the microphones for the recording of the USV's are also recording this frequency and this disturbs the signal and complicates USV analysis. Another possible solution might be an algorithm that corrects the identity swops after video tracking has been done. Recently, such a tool has been developed and validated for video tracking of mice, fruit flies, zebrafish, ants and medaka fish (Perez-Escudero et al., 2014). This tool requires a moderate to high resolution of the video images (150 pixels per animal) in order to assign a fingerprint signature to each animal. This looks like a promising approach to deal with the identity switches intro-

Study	Software	Species	Methodology characteristics	Limitations
De Chaumont et al., 2012	MiceProfiler	Mice	Top view video & automated classification of behaviors	High error rates for correct orientation of mice. Not more than 2 animals possible
Giancardo et al., 2013	Customized software	Mice	Top view video from thermal camera & automated classification of behaviors	High error rates in tracking but multiple mice possible and build in identity correction tool.
Hong et al., 2015	Customized software	Mice	3D images obtained by synced streams from video cameras and depth sensors & Supervised machine learning	Only 2 animals possible, identification is on coat color.
Kabra et al., 2013	JAABA	Mice, Drosophila	Supervised machine learning	Only behavioral classification program.
Matsumoto et al., 2017; Matsumoto et al., 2013	3DTracker	Rats	3D images from 4 depth (Kinect) cameras	Limited to 'simple' social parameters, head-head contact, approach/follow and sexual mounting behavior. Test cage should be adapted to allow infrared recordings without reflection.
Ohayon et al., 2013	MOTR	Mice	Top view video & supervised machine learning (JAABA)	Apply hair bleach to fur of animals to create identification tag.
Unger et al., 2017	Customized algorithms based on MiceProfiler	Mice	Top view video & automated classification of behaviors	Relatively high computing times needed (4 hours for 30 minute video).
Weissbrod et al., 2013	Customized software stand alones (RFID & tracking)	Mice	RFID assisted video tracking & automated classification of behaviors	Sedation animals necessary. Radio frequency in frequency range of USV's.

Table 1: overview of current available automated methods for socially interacting animals

duced by the tracking software. Of course, ideally such a tool is already implemented in any tracking software solution aimed at tracking multiple animals from video.

Due to all the challenges currently faced in full automation of socially interacting rats, one way forward could be the implementation of 'learning' software in behavioral neuroscience. Here, the software is trained by a human observer in recognizing different behavioral interactions between rats, i.e. the detailed and fine grained behaviors performed when rats are in contact, such as during allogrooming or rough and tumble play. This is slowly receiving more attention and there are some first steps made in development of such technology (e.g. Gris et al., 2017; Lorbach et al., 2017). Excluding a human observer completely is also proposed, i.e. via unsupervised machine learning. Here, algorithms classify the data into behavioral categories based on shared common characteristic of data points. It would be very interesting to see whether such a technique would deliver comparable behavioral categories to those classified by human observers, or if completely new behavioral classes would emerge.

The same machine learning technique could in the future also change and improve the analysis of USV's considerably. Some initial steps towards an automated method for USV's analysis are being made (Reno et al., 2013). In this study, different call types are automatically categorized based on call features, e.g. frequency and duration. Replacing subjective judgments of human analyzers with uniform measurements can be achieved with an automated USV analysis program. Additionally, automated call detection will drastically speed up the USV analysis.

CONCLUDING REMARKS

Over the past decades, safety pharmacology testing and high-throughput phenotyping of mutant mice have resulted in a research field in which behavioral analysis is minimized to a few parameters and test conditions are short and simple. Large amounts of results have been generated using this approach, of which most were considered successfully. This approach is also applied to study psychiatric diseases, yet in this field minimizing time and behavioral variability may be counterproductive. Consequently, it is very hard to change animal behavior analysis in such an established conventional field.

During the course of development of the PhenoTyper-9000 with customized tracking software and integrated USV's recordings, I realized that our initial ambition to get a fully automated system to monitor group housed rats was a bridge to far. Therefore, the studies presented in this thesis are a first step in the process of realizing such a system. The findings will hopefully contribute to development of systems feasible for continuous long-term monitoring of group-housed rats. During the last couple of years efforts have been made to realize automated systems to monitor group-housed rodents. I see a few elegant technical advanced techniques in various laboratories described in high impact journals. Also, the first steps in automated classification of the USV's of rats are made.

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NEDERLANDSE SAMENVATTING

Sociaal gedrag is een essentieel onderdeel van het gedrag van mens en dier. In het verleden werd de belangrijke invloed die sociaal gedrag op normale ontwikkeling heeft onderschat. De beroemde experimenten van Harry Harlow in de jaren 50 en 60 van de vorige eeuw, die nu worden beschouwd als onethisch, hebben bijgedragen aan een langzame ommekeer in deze gedachtegang. Uit deze studies bleek namelijk het enorme belang van sociaal contact. In zijn experimenten werden jonge apen sociaal geïsoleerd gehuisvest. Vervolgens werden ze blootgesteld aan surrogaat moeders gemaakt van dekens en zachte materialen of aan surrogaat moeders van metaal. De metalen surrogaat moeder was echter ook de moeder waarbij de jonge apen drinken aangeboden kregen. Ondanks dat deze conditie voorzag in de drinkbehoefte van het jong, was het de zachte moeder waar de jonge apen zich aan vastklampten. Nadat de resultaten van deze studies bekend werden, drong het besef door dat sociaal gedrag een zogenaamde 'ethologische behoefte' is. Hiermee doelen we op gedragingen die cruciaal zijn voor overleving van het individu en derhalve uit zichzelf belonend zijn voor het dier. Ook bij ratten komt sociaal gedrag veelvuldig voor. Overeenkomstig met de apenstudies, werd dit in de wetenschap ook pas halverwege de vorige eeuw echt op waarde geschat. Inmiddels is het algemeen geaccepteerd dat een adequate sociale omgeving significant bijdraagt aan een normale, gezonde ontwikkeling van het individu, zowel van mens als dier.

In het onderzoek naar psychiatrische ziekten wordt onder andere gebruik gemaakt van diermodellen. De rat en de muis worden gebruikt als model voor ziekten, zoals schizofrenie en autisme, met als doel beter te begrijpen hoe deze ziektes zich manifesteren in het brein. Daarnaast is onderzoek aan diermodellen er op gericht effectieve behandelingen te kunnen ontdekken en te ontwikkelen. In dit kader is er momenteel een groeiende interesse in het sociale gedrag van knaagdieren, omdat gedrag wordt gebruikt als uitleesparameter voor het functioneren van de hersenen en dus indicatief kan zijn voor bepaalde hersenaandoeningen. Helaas kampt het knaagdiergedrag onderzoeksveld al enige tijd met geringe vooruitgang. De resultaten uit onderzoek met proefdiermodellen blijken herhaaldelijk slechte voorspellers te zijn. Dit komt veelvuldig aan het licht wanneer bijvoorbeeld nieuwe medicatie wordt getest in humane klinische trials. Dit is te wijten aan de slechte vertaling naar de mens van gegevens verkregen met

proefdieren. Wij zijn van mening dat dit deels komt door gebrekkige kennis en ondermaatse metingen aan het gedrag van proefdieren. Overigens niet omdat proefdieren onvergelijkbaar met mensen zouden zijn. De hedendaagse tendens is dat het gedrag van proefdieren wordt onderzocht door middel van kortdurende testen, waarin gedrag wordt geminimaliseerd tot een of enkele meetparameters. Met name voor complex gedrag zoals sociaal gedrag is dit problematisch, omdat het aantal beperkte parameters niet representatief kan zijn voor de functie die het gedrag heeft. Sociaal gedrag draait om de interacties die de dieren met elkaar hebben, bijvoorbeeld achtervolgingen op hoge snelheid of juiste lage, initiëren van spelgedrag, elkaars vacht groomen, enzovoorts. Een of enkele parameters zijn dus daarmee niet indicatief voor deze variëteit aan gedragingen.

Met de studies in dit proefschrift wil ik bijdragen aan een verbetering van de translatie van proefdierenmodellen naar de mens, door met name de analyse van sociaal gedrag te verbeteren. We beargumenteren dat een verandering in de uitvoering van experimenten waarin gedragsanalyse wordt toegepast, echt nodig is om adequatere parameters van diergedrag te verkrijgen. De belangrijkste twee kenmerken van deze verandering zijn: i) meer aandacht voor ethologie en ii) het invoeren van automatisering. Een ethologische benadering geeft namelijk meer valide metingen van gedrag, omdat het de functionele betekenis, ofwel de biologische relevantie, die het gedrag heeft voor het dier ook integreert in de opzet van het onderzoek aan het desbetreffende diermodel. Als de biologische betekenis van het gedrag in de opzet behouden blijft, faciliteert dit de interpretatie van de gegevens. De setup die ik gebruik geeft bijvoorbeeld veel meer ruimte aan de dieren om vrij te bewegen dan normaliter in de meeste standaard experimenten wordt gehanteerd. Deze ruimte geeft de ratten de mogelijkheid om achter elkaar aan te rennen en elkaar ook te ontwijken, wat vanuit een ethologisch perspectief een logisch gedragsonderdeel is van sociale interactie tussen ratten. De analyse van het ultrasone geluid dat ratten maken is een belangrijk onderdeel van het observeren van sociaal gedrag. Het is namelijk indicatief voor de emotionele staat waarin het dier verkeert. Het is bekend dat geluiden rondom de 22 kHz indicatief zijn voor een negatieve context of toestand, terwijl 55 kHz geluiden juist geassocieerd worden met een positieve toestand of context. Ook fungeren de geluiden mogelijk als communicatieve signalen tijdens sociaal spelgedrag van ratten. Helaas wordt deze expressie van het gedrag nu vaak niet geanalyseerd,

wellicht mede doordat het geluid onhoorbaar is voor mensen. Daarnaast is de analyse een tijdrovend karwei door het hoge aantal ultrasone geluiden (ter illustratie tussen de 500 en 1000 geluiden per 10 minuten) in combinatie met de handmatige controle op de elk individueel geluid. Ook zouden gedragsmetingen, veel meer dan nu gebeurt, geautomatiseerd moeten plaatsvinden. Een computer is namelijk veel beter in staat om complexe gedragingen over een langere tijd te volgen dan een menselijke observator dat kan. Tevens leidt automatisering tot een objectievere weergave. Het is niet gevoelig voor subjectieve interpretatie en fouten. Daarnaast biedt het de mogelijkheid om doorgaans sneller en langer dan een mens te observeren.

De studies beschreven in dit proefschrift zijn gedaan vanuit een ethologisch perspectief en met behulp van geavanceerde software, zoals uitgebreid beschreven in Hoofdstuk 2. Mijn eerste doel was het optimaliseren en valideren van een thuiskooi-situatie waarin sociaal gehuisveste ratten continue gemonitord kunnen worden (Hoofdstuk 3). Met behulp van deze validatie heb ik vervolgens twee veel gebruikte modellen voor de inductie van een afname in sociaal gedrag geëvalueerd (Hoofdstukken 4 en 5).

Hoofdstuk 3 beschrijft onze ethologische benadering en laat zien dat met behulp van slimme parameters, gebaseerd op de natuurlijke variatie van het gedrag, het mogelijk is om sociaal gedrag te observeren. Deze parameters zijn gebaseerd op de variatie van snelheid waarmee de dieren bewegen en de afstand die ze tot elkaar hebben in de ruimte. Daarnaast zijn deze geautomatiseerde parameters gevalideerd met sociaal gedrag verhogende manipulaties, het toedienen van morfine in een lage dosis en door middel van korte sociale isolatie. Een extra verificatie liet zien dat de automatisch verkregen parameters grote mate van overlap vertoonden met hand gescoorde gedragsobservaties.

Vervolgens hebben we deze aanpak gevalideerd met een veelgebruikt farmacologisch model voor afname in sociaal gedrag (Hoofdstuk 4). Het gaat hier om chronische toediening van fencyclidine (PCP) gedurende 2 weken, waarvan in de literatuur gesuggereerd wordt dat dit een afname van sociaal gedrag kan induceren. De resultaten bevestigen dat PCP inderdaad op korte termijn een afname in sociaal interactie gedrag kan induceren, maar zij laten ook zien dat dit

geen permanent effect is. Een permante verandering in de individuele motivatie voor toegang tot de sociale partner was wel aanwezig. Dit doet mij vermoeden dat PCP een afname van de motivatie om sociaal gedrag aan te gaan induceert. De analyse van het ultrasone geluid leverde vooralsnog geen additionele informatie op over veranderingen in sociaal gedrag door PCP. Vervolgens is een diermodel onderzocht dat bestaat uit spel-deprivatie gedurende de kritische periode van ontwikkeling (Hoofdstuk 5). Bij ratten bestaat deze kenmerkende periode, gedurende de leeftijd van drie tot vijf weken, uit een periode waarin spelgedrag tussen jonge dieren zeer veelvuldig voorkomt. Eerder onderzoek duidt erop dat deprivatie van spelgedrag zorgt voor een afname van sociaal gedrag op latere leeftijd. Mijn gedragsanalyse wees echter uit dat volwassen dieren juist een verhoging van motivatie voor sociaal gedrag vertonen, indien zij spel-gedepriveerd zijn. Het blijkt tevens dat speldeprivatie ook een lang aanhoudende (12-uurs) verhoogde activiteit geeft wanneer dieren individueel gemonitord werden.

Het onderzoek in dit proefschrift laat zien dat een ethologische benadering en methode een significante bijdrage levert aan het begrijpen van diermodellen die erop gericht zijn afwijkingen in sociaal gedrag te induceren. Daarmee hoop ik uiteindelijk een verbetering in de translatie van (proef)dier naar mens te bewerkstelligen. De door mij ontwikkelde methode maakt het mogelijk om sociaal gedrag van ratten te meten en te analyseren met gebruik van geautomatiseerde parameters. Deze parameters hebben als belangrijk voordeel dat ze, naast objectiviteit en herhaalbaarheid, gebaseerd zijn op de variatie van het gedrag (snelheid en afstand) en daardoor heeft elk paar ratten zijn eigen drempelwaarde. Met deze benadering blijken de twee onderzochte diermodellen voor gereduceerd sociaal gedrag geen blijvende afname van sociaal gedrag te induceren. Echter, in beide modellen is wel een verandering in de motivatie voor sociaal contact gevonden. In het huidige onderzoek ligt de focus vooral op de analyse van sociale interactie en wordt de motivatie voor het sociale contact vaak buiten beschouwing gelaten. Het zou, gezien deze resultaten, een verbetering zijn als ook het motivationele aspect van sociaal gedrag wordt onderzocht. Het speldeprivatie model geeft in de studie in dit proefschrift andere resultaten, namelijk een toename in motivatie voor sociaal contact. Dit suggereert dat de dieren door de speldeprivatie juist gevoeliger zijn geworden voor sociaal contact. Deze beperking in replicatie geeft aan dat het model gevoelig is voor methodologische ver-

anderingen in de opzet van de studie. In het verleden is er gebruik gemaakt van zeer stimulus arme opgroeicondities in combinatie met speldeprivatie, terwijl de huidige studie verrijkende materialen in de kooi hanteerde tijdens de periode van speldeprivatie. Dit maakt het aannemelijk dat de resultaten van studies met het speldeprivatie model beïnvloed worden door andere gedrag stimulerende omgevingsfactoren. Voor een verbetering van de translationele waarde van dit model is het rapporteren van details omtrent omgevingsfactoren daarom dan ook van cruciaal belang.

Het toepassen van een ethologische benadering tezamen met automatisering van gedragsobservatie heeft behoorlijk wat voeten in de aarde gehad. Mijn eerste doel om 24 uurs-metingen aan sociaal gehuisveste ratten te verrichten moest worden bijgesteld. Voornamelijk vanwege technische beperkingen omtrent de software en ook opslagcapaciteit. Momenteel er is een gestage toename van verschillende geautomatiseerd systemen. Helaas zijn deze, voor alsnog, voornamelijk gericht op de analyse van sociaal gedrag van muizen. Een systeem waarin algoritmes, volledig of gedeeltelijk zelf leren, om zo bepaalde gedragingen van dieren te herkennen van videobeeld, lijkt gezien de groeiende mogelijkheden van zulke zelf-lerende systemen niet ondenkbaar in de toekomst. Ook de analyse van het ultrasone geluid zal hier enorm van profiteren. De huidige analyse is zeer beperkt, doordat het menselijke oog en gehoor beperkt in staat zijn om geluiden in te delen op basis van frequenties, duur en vorm. Zeer recent is aangetoond dat bepaalde type geluiden in het 55 kHz bereik correleren met individuele gedragingen van een rat. In sociale context is echter nog onduidelijk welke type geluiden indicatief zijn voor welk type sociaal gedrag. Gezien de grote aantallen geluiden tijdens sociale interactie, is het zeer aannemelijk dat hier nog veel relevante informatie in verscholen ligt.

Concluderend, er zijn nog vele technologische stappen te zetten. Echter onze studieresultaten laten zien dat er hoop is dat in de nabije toekomst proefdiergedrag zal worden gemeten en geanalyseerd vanuit een ethologische perspectief, waarbij de functionele/biologische betekenis van gedrag kan worden meegenomen en dat moderne software hierbij een uitkomst kan bieden.

CURRICULUM VITAE

Suzanne Peters was born on January 6th 1984, in Elst (Gld), the Netherlands. After her VWO at Olympus College in Arnhem she started the bachelor Biology at the Utrecht University in 2003. This was followed with a master Neuroscience and Cognition, with the track Behavioral Neuroscience. During her master she followed 2 internships at the Utrecht University. The first on the topic of animal welfare and reward sensitivity at the Ethology and Welfare group of the Faculty of Veterinary Medicine. The second was on the genetic influence on alcohol drinking in mice, at the Brain Center Rudolf Magnus. After obtaining her master degree in 2009 she started working at the spin-off company Delta Phenomics founded by Prof. dr. Berry Spuijt and dr. Lucas Noldus. After an initial year as research assistant and management assistant she continued working as scientific researcher at Delta Phenomics till 2014. During this time she performed several studies on rodent social behavior using an ethological approach. The results of these studies are published in scientific journals and presented in this thesis. In 2015, she started as a teacher in the bachelor program Psychobiology at the University of Amsterdam.

Nobody said it was easy
It's such a shame for us to part
Nobody said it was easy
No one ever said it would be this hard
Oh take me back to the start
I was just guessing at numbers and figures
Pulling your puzzles apart
Questions of science, science and progress
Do not speak as loud as my heart

The Scientist - Coldplay

DANKWOORD

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