Beyond Aggression:

Characterizing the Phenotype of the BALB/cJ Mouse

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Abstract

Background: Children with conduct disorder (CD) show high levels of aggression, cognitive impairments and changes in baseline heart rate. Common comorbidities of CD are attention-deficit-hyperactivity disorder (ADHD) and autism spectrum disorder (ASD). Here, we further characterized the behavioural and physiological phenotype of BALB/cJ mice, known for their increased levels of aggression. We investigated if they show symptoms of ASD (social withdrawal) and ADHD (hyperactivity and low temperature) as well as symptoms of CD (cognitive impairments and changes in heart rate).

Method: In experiment 1, social withdrawal was investigated with a modified version of the three-chamber social interaction test. In experiment 2, telemetric devices were implanted to measure locomotion and body temperature for a period of 86 hours. Experiment 3 was an extension of experiment 2 and also involved the investigation of the heart rate. In experiment 4, a virtual environment task was used to test for deficits in learning, attention and cognitive flexibility.

Results: In the social interaction test, BALB/cJ mice showed less interest in an unfamiliar mouse compared to BALB/cByJ mice. Experiment 2 showed that BALB/cJ mice had increased locomotor activity during the active period and a lower body temperature in the non-active period compared to control mice. In experiment 3, it was demonstrated that BALB/cJ mice have a low heart rate given their increased locomotor activity. Experiment 4 indicated deficits in learning, attention and cognitive flexibility.

Discussion: We have further characterized the behavioural and physiological phenotype of BALB/cJ mice, demonstrating that these mice show symptoms of CD, and its associated comorbidities, ASD and ADHD. The model can be used to study brain structures that might give rise to the linked symptoms of CD, ADHD and ASD.

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From an evolutionary perspective, aggression increases an individual's chance of survival. It facilitates the competition for resources and protection of the individual or its offspring (Coppens, De Boer, Buwalda & Koolhaas, 2014). However, to avoid negative consequences, aggressive behaviour needs to be proportionate to the provocation or context and is subjected to strong inhibitory mechanisms (De Boer, van der Vegt, & Koolhaas, 2003). If these inhibitory mechanisms fail, aggressive behaviour can quickly escalate and lose its adaptive function in social interaction (De Boer, Caramaschi, Natarajan, & Koolhaas, 2009). Aggressive behaviour is among the most common causes of referrals to child and adolescent psychiatrists (Gurnani, Ivanov & Newcorn, 2016) and often, conduct disorder (CD) is the diagnosis (Serper, Beech, Harvey & Dill, 2008; Finger et al., 2012; Blair, 2013).

Conduct disorder (CD) is characterized by a persistent pattern of disruptive behaviour, violating the basic rights of others and societal norms (Loeber, Burke, Lahey, Winters & Zera, 2000). Children with CD show high amounts of aggression, fighting, bullying or being cruel to others and animals (Finger et al., 2012). They are less empathic; force someone into sexual activity or run away from home. A number of cognitive impairments are also observed in patients with CD (Blair, 2013). Children with CD have difficulty in forming associations between their behaviour and positive as well as negative consequences. This seems to be due to a reduced sensitivity to reward and punishment, resulting in impaired learning of appropriate behaviour and impaired refraining from inappropriate behaviour (Matthys, Vanderschuren, Schutter & Lochman, 2012). Furthermore deficits in attention, cognitive flexibility and decision-making are observed in children and adolescents with CD. Often, they already show these symptoms at a young age (Blair, 2013).

Prognosis is poor; children diagnosed with CD show high rates of domestic violence, unemployment and homelessness in adulthood and about 54% receive a diagnosis of antisocial personality disorder (APD) later in life (Loeber et al., 2000; Blair, 2013; Noordermeer, Luman & Oosterlaan, 2016). The time of onset and the type of symptoms in CD play a crucial factor for future outcomes. Children that are diagnosed early on with CD and children that display high levels of callous-unemotional (CU) traits are more likely to demonstrate persistent antisocial and/or criminal behaviour during adulthood (Frick & Loney, 1999). The subgroup of children with high CU traits demonstrates a lack of feelings of empathy and guilt, often showing persistent psychopathic traits in adulthood (Blair, 2013). These children are characterized by proactive "cold blooded" aggression, a goal-oriented action motivated by external reward. Proactive aggression is instrumental and requires no provocation or feeling of anger (Evans, Fite, Hendrickson, Rubens & Mages, 2015). In contrast, the subgroup of children with low CU traits frequently displays high levels of anxiety together with bursts of reactive "hot blooded" aggression, which is an emotionally driven and impulsive response to a perceived threat (Loeber et al., 2000).

Neurodevelopmental abnormalities in CD

Patients with CD show decreased cortisol levels at baseline and after stress, as well as low serotonin levels (van Goozen, Matthys, Cohen-Kettenis, Buitelaar & van Engeland, 2000). A malfunctioning hypothalamus–pituitary–adrenal system (HPA axis) and low serotonin levels are thought to relate to social indifference, low emotional reactivity to punishment and impulsivity (Ortiz & Raine, 2004). However, not all patients with CD demonstrate the same pathophysiology and based on the presence of CU traits, two different sets of neurodevelopmental impairments can be observed (Blair, 2013).

Children with high amounts of reactive aggression and low CU traits appear to have an overly responsive basic threat circuitry. This threat circuitry is thought to regulate reactive

aggression and projects from the medial amygdala, via the stria terminalis to the medial hypothalamus and then to the dorsal half of the periaqueductal grey (PAG). Frontal cortical regions, particularly the ventro-medial prefrontal cortex (vmPFC) and the anterior cingulate cortex (ACC) appear to regulate this threat circuitry. If the basic threat circuitry is overly responsive, an individual will be more likely to respond to a perceived threat with a burst of reactive aggression. Children with CD and low CU traits demonstrate increased amygdala responses to fearful expressions (Blair, 2013), and vmPFC and ACC have been found to be hypofunctioning in CD, pointing toward an inadequate regulation of the basic threat circuitry (Hare, Rakimi & Rangel, 2014). Interestingly, a high number of these CD patients also meet the criteria for a mood or anxiety disorder, being in line with the finding of an overly responsive amygdala (Lahey, Loeber, Burke, Rathouz & McBurnett, 2002). These patients often demonstrate physiological hyperarousal, in form of an increased baseline heart rate (Schoorl, van Rijn, De Wied, van Goozen & Swaab, 2015).

In contrast, children with high CU traits show physiological underarousal (sympathetic hypoactivity), in form of a low baseline heart rate compared to healthy children (Beauchaine, Gartner & Hagen, 2000). They also demonstrate decreased amygdala responsiveness to distress cues and decreased striatal and ventromedial prefrontal cortex (vmPFC) sensitivity to reinforcement signals. Decreased amygdala responsiveness is associated with impaired processing of distress cues and this in turn is associated with impaired learning about actions that harm others. Furthermore, decreased sensitivity to reinforcement signals is associated with impairments in decision-making, and children with high CU traits in particular have difficulties with learning from punishment (Blair, 2013).

Common Comorbidities of CD

Autism spectrum disorder. A common comorbidity of CD is autism spectrum disorder (ASD), and about 30% of children with ASD receive a diagnosis of CD. ASD is also

highly comorbid with attention-deficit-hyperactivity disorder (ADHD); children with ASD and comorbid ADHD are even more likely to develop CD (Montes & Halterman, 2007; Guttmann-Steinmetz, Gadow & DeVincent, 2009; Witwer & Lecavalier, 2010). ASD is characterized by severe and pervasive deficits in social interaction and repetitive or stereotyped behaviours (McDougle, Stigler & Posey, 2003; Glickman, 2010). Children with ASD tend to look at and listen to other people less often, make little or inconsistent eye contact and have difficulties in taking the perspective of another person, known as 'theory of mind' deficit (Rehfeldt, Dillen, Ziomek & Kowalchuk, 2007). ASD is diagnosed early in life and symptoms are often already observed in the first 18 months after birth (Ozonoff, Heung, Byrd, Hansen & Hertz-Picciotto). The severity of symptoms in ASD can vary from minimal to profound, prognosis is poor and symptoms are frequently more severe when a comorbid disorder like CD is present (Coplan, 2000; Guttmann-Steinmetz et al., 2009).

A number of abnormalities of brain structure in ASD have been reported and throughout literature the most consistent finding seems to be an increased brain size (Casanova, 2007). Abnormal brain overgrowth appears to happen in the first two years of life and by two to four years of age the most deviant changes are seen in cerebral, cerebellar and limbic structures (Courchesne, 2004). The excessive brain growth is followed by a period of abnormally slow or even arrested growth. It is thought that this abnormal growth pattern interrupts or disrupts circuit formation, resulting in an aberrant connectivity between brain regions. Indeed, decreased size of the corpus callosum and abnormalities in functional connectivity are reported in patients with ASD (Khan et al., 2012; Barttfeld et al., 2013). Furthermore, hypoactivation of the ACC has been linked to social deficits observed in ASD (Di Martino et al., 2009).

Attention-deficit hyperactivity disorder. ADHD is a common comorbidity of CD and ASD, particularly in those children with high anxiety and high levels of reactive

aggression (Loeber et al., 2000). ADHD is characterized by a deficit in attention, increased impulsivity and hyperactivity, and up to 50% of patients with ADHD receive a comorbid diagnosis of CD (Pringsheim, Hirsch, Gardner & Gorman, 2015; Saylor & Amann, 2016). In children with ADHD that develop CD, the age of onset of CD is considerably lower than in children with CD only (Loeber et al., 2000). Furthermore, children with comorbid ADHD demonstrate higher levels of physical aggression, delinquency and more severe symptoms of both CD and ADHD than children with CD or ADHD only (Noordermeer et al., 2016). High levels of impulsivity in children with ADHD or CD contribute strongly to the risk of criminal involvement, even more than early symptoms of CD alone (Babinski, Hartsough & Lambert, 1999).

ADHD is a heterogeneous disorder and several subgroups with different symptom clusters are noted. Structural and functional abnormalities in children and adults with ADHD have been observed in fronto-striatal, fronto-parieto-temporal, fronto-cerebellar and fronto-limbic regions and networks (Cubillo, Halari, Smith, Taylor & Rubia, 2012). Structural abnormalities might be related to a delay in cortical thickness maturation. The peak of this process is delayed by an average of three years in children with ADHD compared to healthy control children and the rate of cortical thinning (reflecting cortical maturation) is inversely associated with levels of hyperactivity and impulsivity (Cubillo et al., 2012). In line with this, it has been demonstrated that cortical thinning in the ACC is related to symptom severity, especially to deficits in attention, inhibitory control and impulsivity (Corbetta & Shulman, 2002; Bledsoe, Semrud-Clikeman & Pliszka, 2013).

The BALB/cJ Mouse Model of CD

In the last two decades, increasingly more knowledge on structural and functional abnormalities in CD, ADHD and ASD has been generated but effective treatments are sparse and long-term prognosis is poor (Casanova, 2007; Esbensen, Greenberg, Seltzer & Aman,

2009; Nestler & Hyman, 2010; Matthys, Vanderschuren & Schutter, 2012). It is hypothesized that CD, ADHD and ASD share a common underlying aetiology and this is supported by genetic studies demonstrating that about 50–72% of the contributing genetic factors overlap (Thapar, Harrington & McGuffin, 2001; Leitner, 2014). Furthermore, several brain structures, such as amygdala, ACC and prefrontal circuits, are affected in all three disorders (Brieber et al., 2007; Blair, 2013). However, it is unknown if these brain structures underlie the co-occurrence of CD, ADHD and ASD. Human neuroimaging studies can guide in identifying neural structures and neuro-circuitry that co-occur in all three disorders but causal relationships between neural structures and behaviour cannot be examined in such studies. Animal models enable us to experimentally manipulate neural structures, observe the effects on behaviour and gain detailed insights into pathophysiological mechanisms (Markou, Chiamulera, Geyer, Tricklebank & Steckler, 2009; Nestler & Hyman, 2010). An animal model that shows symptoms of CD, ADHD and ASD would enable us to investigate which neural structure(s) might underlie the comorbidity of these three disorders.

BALB/cJ mice have been repeatedly used as a model for aggressive behaviour (Dow et al., 2010; Velez, Sokoloff, Miczek, Palmer & Dulawa, 2010). These mice were derived from an initial BALB/c stock, which was established in 1935. Several other laboratories acquired mice of the initial BALB/c stock; maintained them and bred them as independent stocks including BALB/cJ, BALB/cN, and BALB/cByJ. Due to breeding errors introducing new alleles and/or spontaneous mutations, the substrains started to exhibit genetic and phenotypic differences (Velez et al., 2010). For example, BALB/cJ and BALB/cByJ show differences in eleven copy number variants (CNVs) and 38 mRNAs (Velez et al., 2010; Jager et al., unpublished data). BALB/cJ and BALB/cByJ mice also demonstrate differences in their behavioural phenotype; BALB/cJ mice are more aggressive than BALB/cByJ mice. They show high levels of intermale aggression, a shorter latency to attack and a higher incidence of

attack in comparison to BALB/cByJ mice and other mouse strains in the resident intruder paradigm (Dow et al., 2010; Velez et al., 2010). In this paradigm, a mouse – the intruder – is placed in the home cage of another mouse – the resident. The test lasts usually ten minutes and in the first five minutes a Plexiglas separates the resident and the intruder. After removal of the Plexiglas mice are free to interact with each other for another five minutes and the latency to attack as well as the frequency of attacks are scored (Dow et al., 2010; Velez ez al., 2010).

BALB/cJ mice not only attack earlier and more frequently, they also attack vulnerable body parts like the belly. A bite to such body parts has a high chance of being lethal and is considered as "rule breaking" in the animal kingdom (Velez et al., 2010; Jager et al., unpublished data). In comparison to BALB/cByJ mice, BALB/cJ mice demonstrate decreased structural connectivity and decreased gamma-aminobutyric acid (GABA) inhibition in the ACC; changes that have been linked to aggressive behaviour (Jager et al., 2015). The BALB/cJ mouse model thus reproduces the core symptom of CD – increased aggression – and it also shows brain pathology comparable to the human situation (Teng et al., 2016). However, symptoms of common comorbidities of CD, such as ASD and ADHD, as well as cognitive impairments observed in CD, have been insufficiently studied in BALB/cJ mice. Here, we further characterized the behavioural and physiological phenotype of BALB/cJ mice by investigating if these mice show social withdrawal and hyperactivity, major symptoms of ASD and ADHD, and if these mice show symptoms of CD apart from increased levels of aggression. For symptoms of CD, we investigated if BALB/cJ mice demonstrate changes in heart rate, deficits in learning, and deficits in attention and cognitive flexibility. ASD, ADHD and CD are highly prevalent, start early in life and signify a heavy burden for the individual and his/her family. Therefore, finding a good animal model that can capture symptoms of the primary disorder and its associated comorbidities would enable us to explore which neural

structure(s) underlie the high comorbidity of CD, ADHD and ASD, ultimately assisting in the discovery and validation of new therapeutic interventions.

Experiment 1. In order to verify social withdrawal behaviour as a symptom of ASD in the BALB/cJ mouse model, we tested them in a social interaction test. We used a modified version of the three-chamber social preference test. The original test consists of a rectangular arena with three chambers and in one of the end chambers a stimulus mouse is restrained within a clear Plexiglas cylinder. For a period of five minutes a second mouse, the test mouse, can explore the whole arena. In the past, BALB/cJ mice have been reported to spend less time in the chamber with the stimulus mouse compared to other mouse strains; behaviour interpreted as social withdrawal (Brodkin et al., 2004; Fairless et al., 2009). BALB/cJ mice demonstrate a reduced size of the corpus callosum and increased brain size and this has been linked to social withdrawal (Brodkin, 2007; Fairless et al., 2009). However, there are two specific problems associated with previous studies. First, BALB/cJ mice have never been compared to other BALB/c substrains with a similar genetic background. It is known that mice of the BALB/c strain are less social than C57BL/6 mice; therefore, comparing BALB/cJ mice to C57BL/6 mice might lead to an overestimation of effects. Second, the use of a three-chamber apparatus might have a large influence on the behaviour of BALB/cJ mice. The mice are placed into a novel environment and are exposed to an unfamiliar stimulus mouse. Both the stress and novelty may alter the social behaviour of the test mouse, and this may be particularly of importance in BALB/cJ mice, as these mice display high levels of anxiety and are more sensitive to stress than other mice (Crawley et al., 1997; Fairless et al., 2013). Therefore, we created a modified version of the three-chamber test that consists of an arena with a single chamber and cylinders to the right and left side. This arena is similar to the homecage of mice and allows testing of social behaviour in a more familiar environment. We aimed to tackle the following hypothesis:

BALB/cJ mice demonstrate less social interest than BALB/cByJ mice, a behaviour that can be interpreted as social withdrawal.

Experiment 2. In this experiment, we investigated if BALB/cJ mice show signs of hyperactivity commonly associated with ADHD, more specifically, increased locomotor activity. Furthermore, it has been observed that children with ADHD have a decreased core body temperature compared to healthy control children (Dahl & Lewin, 2002; Bijlenga et al., 2013); therefore, we investigated if a decreased core body temperature is seen in BALB/cJ mice as well. To this end, we used radio telemetry. Radio telemetry has the disadvantage of requiring a surgery to implant a transmitter but experiments can be performed in the homecage of the mice and certain transmitters allow the simultaneous measurement of locomotor activity, temperature and biopotentials such as heart rate. Here, we have chosen to implant transmitters that can measure locomotor activity, temperature and heart rate in freely moving animals and recordings were done for 86 hours (Butz & Davisson, 2001). We aimed to tackle the following two hypotheses:

1. *BALB/cJ mice demonstrate increased locomotor activity in comparison to BALB/cByJ mice.*
2. *BALB/cJ mice show a decreased core body temperature in comparison to BALB/cByJ mice.*

Experiment 3. Compared to healthy children, children with CD show changes in baseline heart rate. High CU traits are associated with a low baseline heart rate; low CU traits are associated with a higher baseline heart rate (Beauchaine et al., 2000; van Goozen et al., 2000). The transmitters implanted in experiment 2 also measured heart rate, enabling us to

investigate if BALB/cJ mice show changes in heart rate compared to control mice. Therefore, in this experiment we explored the following hypothesis:

BALB/cJ mice demonstrate changes in heart rate in comparison to BALB/cByJ mice.

Experiment 4. In this experiment we focussed on cognitive impairments seen in children with CD. Specifically, we investigated deficits in learning, attention and cognitive flexibility. To this end, we trained mice on a newly developed visual discrimination task (Havenith et al., in preparation) in a spherical virtual environment set-up for head-restrained mice as described by Schmidt-Hieber and Häusser (2013). The set-up and task allow for moment-by-moment analysis of behaviour and the investigation of deficits in learning, attention and cognitive flexibility. In brief, mice learned to discriminate between sinusoidal horizontal and vertical gratings at orientation differences of 90° down to 5°, with the more horizontal gratings being correct. Mice had to move toward the horizontal targets and were immediately rewarded (soymilk) for correct performance, if the mice moved toward the vertical gratings they would be punished (loud tone and time-out). If BALB/cJ mice needed more time to learn the task than control mice, this would indicate a learning deficit. To investigate deficits in attention, we compared behaviour during so-called attention trials to behaviour in baseline trials. At the start of attention trials a special tone was played and mice learned that fast and correct performance was associated with either double or even triple (very fast performance) reward (more soymilk) but also double punishment (longer punishment tone, longer time-out). We investigated cognitive flexibility by comparing behaviour in consecutive trials with a horizontal target moving to the same side (‘no switch trials) to behaviour in consecutive trials with a target moving to the contralateral side (‘switch trials’). In switch trials, mice need to be able to adapt their behaviour flexibly, and changes in

performance in these trials might indicate deficits in cognitive flexibility. We investigated the following three hypotheses:

1. *BALB/cJ mice need more time to learn the task than other mice*
2. *BALB/cJ mice show an attention deficit.*
3. *BALB/cJ mice demonstrate impairments in cognitive flexibility.*

General Methods

Housing Conditions

All mice were housed individually in an enriched environment (High Makrolon® cages with Enviro Dri® bedding material and Mouse Igloo®) and had free access to dry food and water. They were kept at a reversed 12-12 day-night cycle with sunrise at 7.00pm. Efforts were taken to restrict the number of mice and to keep the discomfort as minimal as possible. All animal procedures, including behavioural tests and surgical procedures were conducted in strict compliance with the European regulations for animal experimentation. The study was approved by the Ethics Committee on Animal Experimentation of Radboud University (RU-DEC).

Experiment 1: Social Withdrawal

Animals

Fifteen-week-old male BALB/cJ (n = 5) and BALB/cByJ (n = 4) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and used as test mice. Male C57BL/6 (n = 2, Charles River Laboratories, Erkrath, Germany) were used as stimulus mice.

Experimental Procedure

Social behaviour was assessed with a modified version of the three-chamber test. The arena (50 cm x 43 cm) consisted of a single chamber with cylinders at the centre of the right

and left side (see Figure 1). Testing was done in the dark and behaviour was video-recorded. At the start of the test a stimulus mouse was randomly placed in one of the cylinders ("social cylinder"). We randomly assigned a stimulus mouse to each test mouse. The test mouse was then placed in the middle arena and was allowed free exploration of the arena and cylinders for a period of five minutes. Both cylinders had many holes, so that the test mouse could sniff the stimulus mouse. The side of the arena with the stimulus mouse was labelled as "social side" and the side with an empty cylinder as "non-social side". There was no barrier or line between the sides. Mice were sacrificed two weeks after the social interaction test.

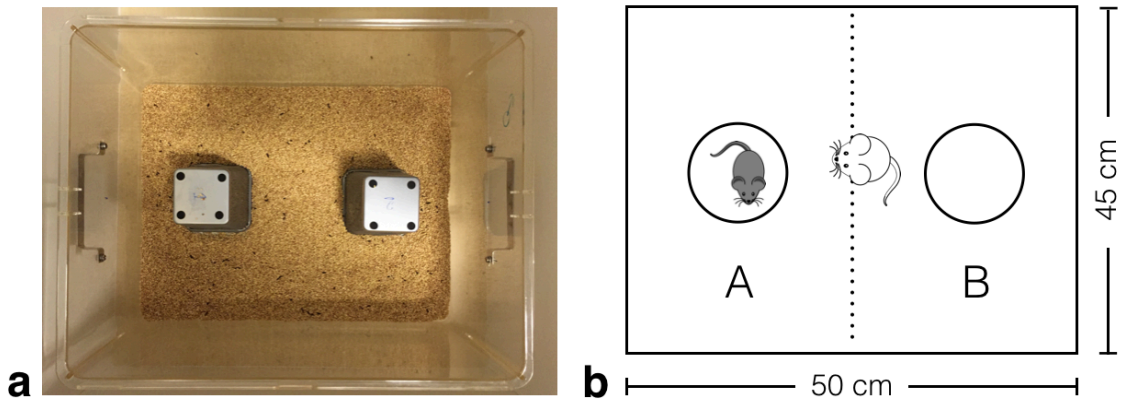

Figure 1a. The arena consisted of a single chamber and two metallic cylinders. The bottom was covered in corn pops to create a more familiar environment.

Figure 1b. A schematic of the arena with the stimulus mouse restrained in the cylinder in the social side (A) and an empty cylinder in the non-social side (B). In this example, the test mouse would be scored as being in the social side.

Data Analysis

We manually scored the time spent in non-social and social side (in seconds). The mouse was scored to be in a side as soon as the head was in that side (see Figure 1b for an example). We also scored "social cylinder investigation", defined as the amount of time (in seconds) that the test mouse sniffed, reared against, and climbed on the walls of the cylinder with the stimulus mouse inside. Climbing on the walls occurred very rarely in both groups and sniffing of the social cylinder was the predominant behaviour. Earlier studies report

social cylinder investigation as a more sensitive and reliable measure for social behaviour than the time spent in social and non-social side (Fairless et al., 2013). For manual scoring The Observer XT software (Noldus Information Technology BV, Wageningen, The Netherlands) was used. The time spent in both sides of the arena was analysed with a repeated measures ANOVA (with side as within-subject factor and group as between-subject factor). Social cylinder investigation was analysed with an independent t-test. All statistical analyses were performed using SPSS21- software (SPSS inc., Chicago, USA).

Results

BALB/cJ mice showed decreased (M = 42.47, SD = 10.36) social cylinder investigation compared to BALB/cByJ mice (M = 75.28, SD = 14.12, p = .004). The data are presented in Figure 2. There were no significant differences in the time spent in both sides of the arena between groups; however, there was an interaction effect for side x group (p = .10). BALB/cByJ mice spent more time in the social side (M = 167.76, SD = 13.27) than in the non-social side (M = 146.13, SD = 13.87), whereas BALB/cJ mice spent more time in the non-social side (M = 148.27, SD = 18.33) than in the social side (M = 161.64, SD = 22.22). The data are presented in Figure 3.

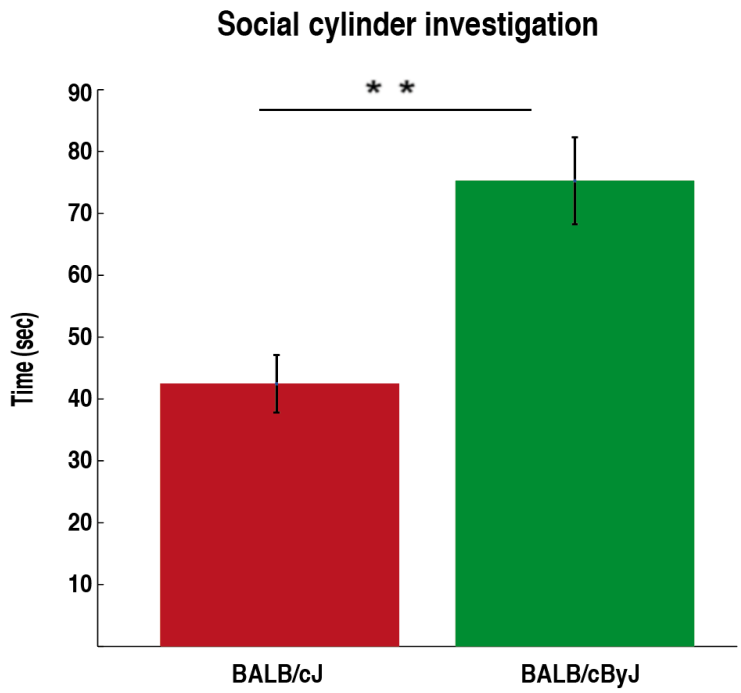

Figure 2. Bar diagram showing the mean and standard error of the mean (SEM) of social cylinder investigation for BALB/cJ and BALB/cByJ mice. BALB/cJ mice spent significantly less time investigating the social cylinder than BALB/cByJ mice.

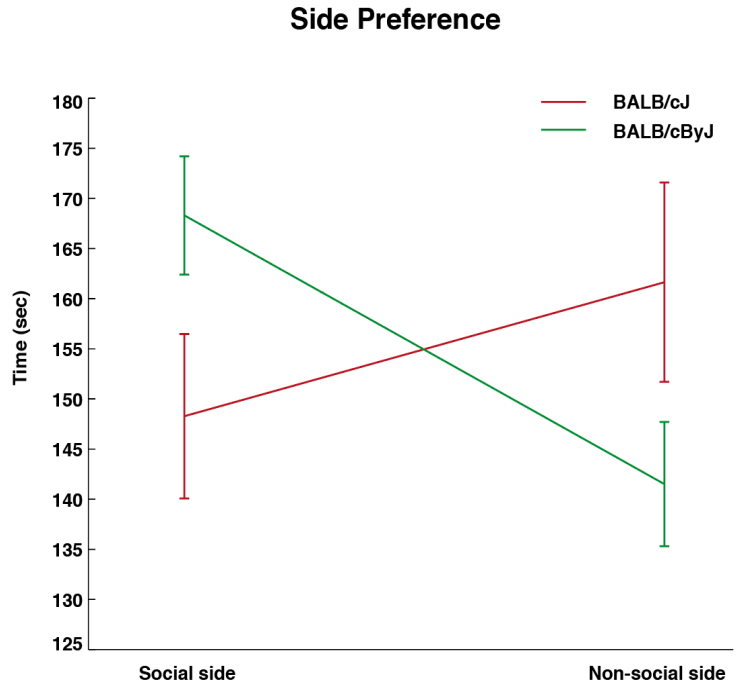

Figure 3. Interaction plot showing the mean and standard error of the mean (SEM) of social and non-social side investigation for BALB/cJ and BALB/cByJ mice. BALB/cJ mice spent less time in the social side than in the non-social side, the reversed pattern was observed for BALB/cByJ mice.

Discussion

The results of this experiment confirm our hypothesis that BALB/cJ show social withdrawal. We demonstrated that BALB/cJ mice are less interested in a stimulus mouse than BALB/cByJ mice, even when tested in a familiar environment. The arena we used closely resembled the homecage environment and did not require the mice to explore three different chambers. We also found a reversed side preference; BALB/cJ mice spent more time in the non-social side whereas BALB/cByJ mice spent more time in the social side. It has been repeatedly demonstrated that BALB/cJ mice show high levels of anxiety (e.g. Brodkin, 2007); therefore, we aimed to reduce anxiety induced by a new environment. The fact that BALB/cJ mice did not differ from BALB/cByJ mice in exploration behaviour (both groups investigated both sides of the arena), illustrates that BALB/cJ mice were not too anxious to investigate the arena. In this way, we were able to demonstrate that reduced social interest of BALB/cJ mice, interpreted as social withdrawal, is not (only) due to being too anxious to investigate the arena. Furthermore, by comparing BALB/cJ mice to a proper control group, the BALB/cByJ strain, we avoided on overestimation of effects as frequently observed in literature. BALB/cJ mice are usually compared to C57BL/6 mice, although it is known that C57BL/6 mice are more social than most other strains and that mice of the BALB/c strain in general show less social interest than other mice (Brodkin, 2007; Fairless et al., 2013).

We need to note that apart from the utilized arena, there are a few other methodological differences between our study and previous reports. We housed both BALB/cJ and BALB/cByJ mice individually while previous studies housed mice in groups. By housing them individually we prevented high levels of intermale aggressive behaviour in BALB/cJ mice and to account for individual housing effects we also housed our control group individually. It is known that individual housing can increase aggressive behaviour but it is unknown how individual housing affects behaviour in tests where test and stimulus mouse

cannot interact freely, like the three-chamber social preference test and the arena used in this study (Beery & Kaufer, 2015). Follow-up studies should investigate if individual housing has any effects on social behaviour in tests like the three-chamber social preference test.

Furthermore, previous studies usually tested BALB/cJ mice at young age (around 4 weeks, corresponding to pre-pubescence) whereas we have chosen to test our mice at a later stage (15 weeks), corresponding to adulthood (Kumar et al., 2012). In patients with ASD, deficits in social interaction persist into adulthood (Bejerot, Eriksson & Mörtberg, 2014). Here, we have not only demonstrated that BALB/cJ mice show social withdrawal but also that they demonstrate decreased social interest in adulthood, recapitulating the human situation.

Experiment 2: Hyperactivity & Temperature

Animals

Six-week-old BALB/cJ (n = 5) and BALB/cByJ (n = 6) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were housed individually. Surgery was performed at seven weeks of age and mice were allowed two weeks of recovery. Experimental recordings were performed at nine weeks of age.

Transmitter Preparation

Transmitters were purchased from Data Sciences International (DSI^{TM}, St. Paul, MN, USA). The transmitters model PhysioTel ETA-F10 was used, allowing simultaneous recordings of heart rate, temperature and locomotor activity. These transmitters operate on radio frequency, have a battery life of about 2 months and are placed intraperitoneally. They can be switched on and off with a magnet, allowing full recovery of the animal before starting experiments without consuming battery life. The transmitters consist of an insulated red (positive) and an insulated white (negative) electrode wire. Prior to surgery, the transmitters needed to be prepared for insertion. In a first step, the wires were shortened to a length

appropriate for the size of the mouse (red: 2.5 cm, white: 5 cm). Then, 1 cm from the distal part of each electrode wire, the insulation was scored with a sharp scalpel blade and pulled away from the wire, leaving an approximately 3 mm large opening. Next, the insulation tubing around the opening was fixed with non-absorbable threads (at each side of the opening). The excess insulation that extended beyond the distal end of the wire was cut. Transmitters were sterilized over night in 2 % glutaraldehyde (Helipur H Plus N; B. Braun, Melsungen) and washed 3 times in sterile sodium chloride (NaCl) before implantation.

Surgical Procedure

Before surgery mice were weighed and transferred to a clean homecage. Surgery was performed under sterile conditions. Anaesthesia was induced and maintained using isoflurane (3 % and 1.5 - 1.8 %, respectively). The mouse was fixated with tape on all four legs ventral side up on a temperature-controlled surgery stage, adjusted to 36.5°C. The abdomen and chest of the mice were shaved and the skin was disinfected with Iodine sterilization solution. Next, a longitudinal and medial abdominal skin incision of 1.8 to 2 cm was performed. The incision extended from the navel area to about 1 cm below the caudal tip of the sternum. Using a blunt metal probe moistened with physiological solution (0.9 % sterile NaCl), skin and underlying muscle tissue were separated. Then, a subcutaneous tract towards the right front leg and the left hind leg was opened for the electrode wires. According to the first skin cut, a cut of muscle tissue was performed (about 1.5 cm long). The wound area and tissue were kept moist with sterile NaCl. The rinsed transmitter was then placed into the abdominal cavity with the electrode wires positioned anteriorly. Next, using a sharp probe, a little opening left from the abdominal cut, at chest-level, was made (through the muscle tissue). Through this opening the red electrode wire was guided and non-absorbable threads were used to fixate the electrode on the muscle tissue. The muscle tissue was then closed with absorbable threads. The white wire was placed in a loop under the skin and fixated to the muscle tissue below the costal arch

(using non-absorbable threads; see figure 4 for a schematic). The two plastic fixation straps of the transmitter were connected to the muscle tissue using non-absorbable threads. Then, the muscle tissue was closed with absorbable threads. Before closing the skin, the transmitter was switched on with a magnet to assure proper function and signal. The skin was closed using three to four wound clips. Analgesia was provided by subcutaneously injecting rimadyl (Carprofen 5% with Ethanol 10%; 5-10 mg/kg) directly after surgery and twice a day for two days post-surgery. After surgery, mice were kept overnight in a warming chamber (38.5°C) for recovery. The weight and the general condition of the mice were checked daily. Wound clips were removed 10 days post-surgery.

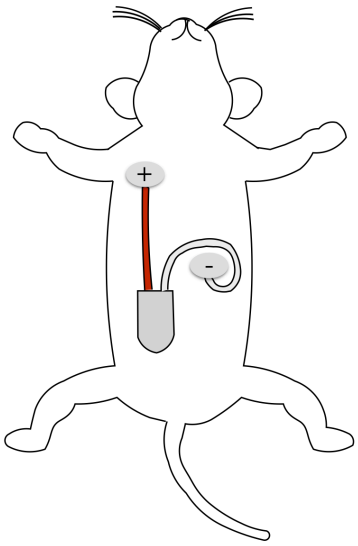

Figure 4. Schematic of the transmitter placement. The transmitter was placed abdominally. The positive wire (red) was placed at chest-level; the negative wire (light-grey) was placed in a loop just below the costal arch.

Experimental Procedure

Two weeks after surgery, mice were transferred to a separate room (same reversed day-night cycle as in the housing room, with sunrise at 7.00pm). Their cages were placed on receiver plates (PhysioTel®, DSI^{TM}) that collected the signal from the transmitters. To initiate data collection, the transmitter was switched on by touching the mouse with a magnet (possible through the cage). Ponemah Software (DSI^{TM}) was used for the detection, collection

and initial analysis of signals. This program collects data signals sent to the computer from the receiver plates via a Data Matrix (Matrix 2.0, DSI^{TM}). Data were collected at regular intervals (every 5 seconds). Recordings were started at 5.00pm and lasted 86 hours. During this period no one was allowed entrance to the room, preventing any effect of the experimenter on the mice. Mice were provided with enough food and water for this period. At the end of the experiment, mice were brought back to their original room and were sacrificed two days after the recordings.

Data Analysis

The first two hours of the recordings were not analysed to account for possible stress due to transportation. Data were analysed with start of the non-active phase (7.00pm, lights on) for a total of 84 hours (= 3.5 days). Ponemah Software (DSI^{TM}) initially pre-processed the data in data intervals of five minutes (the user can manually change these intervals). Temperature was measured in °C per minute and locomotor activity in counts per minute. Initially, for each hour we calculated a mean for temperature and locomotor activity. These data were then used to compute the mean for all non-active (4 in total) and the mean for all active phases (3 in total). The data per phase were analysed with repeated-measures ANOVAs (phase as within-subject factor and group as between-subject factor) for temperature and locomotor activity. Post-hoc tests were performed when necessary. The data per hour were also analysed with repeated-measures ANOVAs (hour as within-subject factor and group as between-subject factor) and post-hoc tests were done when necessary. All statistical analyses were performed using SPSS21- software (SPSS inc., Chicago, USA).

Results per Phase

Locomotor activity. Both BALB/cJ and BALB/cByJ mice were more active during the active phase than during the non-active phase (p = .002). There was an interaction effect of phase x group, demonstrating that BALB/cJ mice were more active than BALB/cByJ mice

during the active phase (p = .06). The data are presented in Figure 5. The means and standard deviations can be found in Table 1.

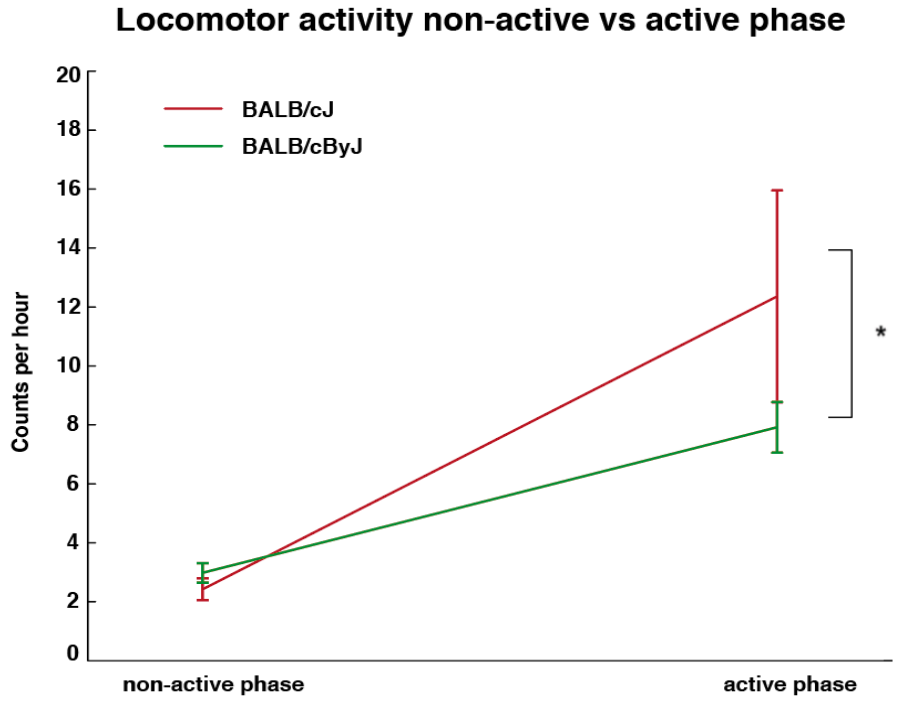

Figure 5. Plot showing the mean and SEM of locomotor activity in non-active and active phase for BALB/cJ and BALB/cByJ mice. Both groups show more activity in the active phase than the non-active phase. BALB/cJ mice are more active in the active phase than BALB/cByJ mice.

Table 1

Locomotor activity non-active vs active phase

Locomotor activity	*M* non-active phase	*M* active phase	*SD* non-active phase	*SD* active phase
BALB/cJ	2.4	12.4	5.1	10.8
BALB/cByJ	2.9	7.9	5.3	8.1

Note. M = Mean, SD = standard deviation.

Temperature. Both groups demonstrated a lower temperature during the non-active phase than the active phase (p = .000). BALB/cJ mice showed a lower temperature than BALB/cByJ mice (trend, p = .091). There was an interaction effect of phase x group, demonstrating that BALB/cJ mice had a lower temperature than BALB/cByJ mice during the

non-active phase (p = .02). The data are presented in Figure 6. The means can be found in Table 2.

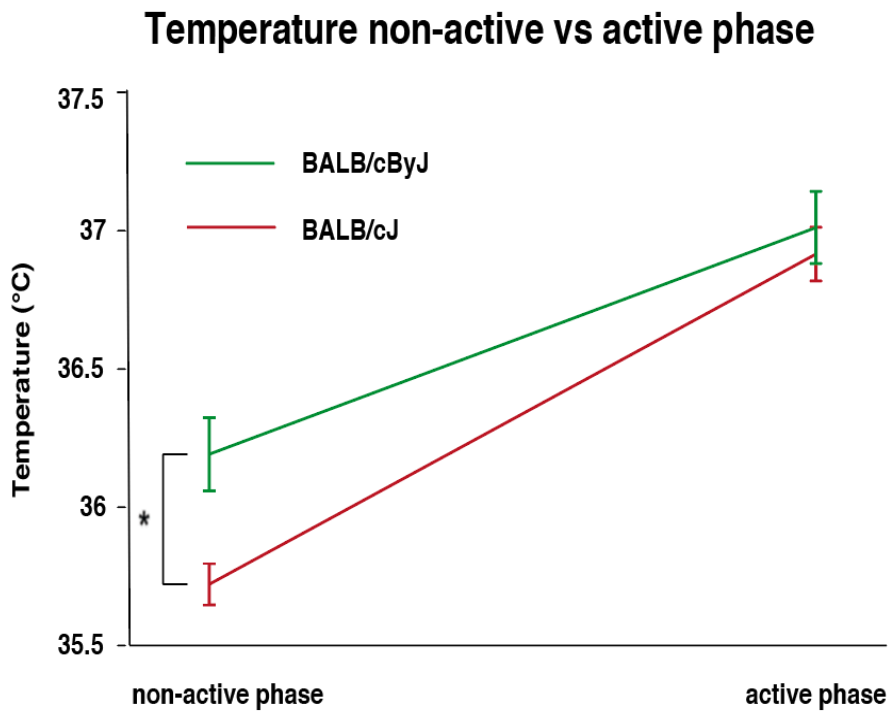

Figure 6. Plot showing the mean and SEM of temperature in non-active and active phase for BALB/cJ and BALB/cByJ mice. Both groups show a lower temperature in the non-active phase than the active phase. BALB/cJ mice have a lower temperature in the non-active phase than BALB/cByJ mice.

Table 2

Temperature non-active vs active phase

Temperature	*M* non-active phase	*M* active phase	*SD* non-active phase	*SD* active phase
BALB/cJ	35.7	36.9	0.60	0.88
BALB/cByJ	36.2	37.0	0.58	0.84

Note. M = Mean, SD = standard deviation.

Results per Hour

Locomotor activity. In both groups, locomotor activity was higher during the active than the non-active hours (p = .000). There was an interaction effect of hour x group (p = .036). Based on the results of the data per phase, we decided to do post-hoc tests only for the

hours in the active phase. In these hours, BALB/cJ mice were more active than BALB/cByJ mice (all $p < .05$). The data are presented in Figure 7.

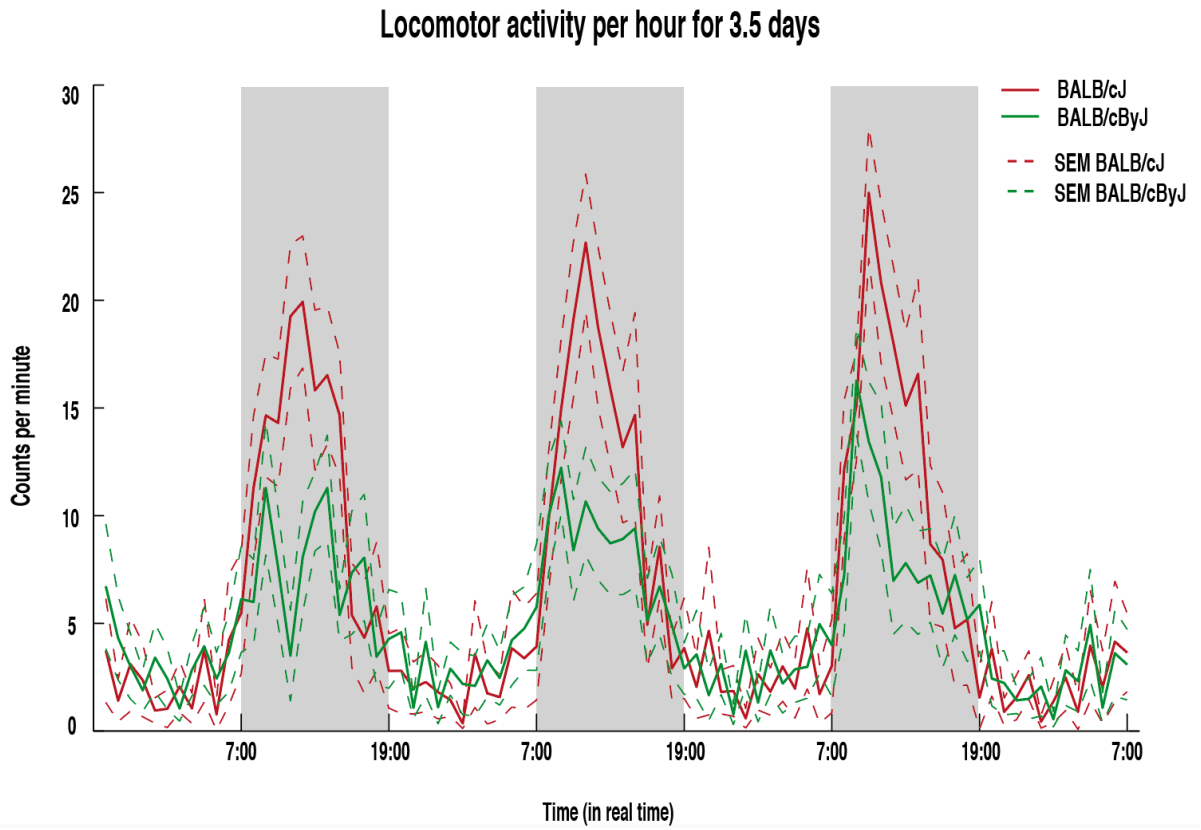

Figure 7. Graph showing the mean and SEM of locomotor activity per hour for 3.5 days. Grey shaded areas indicate the active hours (lights off). Both groups show a higher locomotor activity in the active hours than the non-active hours. BALB/cJ mice are more active in the active hours than BALB/cByJ mice.

Temperature. In both groups, temperature was lower during the non-active hours than the active hours (p = .000). BALB/cJ mice showed a lower temperature than BALB/cByJ mice (trend, p = .07). There was an interaction effect of hour x group (p = .025). As the data per phase had indicated that BALB/cJ mice only demonstrated a lower temperature than BALB/cByJ mice in the non-active phase, we decided to restrict post-hoc tests to the non-active hours. In these hours, BALB/cJ mice had a lower temperature than BALB/cByJ mice. The data are presented in Figure 8.

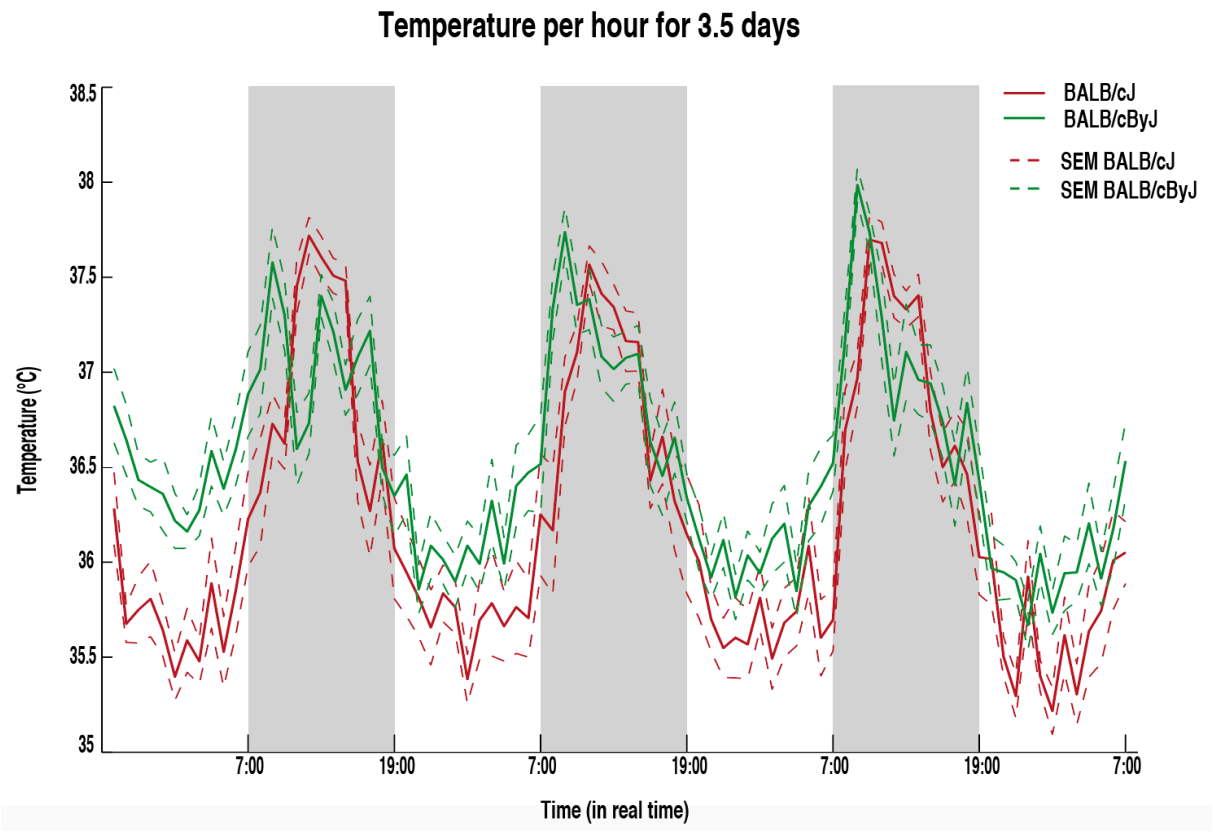

Figure 8. Graph showing the mean and SEM of temperature per hour for 3.5 days. Grey shaded areas indicate the active hours (lights off). Both groups show a lower temperature in the non-active hours than the active hours. BALB/cJ mice have a lower temperature in the non-active hours than BALB/cByJ mice.

Discussion

In experiment 2 we investigated if BALB/cJ mice show hyperactivity (increased locomotor activity) and a lower body temperature; symptoms observed in patients with ADHD. We implanted transmitters and took measurements for a period of 86 hours. We found that BALB/cJ mice showed increased locomotor activity in comparison to BALB/cByJ mice and a lower (nocturnal) body temperature than BALB/cByJ. Multiple lines of research point toward a role of dopamine in ADHD, more concrete, a dopamine dysfunction in the mesocortical, mesolimbic and nigrostriatal pathways (Sonuga-Barke, 2005). In patients with ADHD, there seems to be increased dopamine reuptake by dopamine transporters (DAT), resulting in decreased extracellular dopamine (Volkow et al., 2001; Gold, Blum, Oscar-

Berman & Braverman, 2014). Symptoms of hyperactivity have been related to the nigrostriatal pathway, which is involved in cognitive and voluntary movement control (Aguiar, Eubig & Schantz, 2010) and projects from the substantia nigra to the striatum (Cho, Baek & Baek, 2014).

Dopamine levels can also be regulated by serotonin, and serotonin dysfunction has been related to ADHD and hyperactivity (Quist et al., 2003). For example, studies in rodents and humans demonstrated that polymorphisms in the 5-HT1B receptor are associated with hyperactivity (Quist et al., 2003; Guimares et al., 2009). Deletion of the 5-HT1B receptor in mice resulted in hyperactivity, increased exploratory activity and increased aggressive behavior and patients with ADHD frequently have the G861C polymorphism of the 5-HT1B receptor, which is associated with reduced 5HT1B receptors (Huang, Grailhe, Arango, Hen & Mann, 1999; Quist et al., 2003). Interestingly, polymorphisms in the TPH2 gene, coding for the rate-limiting enzyme in the synthesis of 5-HT, have been associated with ADHD and BALB/cJ mice have a polymorphism in the TPH2 gene as well (Biskup et al., 2012). Therefore, it might be that a serotonin dysfunction in BALB/cJ mice influences the dopamine system, resulting in symptoms of ADHD, such as increased locomotor activitiy. Furthermore, the lower body temperature we observed in BALB/cJ mice is also observed in children with ADHD and has been linked to low levels of serotonin and dopamine (Catalina, Milewich, Frawley, Kumar & Bennett, 2002). Children with ADHD often have sleep problems (Stein, 2009) and waking up during a period of low body temperature has been related to sleep deprivation and attention deficits (Dahl & Lewin, 2002).

Increased locomotor activity in BALB/cJ mice might be a method to augment their low body temperature, instead of being a sign of hyperactivity. However, one would then expect to see increased levels of locomotor activity especially during the non-active phase, as the temperature of BALB/cJ mice is significantly lower during the non-active phase. In the

non-active phase there is no difference in locomotor activity between BALB/cJ mice and BALB/cByJ mice. It is known that stimulant medication used for the treatment of ADHD decreases symptoms of hyperactivity and there are indications for increases in body temperature in patients taking stimulant medication (Schacher, Tannock, Cunningham & Corkum, 1997; Lakhan & Kirchgessner, 2012). However, patients that misuse their prescribed stimulants (e.g. take increased doses) show increased hyperactivity and increased body temperature at the same time (Lakhan & Kirchgessner, 2012), and healthy individuals that make use of stimulant medication also show increased activity and increased body temperature (Pigeau et al., 1995). Therefore, hyperactivity in ADHD does not seem to be a method to augment body temperature. To definitely test whether a low body temperature causes increased locomotor activity in BALB/cJ mice, one could house BALB/cJ mice in a heat chamber and observe if they still show signs of hyperactivity.

Experiment 3: Heart Rate

This experiment was an extension of experiment 2. The same mice were used and data were acquired simultaneously with the data of experiment 2. Method and data analysis were performed as described in experiment 2 and heart rate was measured in beats per minute. In addition, a linear regression was fitted to examine the correlation between heart rate and locomotor activity.

Results per Phase

Heart rate was higher during the active phase than the non-active phase in both groups (p = .001). There was neither a difference between groups nor a phase x group interaction effect. The data are presented in Figure 9. The means can be found in Table 3.

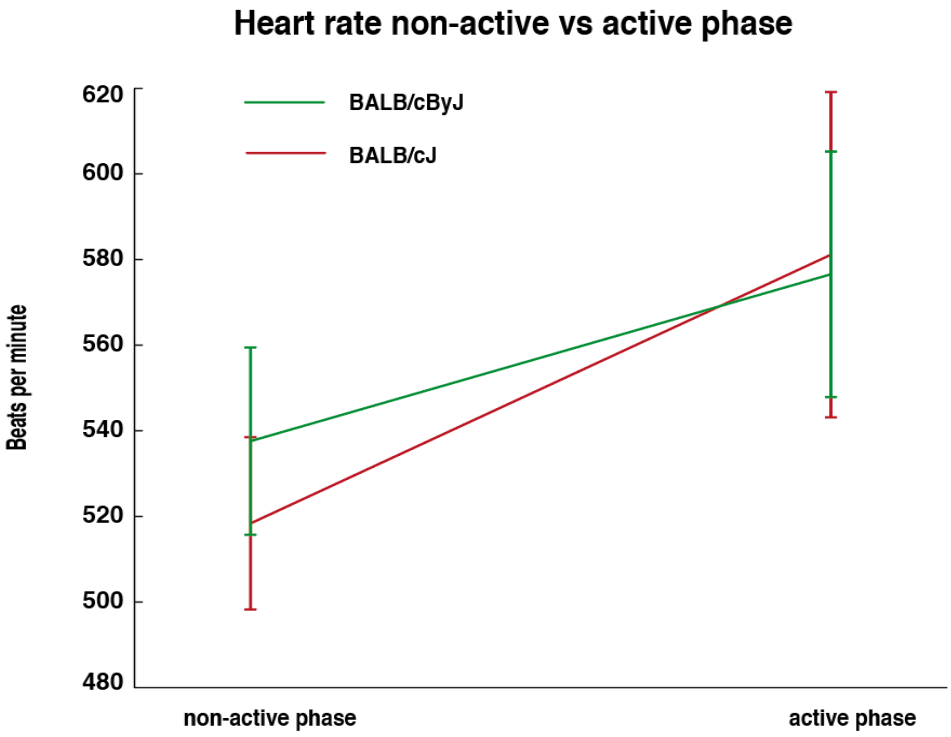

Figure 9. Plot showing the mean and SEM of heart rate in non-active and active phase for BALB/cJ and BALB/cByJ mice. Both groups show a lower heart rate in the non-active phase than the active phase.

Table 3

Heart rate non-active vs active phase

Heart rate	*M* non-active phase	*M* active phase	*SD* non-active phase	*SD* active phase
BALB/cJ	517.3	581.1	56.7	68.2
BALB/cByJ	537.6	576.6	60.0	60.0

Note. M = Mean, SD = standard deviation.

Results per Hour

Heart rate was lower during the non-active hours than the active hours, this was observed for both groups (p = .000). There was neither a difference between groups nor an hour x group interaction effect. The data are presented in Figure 10.

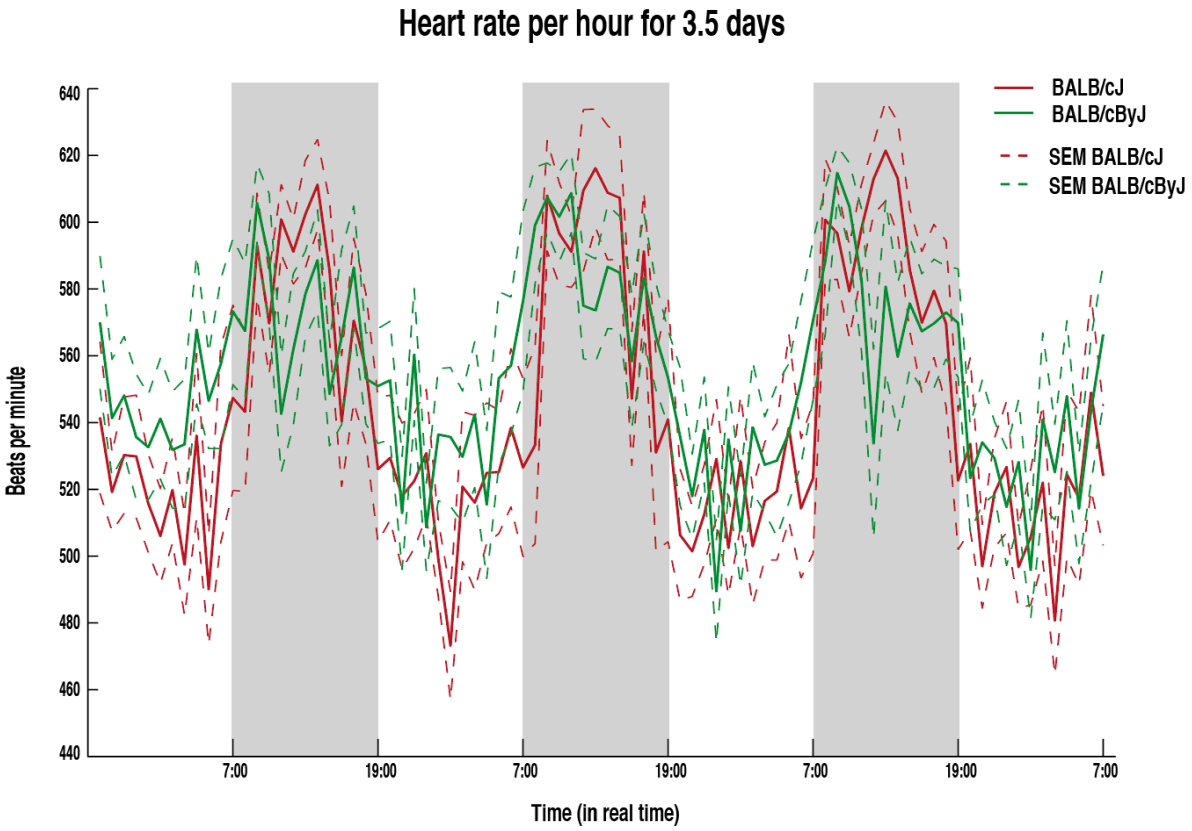

Figure 10. Graph showing the mean and SEM of heart rate per hour for 3.5 days. Grey shaded areas indicate the active hours (lights off). Both groups show a lower heart rate in the non-active hours than the active hours.

Linear Regression

Heart rate and locomotor activity were highly positively correlated in both groups (BALB/cJ: r = 0.91, BALB/cByJ: r = 0.93). This means that when locomotor activity increased, heart rate increased too. However, the slopes between the groups were different (p = .02), with the BALB/cByJ having a steeper slope. This means that the increase in heart rate with increasing levels of locomotion was smaller for BALB/cJ mice than BALB/cByJ mice. The data are presented in Figure 11.

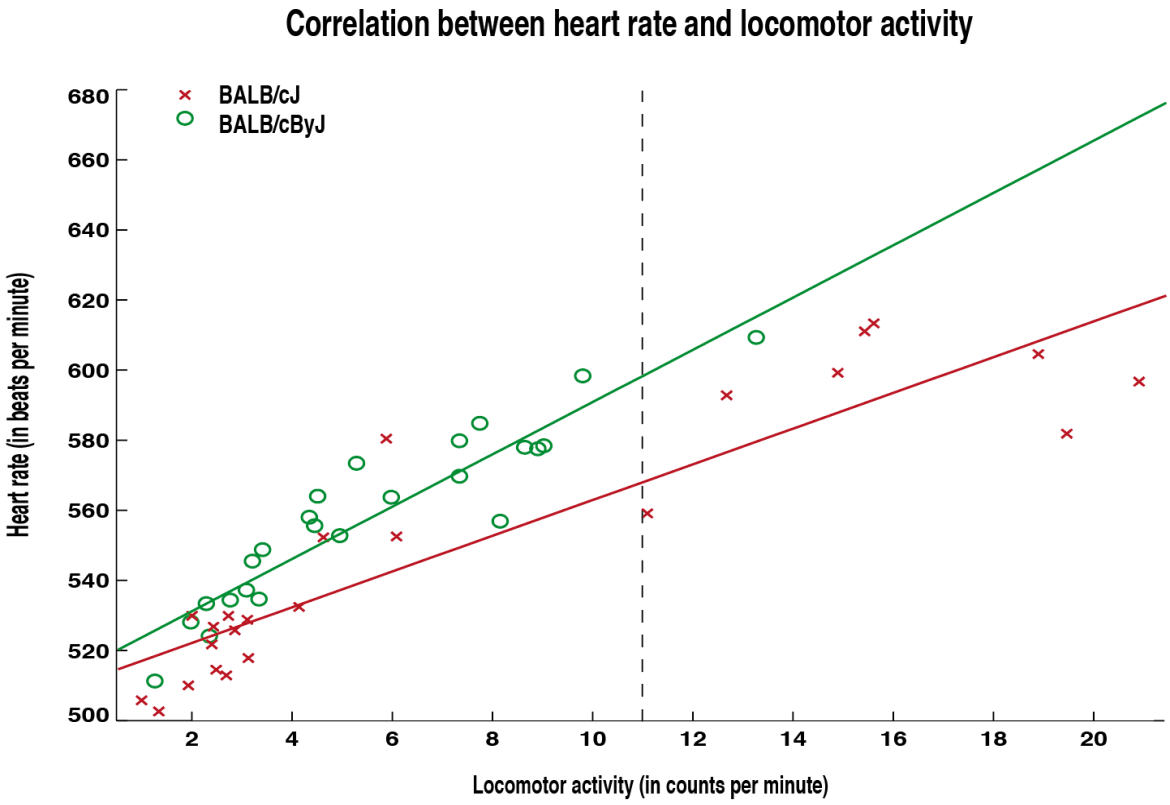

Figure 11. Graph showing the correlation between locomotor activity (x-axis) and heart rate (y-axis) for both BALB/cJ and BALB/cByJ mice. In both groups heart rate positively correlates with locomotor activity. The increase in heart rate with increasing levels of locomotion is smaller for BALB/cJ mice than BALB/cByJ mice as can be seen in the difference in the steepness of the slope between the groups.

Discussion

In experiment 3 we investigated if BALB/cJ mice show a decreased baseline heart rate, as observed in children with CD (van Goozen et al., 2000). We used the data of experiment 2, as the transmitters also measured heart rate. At first, we did not find differences in baseline heart rate between BALB/cJ and BALB/cByJ mice. This might be due to the rather small N (5 BALB/cJ and 6 BALB/cByJ) and the relatively large standard variation between the BALB/cJ mice during the active period (see Table 3). Heart rate increases with activity and is usually lower during the night (Bonnet & Arand, 1997), which we have also seen in both groups. Given the high locomotor activity of BALB/cJ mice one would expect to

see a correspondingly high heart rate. However, when we correlated heart rate with locomotor activity we were unable to observe this correspondingly high heart rate in BALB/cJ mice. In both groups, locomotor activity strongly and positively correlated with heart rate but the slope for the BALB/cByJ mice was significantly steeper than for the BALB/cJ mice. This means that for BALB/cJ mice the extremely high levels of locomotor activity were not associated with extremely high levels of heart rate, as one would expect. Take for example the highest locomotor activity of the BALB/cByJ mice, which was about 14 counts per minute. The corresponding heart rate for this data point was about 610 beats per minute. This is also the highest heartbeat per minute observed in BALB/cJ mice even though locomotor activity levels were as high as 24 counts per minute. Therefore, we can conclude that BALB/cJ mice show a low heart rate given their high locomotor activity levels.

A lower baseline heart rate is observed in children and adolescents with CD and high CU traits (van Goozen et al., 2000). A lower baseline heart rate is an indicator of physiological underarousal and underaroused individuals might engage in stimulation- or risk seeking to raise their autonomic activity to more optimal levels, reinstating homeostasis (Baker et al., 2009). Low levels of arousal might result in a lack of fear, which in turn can result in impaired learning from threat, such as punishment. In the end, children with low baseline heart rate may show higher levels of aggression, and rule breaking, as observed in children with CD. However, children with low CU traits and high levels of anxiety show an increased baseline heart rate, which is an indication of hyperarousal and seems to be related strongly to reactive aggression (Schoorl et al., 2015). This distinction within the group of children with CD could also be present in BALB/cJ mice, explaining the fact that there was no overall difference in heart rate between BALB/cJ and control mice. It might be that highly anxious BALB/cJ mice show an increased heart rate whereas less anxious BALB/cJ mice

show a decreased heart rate. In future studies we should assess anxiety levels in these mice and correlate these to their heart rate.

Currently, we are focussing on analysing heart rate variability in BALB/cJ mice, which can be calculated as the absolute mean successive difference in R-R intervals and/or with a power spectrum analysis. In children with CD a decreased heart rate variability is observed (Beauchaine et al., 2000). Decreased heart rate variability is general, is associated with maladaptive reactions to environmental challenges and inability to modulate affective responses and decreased heart rate variability in CD is correlated with antisocial behaviour and criminal involvement (Srinivasan, 2007). However, decreased heart rate variability is mostly associated with reactive aggression, which is mainly seen in children with CD and low CU traits. Lately, it has been shown that children with CD and high CU traits have higher heart rate variability (Schoorl et al., 2015). In contrast to low heart rate variability, high heart rate variability is not associated with an inability to modulate affective responses (Williams et al., 2015). Children with high CU traits do not show problems regulating their affective responses; instead they demonstrate proactive aggression (Dadds et al., 2015). Given this distinction within the group of CD children, we do not expect to find differences in heart rate variability between BALB/cJ mice and control mice. It might be necessary to repeat the experiment and to additionally collect data on anxiety levels in BALB/cJ mice. Therefore, acquiring a larger data set on heart rate in BALB/cJ mice together with data on their anxiety levels will enable us to extend our findings on physiological underarousal and hyperarousal in the BALB/cJ strain.

Experiment 4: Cognitive Deficits

Animals

One six-week-old male BALB/cJ (n = 1) was obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and six-week-old male C57BL/6 (n = 5) were obtained from Charles River Laboratories (Erkrath, Germany).

Virtual-reality Environment

The virtual-reality system was centred around an air-supported Styrofoam ball (diameter: 20cm) that was used as a spherical treadmill for head-restrained mice. A projector (Optoma; brightness: 65, contrast: 33, keystone: 40) screened the image, which in turn was reflected by a mirror (placed underneath the mouse, diameter: 38cm), onto a spherical dome screen (diameter: 49cm). The screen covered 270° of the mouse's horizontal field of view (relative to the mouse's head). The whole set-up was grounded on a 1m x 2m table (Nexus, Thorlabs; see Figure 12).

Rotations of the ball were read out with two optical computer mice (Logitech), positioned at a 90 degree angle along the horizontal midline of the ball - one behind the animal, and one to its right, corresponding to the readout of forward and sideways movements. Readouts of the ball movement were made at a frequency of 60Hz, recorded and translated into movement in the virtual environment using scripts provided by Gnoom (https://github.com/neurodroid/gnoom) in a custom-programmed Blender Game Engine (http://www.blender.org). Rewards were delivered by a valve, driven by an arduino TTL pulse, which was attached to a tube. The tube was placed into a metal holder, and positioned directly in front of the animal's mouth so that the mouse could lick the reward from the tube.

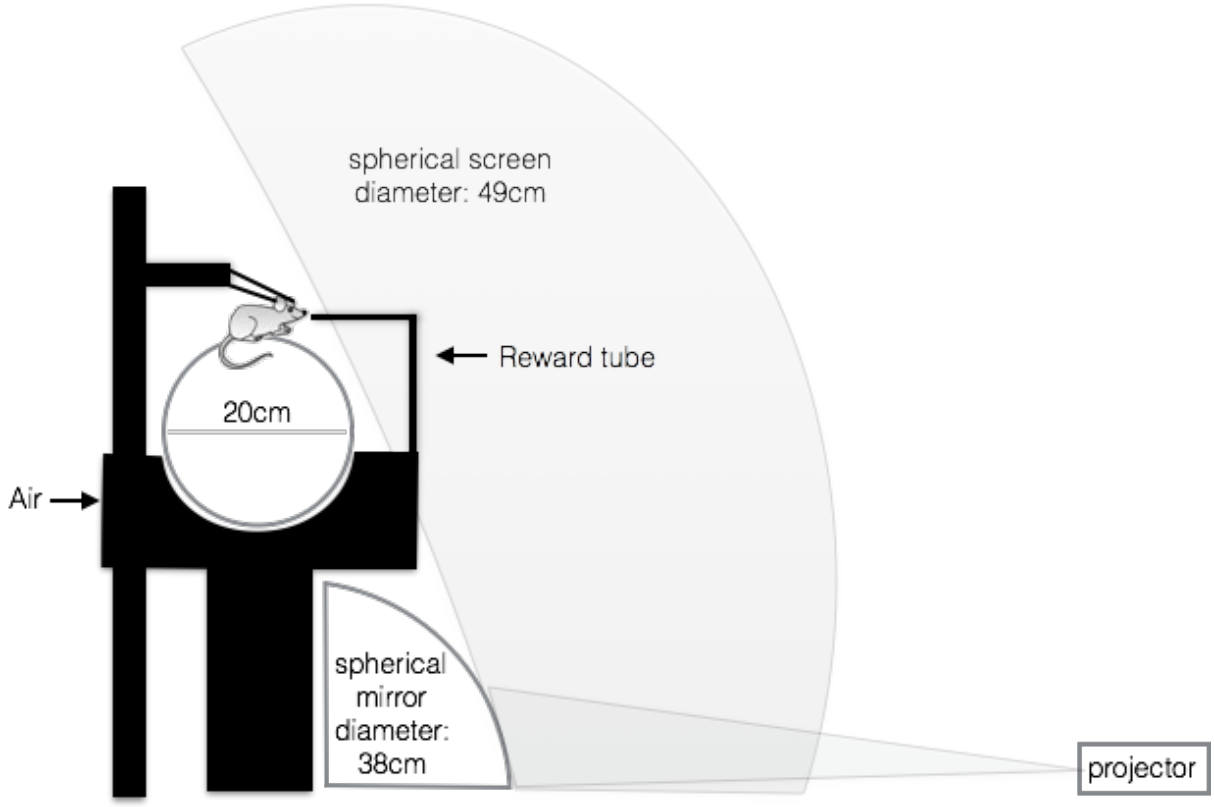

Figure 12. Set-up of the virtual-reality system. The mouse was head-restrained on an air-supported Styrofoam ball.

Surgical Procedure

Surgery was performed once the mice were eight weeks old. To implant the headplate, anaesthesia was induced using isoflurane, and maintained with a mixture of Ketamine (525µl) and Dextomidor (700µl) and saline (275µl), injected intraperitoneally (2.14µl per gram). After surgery, mice were injected with a 'wake mix' containing an α2-adrenergic antagonist (Antisedan, Zoetis; 1ml of Antisedan contains 5.0 mg atipamezole hydrochloride, 1.0 mg methylparaben (NF), 8.5 mg sodium chloride (USP), and water for injection). Analgesia was provided by adding rimadyl (Carpofen 5% with Ethanol 10%, Pfizer; 18.75mg per 300ml water) to the drinking water 2 days prior to surgery until 5 days post surgery. Additional local analgesia was provided during the surgery by treating the skin in the ears and on the skull with lidocaine cream (EMLA). An aluminum headplate (0.2 gram) was then implanted in the

following steps: First a circular portion of skin was removed on top of the skull, and the surrounding skin was glued to the skull with small amounts of superglue. The skull was then cleaned of tissue using a scalpel blade, and the headplate was attached using Superbond (Sun Medical Co., Ltd). In this case, since no additional chronic implants (e.g. imaging windows) were necessary, the open center of the headplate was simply covered using either more Superbond or a layer of Kwikcast (World Precision Instruments).

Pretraining Protocol

Before headplate implantation, mice were housed in groups in an enriched environment (High Makrolon cages with Enviro Dri bedding material and Mouse Igloo) and had free access to dry food and water. They were kept at a regular 12-12 day-night cycle with sunrise at 7.30 am. After headplate implantation, mice were kept overnight in a warming chamber (38.5°C) for recovery, and then transitioned to individual housing with a reversed 12-12 day-night cycle (sunrise at 7.30pm) and an added running wheel in the cage (Fast-Tracs). Mice were allowed at least 1.5 weeks of recovery. Five days prior to training, animals were put on a food restriction schedule designed to reduce their body weight by 20%. This entailed that mice received 2.0-2.5 grams of dry food per day. When mice reached 80% of their initial weight, they were habituated to handling by being put on a soft cloth for several minutes, and repeatedly immobilised ('squeezed') gently for a few seconds and then fed with soymilk (3.05 gram soy powder and 0.3 gram sugar dissolved in 20 ml water) from a pipette. When animals showed no sign of distress at being handled (after 1-3 handling sessions), task training began. During the weekends, the amount of food was elevated for one day and then again lowered; this was done to keep up the mice's metabolism. One hour before each training session, mice were additionally water deprived. The discomfort of the mice was kept as minimal as possible.

Training Protocol

In this task, mice were initially presented with a grey target wall located in the centre of the virtual environment and once they moved toward the target, they crossed an invisible trigger zone and the target moved either to the left (40% of trials), centre (20%) or right (40%), displaying a circular sinusoidal grating (see Figure 13). Mice were trained to move toward horizontally oriented circular sinusoidal gratings and to avoid vertically oriented circular sinusoidal gratings. The training consisted of several stages, programmed using Blender. The first training stage consisted of a dark corridor (with walls on both sides) where the target appeared as soon as mice initiated (by moving) the (invisible) trigger zone. Whenever the mice ran through the target, a reward was immediately delivered to the tube in front of the mouse's mouth. As soon as the mice learned to run independently (without assistance of the researcher) on the ball, the second training stage was started. During the second training stage, the corridor was removed and the target was freestanding. When the target appeared (initiated by running), the mice had to run straight to get the target and the associated reward. The third training stage was started once mice learned to run straight. In the third stage the trials, targets were again freestanding and moved to different locations: to the left, right and middle of the screen. When mice learned to steer to either the left/right or to run straight (middle target) and no biases to one of the sides were present, the fourth training stage was started. In this stage, a low-contrast distractor was added and targets moved either to the left, center or right. Mice had to learn that running through this distractor was associated with a punishment: an 'air puff' tone was played and to initiate the next trial, mice had to run through a dimly lit punishment corridor (time-out). In the fifth stage, the distractor had the same contrast as the target and multiple stimulus difficulties were implemented. The distractor and target got progressively more similar by changing the difference in degree of the distractor and target (initially 90° difference, the difference can be lowered to 30° in this

stage). Again, if the mouse had hit the distractor, a punishment tone was heard and a time-out (corridor) was induced. In the last stage of the training the difference in degree of the target and distractor was lowered until 5° (see Figure 12). During all stages of training a tone was heard at the beginning of each trial to indicate the availability of reward for a correct response. The mice learned that at the beginning of trials they needed to run straight, otherwise the grey wall would not start moving and the target would not appear. This was done to ensure that mice would only decide for one direction once the target and distractor appeared. From the fifth stage of training on, another type of trials was added: attention trials, which are trials that started with a second tone (attention cue), indicating the availability of more reward. These were speed trials, meaning that whenever the mice ran fast enough through the target, twice or even triple (for very fast performance) the amount of soymilk as in a normal trial would be given. However, the same was true for punishment, if the distractor was hit, a twice as long punishment tone and time-out were given.

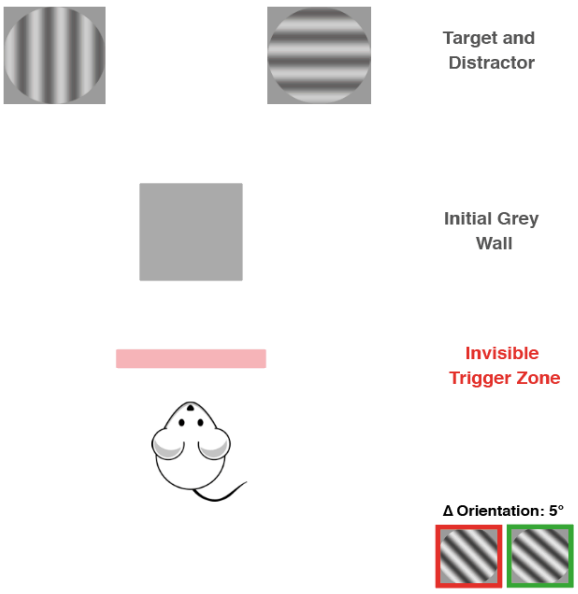

Figure 13. Schematic representation of the task. When the mouse crossed and invisible trigger zone, a zero-contrast target at the centre moved to the left, middle or right and displayed a circular grating stimulus. The targets were the more horizontal gratings. Distractors always moved in the opposite direction of the target. In the

right bottom of the figure an example of the hardest stimulus difficulty is presented, with a 5° orientation difference between target and distractor (green framed one is correct).

Data Analysis

Data were initially processed with analyses designed by M. N. Havenith (Havenith, Zijderveld, van Heukelum, Tiesinga & Glennon, in preparation). A number of different behavioural read-outs are possible, divided into three categories: accuracy, speed and motivation/expectancy. For accuracy, we calculated a hit index (1 for correct trials, 0 for undecided trials, and -1 for incorrect trials) and the lateral distance from the target at the end of the trial (target distance). Reaction time and the moment the mice reached the goal (target or distractor) were used as measures for speed. To do so, we first assessed for each trial the most abrupt change in running direction, in other words the point when the mice first decided to move in one specific direction and this point was then defined as reaction time. From this point on, we calculated at what moment mice reached the goal, defined as goalreachtime. Licking behaviour and running speed were used as measures for motivation and expectancy. Concerning licking behaviour, we calculated the average timing of licks compared to the moment the mice reached the target (lick position). This was used as a measure of expectation and confidence; a mouse that knows the task will already lick before reaching the target, expecting an upcoming reward. Running speed can be used to investigate the mice's motivation to receive the reward as fast as possible.

To investigate attention, we computed the measures of the three categories for attention trials and compared it to baseline trials without an attention cue across the different stimulus difficulties, generating psychophysical tuning curves. We decided to not look at all the different difficulties individually but instead looked at 90° difference, 70-50° difference, 30-20° difference and 10-5° difference, due to a low number of trials for some of the difficulties. Data were analyzed with a 2-factor ANOVA (factor 1 = cue/no cue, factor 2 =

stimulus difficulty). This was done for the data on C57BL/6 mice and these data were then used as a 'baseline' to which we compared the data of the first BALB/cJ mouse. We did the same for no switch and switch trials to examine cognitive flexibility (for the 2-factor ANOVA: factor 1 = switch/no switch, factor 2 = stimulus difficulty).

Preliminary Results: Learning

The first BALB/cJ mouse we tested needed overall more trials to learn all the stages of training (see Figure 14). The most striking difference seems to be in stage 5, where the full contrast distractor is introduced. The BALB/cJ mouse needed more trials to complete this training stage than the C57BL/6 mice.

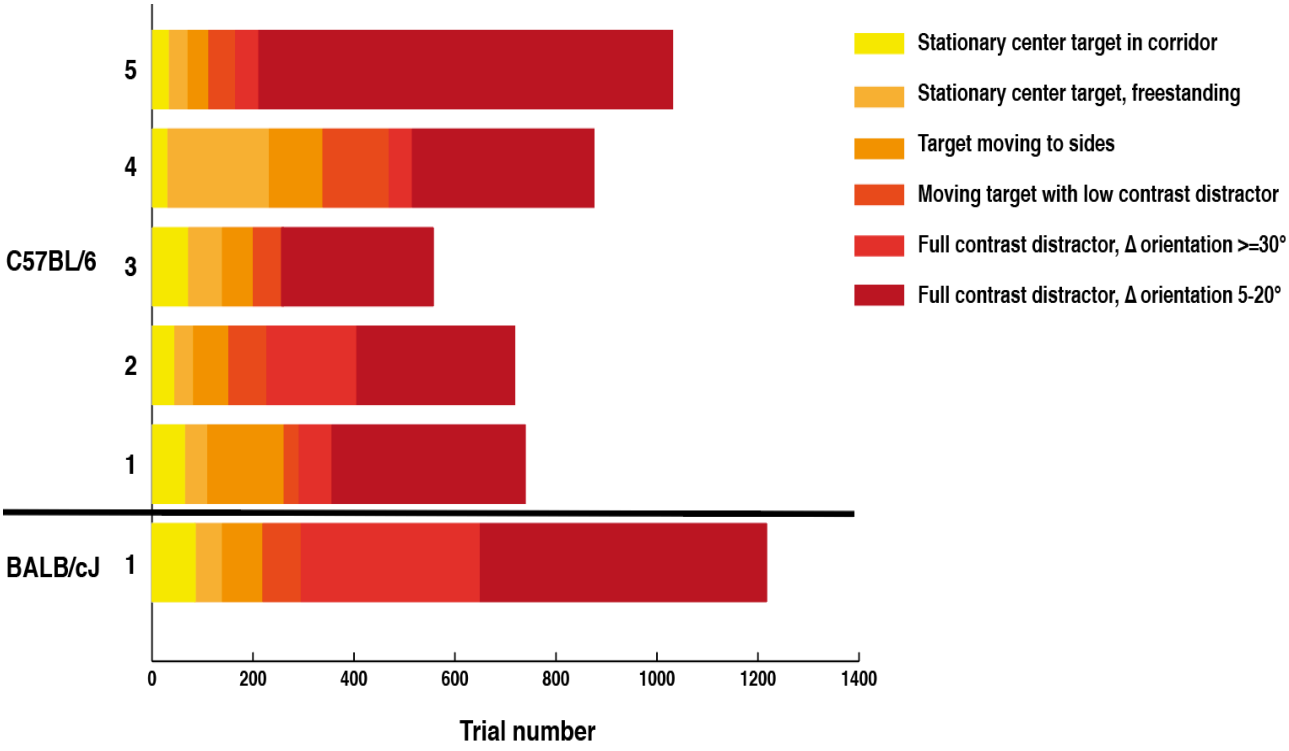

Figure 14. Diagram showing the number of trials mice needed for the different training stages. C57BL/6 mice needed overall less time to complete all training stages than the tested BALB/cJ mouse; this difference is most obvious for training stage 5.

Preliminary Results: Attention

Accuracy. C57BL/6 mice did not show difference in hit index between attention and baseline trials, but hit index decreased with stimulus difficulty (p = .041). Their distance to the target decreased in attention trials compared to baseline trials (cue: p = .002). When we compared the first BALB/cJ mouse to these data, it seemed that there was no difference for hit index; however, the BALB/cJ mouse showed the reversed pattern for target distance (increase in attention trials compared to baseline trials, see Figure 15).

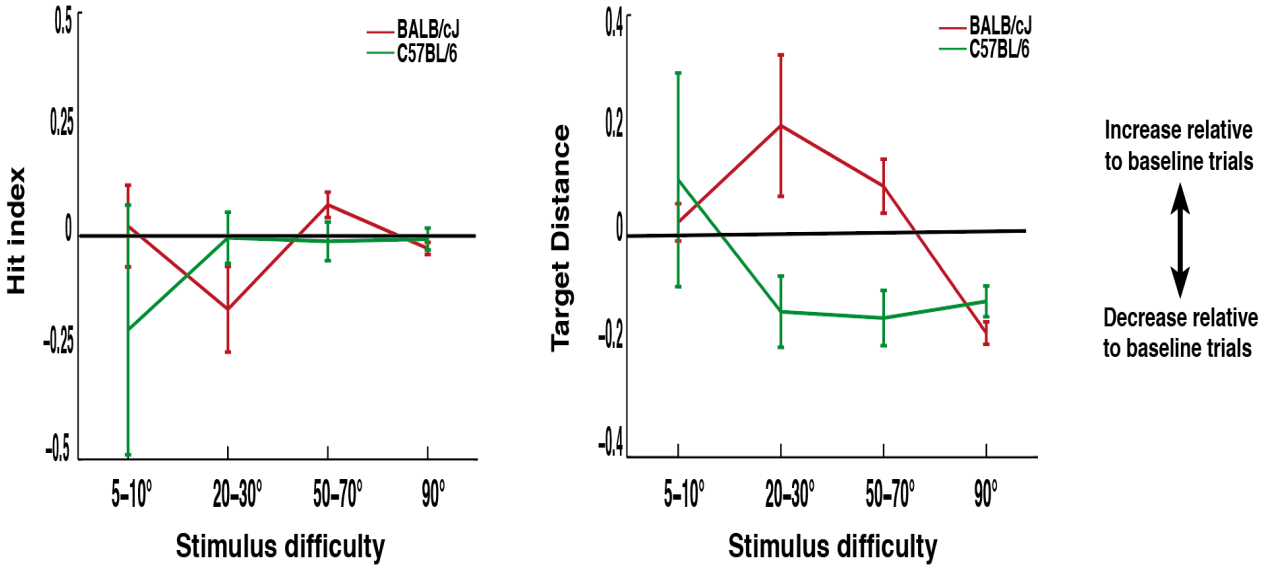

Figure 15. Tuning curves for hit index and target distance. There was no difference in hit index for attention trials compared to baseline trials in C57BL/6 mice but there was a decrease in target distance for attention trials. The BALB/cJ mouse appeared to show the reversed pattern for target distance but there appeared to be no difference in hit index.

Speed. There was no difference in reaction time between attention and baseline trials for C57BL/6 mice but the mice reached the goal earlier in attention trials (cue: p = .07). Compared to C57BL/6 mice, the BALB/cJ mouse tended to reach the goal later in attention trials but there seemed to be no difference for reaction times (see Figure 16).

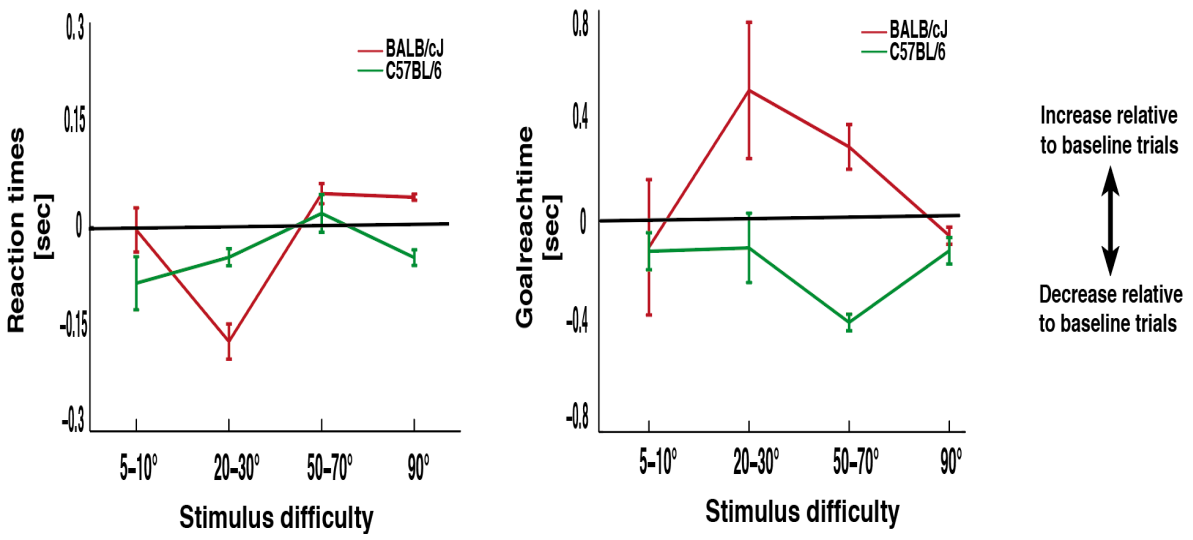

Figure 16. Tuning curves for reaction time and goalreachtime. There was a decrease in goalreachtime for attention trials compared to baseline trials in C57BL/6 mice. The BALB/cJ mouse appeared to show the reversed pattern for goalreachtime but there appeared to be no difference in reaction time.

Motivation/Expectancy. There were no differences for running speed and lick location between attention and baseline trials in C57BL/6 mice. It seemed that the BALB/cJ mouse ran slower and licked later in attention trials compared to baseline trials (see Figure 17).

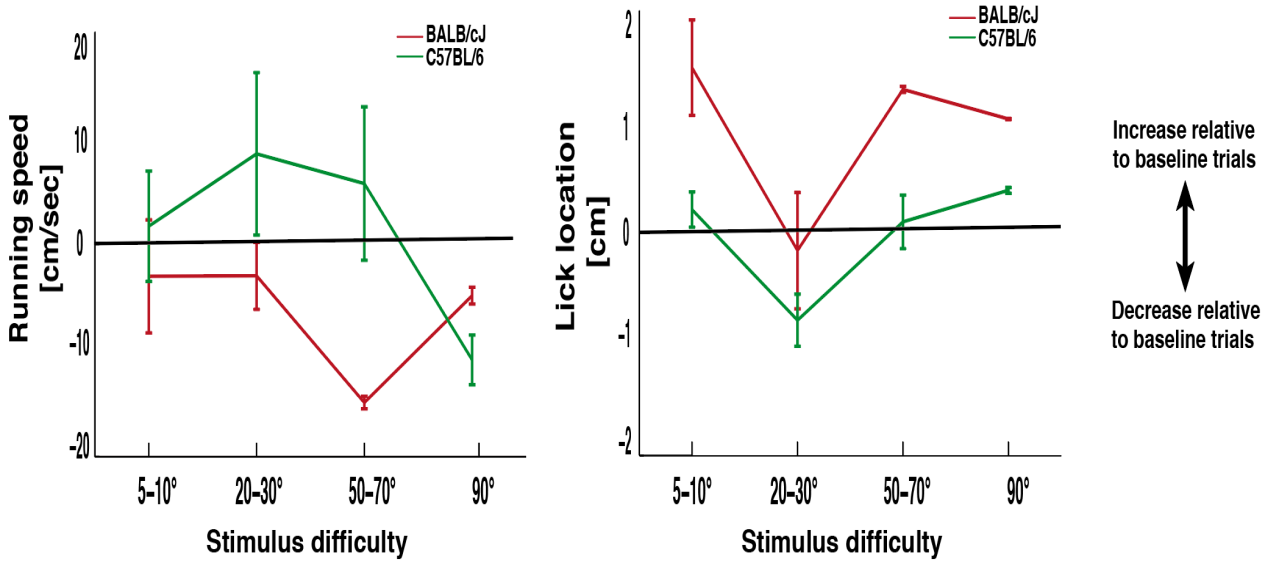

Figure 17. Tuning curves for running speed and lick location. There were no difference in running speed and lick location for attention trials compared to baseline trials in C57BL/6 mice .The BALB/cJ mouse seemed to lick later and to run slower in attention trials.

Preliminary Results: Cognitive Flexibility

Accuracy. For C57BL/6 mice, the hit index was lower (switch: p = .005) in switch trials compared to baseline trials, and hit index decreased with increasing stimulus difficulty (p = .03). There was no difference in target distance, neither for switch nor stimulus difficulty. There seemed to be no difference between the BALB/cJ mouse and the C57BL/6 mice for target distance, but the BALB/cJ mouse tended to show inconsistent behaviour for hit index across the different stimulus difficulties (see Figure 18).

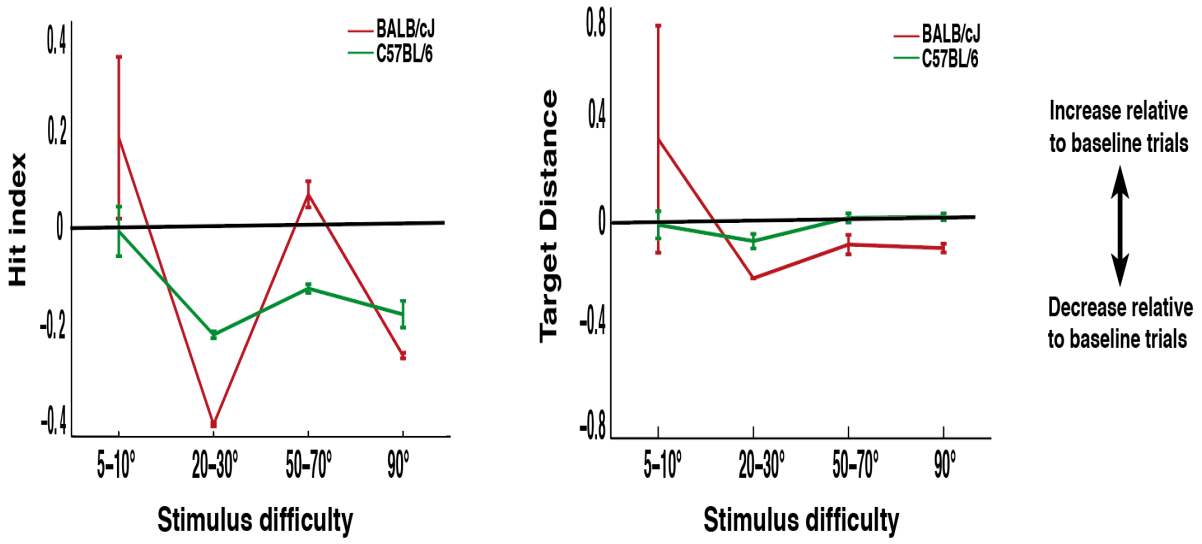

Figure 18. Tuning curves for hit index and target distance. There was no difference in target distance for attention trials but there was a decrease in hit index for switch trials (C57BL/6). The BALB/cJ mouse appeared to have a very inconsistent hit index for switch trials across the different stimulus difficulties.

Speed. C57BL/6 mice reacted slower in switch trials compared to baseline trials (switch: p = .02). There was no difference in goalreachtime between switch and baseline trials. For the BALB/cJ mouse there seemed to be an increase in goalreachtime and a decrease in reaction time for the easier stimulus difficulties (see Figure 19).

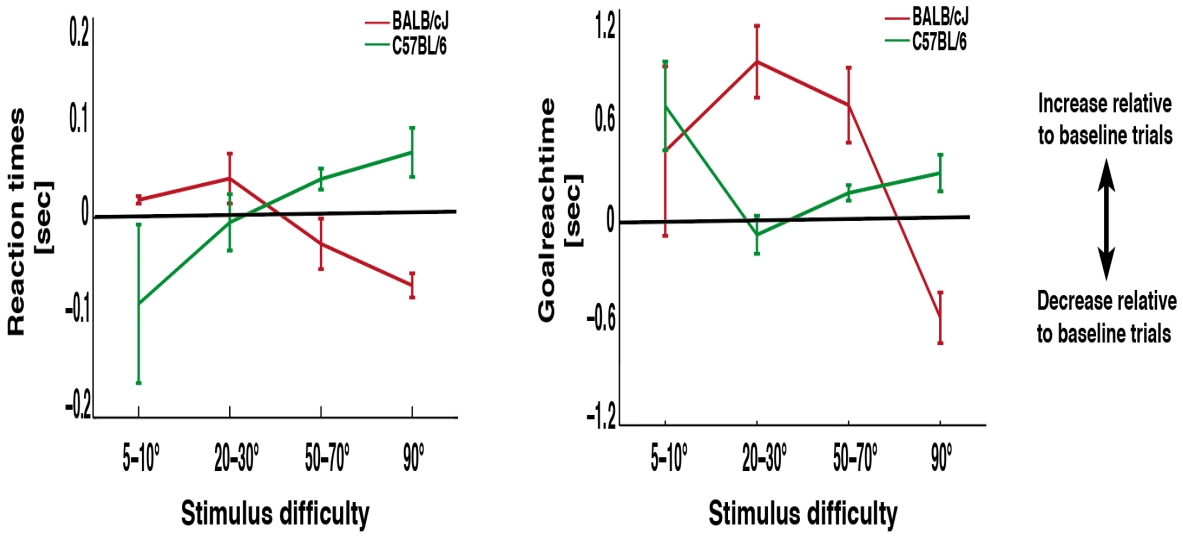

Figure 19. Tuning curves for reaction time and goalreachtime. There was an increase in reaction time for switch trials compared to baseline trials in C57BL/6 mice. The BALB/cJ mouse appeared to reach the goal later in switch trials but seemed to react faster for the easier stimulus difficulties.

Motivation/Expectancy. C57BL/6 mice ran faster in switch trials compared to baseline trials (switch: $p = .01$). The mice licked later in the most difficult trials (stimulus difficulty: $p = .08$) but this effect seemed not to be present in the BALB/cJ mouse. The BALB/cJ mouse tended to show a very inconsistent running speed for the switch trials across the different stimulus difficulties (see Figure 20).

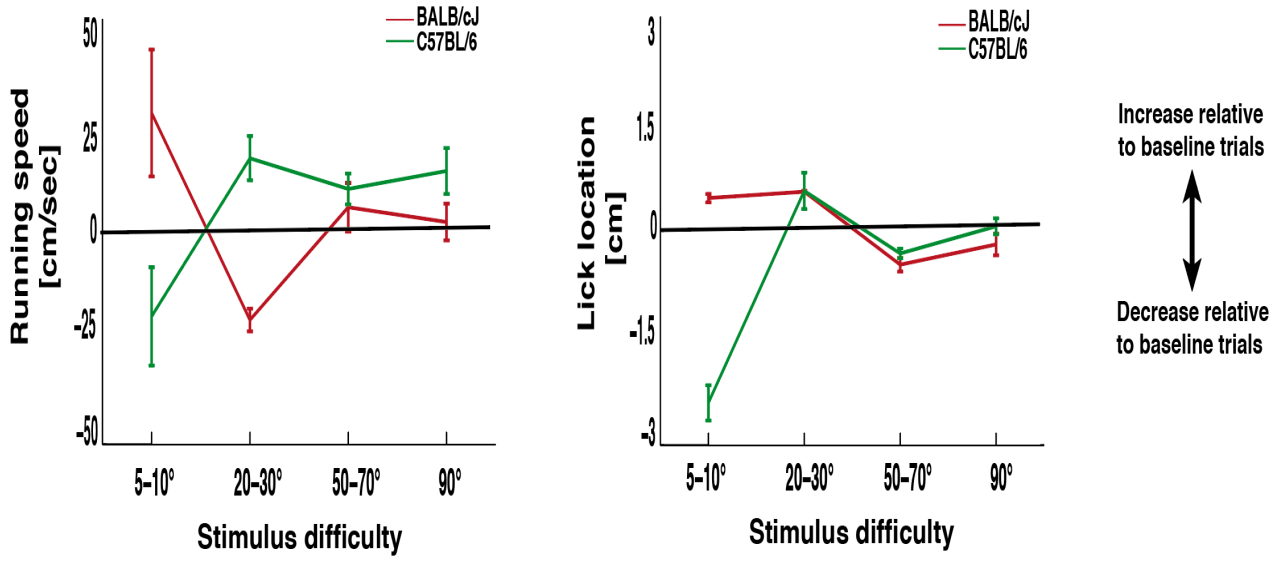

Figure 20. Tuning curves for running speed and lick location. C57BL/6 mice ran faster in switch trials compared to baseline trials and licked later for the most difficult trials. The BALB/cJ mouse tended to show inconsistent running speeds for switch trials across the different difficulties.

Discussion

In experiment 4, we investigated if BALB/cJ mice show cognitive impairments, as observed in children with CD (Blair, 2013). More specifically, we tested for deficits in learning, attention and cognitive flexibility. To do so, we utilized a virtual reality environment for head-restrained mice and trained the mice on a visual discrimination task (Havenith et al., in preparation). We had a number of behavioural measures, divided into the categories accuracy, speed and motivation/expectation. It has to be noted that we only managed to train one BALB/cJ mouse in time and that our control group consisted of C57BL/6 mice, which might not be a proper control group for BALB/cJ mice. We also need to note that we tried to train three other BALB/cJ mice in the virtual environment. These mice were 15-weeks old and had been housed isolated for more than eight weeks. They were more difficult to handle and showed aggressive behaviour during task performance (i.e. tail rattle), and they refused to run on the ball. We suppose that their age and their increased levels of aggression were the reasons for their inability to perform the task. In C57BL/6 mice we see that with age it becomes more difficult to train them and it might be that this effect is even stronger in BALB/cJ mice, especially due to their high levels of aggression. In future experiments we will again train younger BALB/cJ mice to test whether age is an important factor for BALB/cJ mice.

Yet, we still found some indications for cognitive impairments in BALB/cJ mice. We demonstrated that the BALB/cJ mouse needed overall more time to learn the task, especially during the stage when the full contrast distractor was introduced. In this stage, learning from punishment gets progressively more important as also the double punishment for attention

trials is introduced. These results seem to indicate a learning deficit in BALB/cJ mice but await further confirmation. In further experiments, it will also be necessary to disentangle whether this learning deficit is related to deficits in punishment processing or reward processing (or both). For example, we could train some mice on a protocol without punishment and other mice on a protocol with increased punishment to address this issue.

C57BL/6 mice showed a comparable hit index for attention trials compared to baseline trials. However, their distance to the target decreased, reflecting that they performed more accurately in attention trials. This also means that distance to the target was a more sensitive measure for accuracy than hit index. C57BL/6 mice also reached the goal earlier in attention trials compared to baseline trials, indicating that they understood the rule behind attention trials. The BALB/cJ mouse seemed to reach the goal later, lick later and have a larger distance to the target in attention trials. There seemed to be no difference in hit index for attention trials compared to baseline trials in the BALB/cJ mouse, meaning that changes in behaviour cannot be explained by overall worse performance of the BALB/cJ mouse in attention trials. It seems that the BALB/cJ mouse was less sure about his choices, as he licked later and reached the goal later. The differences between the BALB/cJ mouse and the C57BL/6 mice seemed not to be related to the different stimulus difficulties as the differences in behaviour were observed across the difficulties. These results point toward an attention deficit.

Cognitive flexibility was measured as the difference in performance between switch and no switch trials (baseline). For switch trials, C57BL/6 mice showed a decrease in hit index and an increase in reaction time. However, they ran faster in switch trials, which might indicate a trade-off between slower reaction time and higher running speed. Considering the fact that switch trials could also be attention trials at the same time, reacting later would mean less reward. Therefore, increasing the running speed might be a method to account for late reactions. Analysing switch trials with and without attention cue separately will enable us to

investigate this more closely. In general, the BALB/cJ mouse tended to show inconsistent behaviour for switch trials across the different stimulus difficulties. For example, the hit index of the BALB/cJ mouse for switch trials compared to baseline trials varied a lot across the different difficulties. This varied behavioural pattern was also observed for running speed. Only for goalreachtime there seemed to be a decrease in switch trials across all difficulties. This inconsistency across stimulus difficulties might indicate that cognitive flexibility in the BALB/cJ mouse is strongly affected by the difficulty of the task. BALB/cJ mice might experience difficult trials (i.e. differences =< 30°) as even more cognitively demanding in switch trials, resulting in their more variable performance. Testing more BALB/cJ mice in the virtual environment and comparing them to BALB/cByJ mice will show if the cognitive flexibility of these mice is indeed strongly affected by task difficulty.

From previous studies and also from work in our lab it is known that BALB/cJ mice show high levels of anxiety. Anxiety seemed to be a problem during the first session in the virtual environment (e.g. the mouse froze quite a long time before starting to run) but during later sessions anxiety did not seem to be a problem; however, it might be a good idea to measure levels of anxiety with heart rate recordings, for example before and after putting the mouse on the ball (Gaburro et al., 2011). Another point is the visual acuity of BALB/cJ mice; mice of the BALB/c strain have a lower visual acuity than C57BL/6 mice, which might influence their performance (Yeritsyan, Lehmann, Puk, Graw & Löwel, 2012). However, the BALB/cJ mouse learned the task, and as the target and distractor are randomly distributed across both left and right side, the mouse would have shown chance-level performance if he only ran to one side. Therefore, it seems that these mice have enough visual acuity to learn the task but testing BALB/cByJ mice and comparing their behaviour to BALB/cJ mice will give us more information about this.

General Discussion

The current study served to characterize the behavioural and physiological phenotype of BALB/cJ mice. These mice are highly aggressive, and aggression is a cardinal symptom of CD (Velez et al., 2010; Blair, 2013). However, it was unknown whether BALB/cJ mice also demonstrate symptoms of ADHD and ASD, common comorbidities of CD, as well as changes in physiological arousal (low or high heart rate) and cognitive impairments, as observed in CD. Therefore, we further characterized the behavioural and physiological phenotype of BALB/cJ mice and designed four experiments that enabled us to explore if BALB/cJ mice show symptoms of ASD, ADHD and CD.

In experiment 1, we examined social withdrawal, a prominent symptom of ASD. We designed a one-chambered apparatus that closely resembled the homecage and demonstrated that BALB/cJ mice are less interested in a stimulus mouse than BALB/cByJ mice, a behaviour interpreted as social withdrawal. In experiment 2, we implanted a transmitter to examine whether BALB/cJ mice show hyperactivity and decreased core body temperature, symptoms of ADHD and we demonstrated that BALB/cJ mice show hyperactivity and a lower (nocturnal) body temperature. As the transmitter also measured heart rate, experiment 3 tested whether BALB/cJ mice show changes in heart rate, which are observed in children with CD. We could not find any differences in baseline heart rate between BALB/cJ mice and their control group, BALB/cByJ mice. However, given their high locomotor activity, BALB/cJ mice showed a relatively low heart rate. In experiment 4 we focussed on cognitive impairments, more specifically, a deficit in learning, attention and cognitive flexibility. To do so, we trained mice on a visual discrimination task in a virtual environment. Preliminary results indicated deficits in learning and attention and some changes in cognitive flexibility, but the results await further confirmation.

Our results show that BALB/cJ mice not only demonstrate increased aggression, a core symptom of CD, but also symptoms of common comorbidities such as ASD and ADHD in form of social withdrawal, hyperactivity and a lower body temperature. We also demonstrated that BALB/cJ mice seem to show underarousal in form of a low heart rate and found indications for cognitive impairments, as observed in CD. One might argue that the observed symptoms also appear in other diseases. For example, deficits in cognitive flexibility are observed in a number of diseases, ranging from depression to obsessive-compulsive disorder (Fossati, Ergis & Allilaire, 2002; Chamberlain, Fineberg, Blackwell, Robbins & Sahakian, 2006). However, it is highly likely that psychiatric disorders share partly similar origins, which would also explain the high comorbidity of several disorders (Pettersson, Larsson & Lichtenstein, 2016). Therefore, we argue that we highlighted a particular constellation of symptoms, and that the BALB/cJ model seems to be well suited to study brain structures that might underlie the linked symptoms of CD, ADHD and ASD. Ultimately, this could aid in the discovery of new treatments for children and adolescents that suffer from CD with comorbid ADHD and/or ASD.

A Candidate Brain Structure For the Link Between CD, ADHD and ASD

A brain structure that mediates the link between CD, ADHD and ASD could be the ACC. The ACC is highly preserved across mammals and regulates cognitive processes ranging from sensory to autonomous functions (Barbas & Zikopoulos, 2007). The ACC can be subdivided into a dorsal and a ventral part. These subdivisions have been viewed as functionally segregated, with dorsal ACC being involved in cognitive control and ventral ACC being involved in emotional control (Etkin, Egner & Kalisch, 2011). However, ventral and dorsal ACC are also jointly involved in emotion and cognition, as well as being highly interconnected anatomically (Jones, Groenewegen & Witter, 2005). In line with these observations, CD, ADHD and ASD occur with high comorbidity and all three disorders show

an involvement of the ACC (Blair, 2013; Bledsoe et al., 2013; Di Martino et al., 2009). Furthermore, the ACC has extensive projections throughout the whole brain and projects to prefrontal regions (attention and social deficits), the striatum (hyperactivity), and hypothalamus (body temperature), regions that have been implicated in the symptoms of CD, ASD and ADHD (Blair, 2013; Bledsoe et al., 2013; Di Martino et al., 2009).

As it is known, that 75% of the mRNAs that have been found to be differentially expressed in BALB/cJ mice and BALB/cByJ mice, relate to GABAergic functioning, it would be interesting to investigate the role of GABA-ergic signalling in ACC in BALB/cJ mice. In a future study, we want to examine local GABAergic populations within both dorsal and ventral ACC circuits, manipulate them in behaving mice and observe the effects on behaviour. This will be performed in the virtual environment, which we already used to test cognitive performance in BALB/cJ mice. We are particularly interested in the role of the ACC in attention, as our preliminary results indicate that BALB/cJ mice show an attention deficit and we hypothesize that attention might mediate the linked symptoms of CD, ADHD and ASD.

Attention Might Mediate the Link Between CD, ADHD and ASD

Children with ADHD that develop CD, show a considerably earlier onset of CD symptoms and more severe symptoms than children with CD only (Loeber et al., 2000). Furthermore, children with ASD that also receive a diagnosis of ADHD, have a higher chance to develop CD (Montes & Halterman, 2007). This suggests that symptoms of ADHD, like impulsivity, hyperactivity or inattention, might play a role in the link between CD, ADHD and ASD. The focus has long been on impulsivity, as high levels of impulsivity in children with ADHD or CD contribute strongly to the risk of criminal involvement, even more than early symptoms of CD alone (Babinski, Hartsough & Lambert, 1999). However, to date it is

unknown if high levels of impulsivity are a common cause of ADHD, ASD and CD, explaining the high comorbidity of these three disorders.

More recently, the focus has been shifted toward inattention as a possible mediator of the relation between CD, ADHD and ASD. Attention enables us to selectively concentrate on certain aspects of information, suppressing distracting or irrelevant information (Kim, Ährlund-Richter, Wang, Deisseroth & Carlén, 2016). Social situations in general, and even more so ambiguous social situations, require high levels of attention. If a person is non-attentive to subtle cues during interactions (e.g. tone and facial expression), situations can be interpreted as hostile leading to an (unprovoked) outburst of aggression (Evans et al., 2015). It is known that patients with ASD, ADHD as well as CD have difficulty understanding social cues and that they tend to interpret ambiguous social situations as hostile (Evans et al., 2015). It might be that being inattentive to subtle cues leads to a misinterpretation of a situation, which results in aggressive behaviour. Indeed, causal modelling in a population of ADHD patients suggests that inattention is causal to aggression (Heskes, unpublished data). This also implies that inattention should precede aggression, being in line with the fact that children with ADHD, who develop CD, have an earlier onset of CD symptoms than children with CD only.

The Prefrontal Cortex and Attention

The prefrontal cortex, intraparietal cortex and temporoparietal cortex are generally seen as 'the' brain areas involved in attentional control (Corbetta & Shulman, 2002). Intraparietal cortex and the dorsal part of prefrontal cortex (dorsolateral prefrontal cortex – dlPFC) are more involved in top-down attention, the preparation and application of a goal-directed selection for stimuli and responses. vmPFC and temporoparietal cortex are specialized in detecting behaviourally relevant stimuli, especially if these stimuli are salient or unexpected (Corbetta & Shulman, 2002). Recently, Kim et al. (2016) demonstrated that

gamma-Aminobutyric acid (GABA-ergic) interneurons expressing parvalbumin (PV) in prefrontal cortex are involved in sustaining and directing attention. Mice performed a 3-choice-serial reaction-time task (modification of the 5-choice-serial reaction time task) and neuronal responses in prefrontal cortex were recorded. In the 3- (or 5) choice task, the animal needs to sustain and divide its attention across a row of three (or five) screen locations to detect and respond to a brief visual stimulus in order to receive a reward. At the start of each trial the activity of the PV neurons increased and this heightened activity was sustained during the whole delay period (i.e. until presentation of the stimulus). The activity increased even more when the animal was about to perform correctly. This means that a high and sustained activity of PV neurons at the start of a trial predicts whether the animal will perform correctly, more than 2.5 seconds before presentation of the stimulus. The activity of the PV neurons neither correlated to the motivational state of the animal or motor behaviour and it can be concluded that prefrontal PV neurons are involved in attentional control.

Patients with ADHD and patients with ADHD and cormorbid ASD and/or CD show decreased activity during tasks involving attentional processing (e.g. measures of sustained attention comparable to the 5-choice-serial reaction time task as used in rodents) in regions such as dlPFC and intraparietal cortex (Dickstein, Bannon, Castellanos & Milham, 2006). Reduced activation of dlPFC during attentional processing has also been observed in patients with CD only (Rubia et al., 2009). Furthermore, the dlPFC projects to vmPFC and ACC, brain areas that have been implicated in the control of aggressive behaviour and both ACC and vmPFC have been found to be hypofunctioning in ADHD, ASD and CD (Hare, Rakimi & Rangel, 2014; Blair, 2013). It is know that the connectivity between dlPFC and vmPFC is associated with computation of stimulus features (Hare et al., 2014). The connections between ACC and dlPFC are mostly excitatory, and important in the adaptation of behavioural responses (Medalla & Barbas, 2012). Possibly, a hypofunctioning dlPFC cannot communicate

efficiently with ACC and vmPFC about features of a stimulus and the necessity of adapting behavioural responses. ACC and vmPFC might then show attenuated activity and cannot exert control over the basic aggression circuitry running from medial amygdala to medial hypothalamus and to the dorsal half of the periaqueductal grey (Blair, 2013). An attention deficit (related to a hypoactive dlPFC) might lead to a misinterpretation of stimulus features (related to a hypoactive vmPFC) that in turn could lead to aggressive behaviour (less control over basic aggression circuitry). Subsequently, this aggressive behaviour will not be adapted because control mechanisms fail (hypoactive ACC).

Future Studies: The Role of ACC in Attention

As the ACC projects to and influences prefrontal regions, we hypothesize that PV neurons within ACC might influence attentional processing. When these neurons are dysfunctional, attentional deficits could arise and, as explained in the previous section, might give rise to symptoms of CD and ASD. With the BALB/cJ model we can investigate this hypothesis, by recording from ACC and by manipulating ACC during tasks that require attention and tasks that are focused on social and aggressive behaviour. Furthermore, we can tackle the hypothesis of inattention being causal to aggression. By administering an attention training to BALB/cJ and subsequently testing their aggressive behaviour, we can explore if trained mice show less aggression.

Conclusion

Here, we have demonstrated that aggressive BALB/cJ mice show social withdrawal, locomotor hyperactivity and decreased (nocturnal) body temperature, symptoms of ASD and ADHD, respectively. Furthermore we have shown that BALB/cJ mice seem to have a low heart rate (given their locomotor activity), which is a sign of underarousal, and we found indications for cognitive impairments in form of a deficit in learning, attention and cognitive flexibility. This means that BALB/cJ mice show symptoms of CD, ADHD and ASD,

recapitulating the clinical situation. Having validated the behavioural and physiological phenotype of BALB/cJ mice, we can use the model to study brain structures that might give rise to the linked symptoms of CD, ADHD and ASD.

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