Snap25 heterozygous knockout mice as a model for attention deficit/hyperactivity disorder (ADHD)

Heterozygote *Snap25* Knockout-Mäuse als Modell für Aufmerksamkeitsdefizit- / Hyperaktivitätssyndrom (ADHS)

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Julius-Maximilians-Universität Würzburg,

Section Neuroscience

submitted by

Antonia Post

from

Augsburg, Germany

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Submitted on: ……..……..

Members of the *Promotionskomitee*:

Chairperson: Prof. Dr. Michael Sendtner

Primary Supervisor: Prof. Dr. Klaus-Peter Lesch

Supervisor (Second): Prof. Dr. Paul Pauli

Supervisor (Third): Prof. Dr. Erhard Wischmeyer

Date of Public Defence: ……..……

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Zusammenfassung

SNAP25 (Synaptosomal assoziiertes Protein, 25 kDa; Teil des SNARE Komplexes) ist an der Fusion von synaptischen Vesikeln mit der präsynaptischen Zellmembran beteiligt, und somit notwendig für die Regulation der Neurotransmitter-Ausschüttung. Außerdem wird eine wichtige Funktion bei dem Wachstum von Axonen und synaptischer Plastizität diskutiert. In Humanstudien wurden wiederholt verschiedene Einzelnukleotid-polymorphismen von *SNAP25* mit Aufmerksamkeitsdefizit- / Hyperaktivitätssyndrom (ADHS) assoziiert. In der vorliegenden Studie wurden heterozygote *Snap25* knockout Mäuse als Modell für ADHS untersucht.

Heterozygote (+/-) *Snap25* knockout Mäuse und ihre wildtypischen Wurfgeschwister wurden unter Kontrollbedingungen großgezogen oder einer maternalen Separation (MS) unterzogen. Beginnend im Alter von etwa 2 Monaten wurden diese Mäuse verschiedenen Verhaltenstests unterzogen: in einem wiederholten Langzeit-Open-Field (OF) Test wurde Aktivität untersucht, Aufmerksamkeitsdefizite und Impulsivität mit dem 5 Choice Serial Reaction Time Task (5CSRTT), angst-ähnliches Verhalten in der Light-Dark Box (LDB) und depressions-ähnliches Verhalten im Porsolt Forced Swim Test (FST). Die Gehirne dieser Mäuse wurden anschließend auf die Expression verschiedener ADHS bezogener Gene in einer quantitativen Real-Time-PCR (qRT-PCR) untersucht. Eine zusätzliche Gruppe weiblicher Mäuse (+/+; +/-) durchlief einen einstündigen OF Test nach oraler Gabe von 45 mg/kg Methylphenidat (MPH) oder Placebo.

Um eine optimale Dosierung für MPH in diesem Experiment zu finden, wurde eine Pilotstudie durchgeführt. Hierbei wurden wildtypische C57/BL6 Mäuse in einem Langzeit OF Test mit Gabe unterschiedlicher Dosierungen von MPH, sowohl oral als auch intraperitoneal (i.p.), untersucht. Im Anschluss wurden die Gehirne dieser Tiere auf Neurotransmitter-konzentration geprüft. Diese Pilotstudie ergab als optimale Dosierungen von MPH auf Verhaltensebene 7.5-15 mg/kg i.p. und 30-60 mg/kg oral. Allerdings waren die neurochemischen Effekte der beiden unterschiedlichen Applikationsarten größtenteils verschieden.

In der *Snap25* Studie zeigten ungestresste Kontroll-Tiere einen leicht hyperaktiven Phänotyp in dem zweiten von zwei Langzeit-Open-Field Tests (60 min) im Abstand von 3

Wochen. Bei Betrachtung aller Gruppen ergab sich auch eine signifikante Interaktion von Stress und Genotyp in der zweiten Testung, und zwar dahingehend, dass MS Tiere grundsätzlich aktiveres Verhalten zeigten, ohne Genotypen-Unterschiede. In der Anfangsphase des 5CSRTT lagen nur signifikante Haupteffekte für Stress vor, gestresste Tiere hatten größere Probleme im Meistern der Aufgabe als Wildtypen. Erst im sogenannten Test-Trial am Ende der Versuchsreihe ergaben sich signifikante Haupteffekte für den Genotyp. Heterozygote *Snap25* knockout Mäuse zeigten beispielsweise weniger korrekte Reaktionen und konsumierten auch weniger Belohnungspellets direkt im Anschluss an eine korrekte Reaktion als Wildtypen. In der LDB brauchten +/- Mäuse wiederum weniger Zeit als Wildtypen, um den erleuchteten Teil der Arena zu betreten, und zeigten dadurch ein reduziertes Angst-ähnliches Verhalten. Im Gegensatz dazu ergab sich ein erhöht Depressions-ähnliches Verhalten für männliche heterozygote *Snap25* knockout Mäuse im FST. Auf der Genexpressions-Ebene hatten +/- Mäuse niedrigere Expressionslevels von *Maoa* und *Comt* und höhere Expressionslevels von *Nos1* als Wildtypen. Abschließend zeigte sich eine erhöhte Reaktion auf MPH bei heterozygoten Mäusen.

Zusammenfassend zeigen heterozygote *Snap25* knockout Mäuse einige Charakteristika von ADHS auf Verhaltensebene, wie zum Beispiel eine leichte Hyperaktivität in bekannter Umgebung, Schwierigkeiten im Erlernen einer gestellten Aufgabe und sogar Verhaltensweisen, die auf eine Abneigung gegenüber Verzögerungen hindeuten. Zusätzlich kommt es aufgrund des Knockouts zu veränderten Expressionslevels verschiedener ADHS assoziierter Gene. Auch wenn die erhöhte Verhaltensreaktion von +/- Mäusen auf MPH nicht die erwartete Reaktion eines ADHS Modells darstellt, deutet sie dennoch auf ein Ungleichgewicht des dopaminergen Systems im Gehirn hin, das bei ADHS eine wichtige Rolle spielt.

Summary

SNAP25 (Synaptosomal-Associated Protein of 25 kDa; part of the SNARE complex) is involved in the docking and fusion of synaptic vesicles in presynaptic neurons necessary for the regulation of neurotransmitter release, as well as in axonal growth and synaptic plasticity. In humans, different single nucleotide polymorphisms of *SNAP25* have repeatedly been associated with attention deficit/hyperactivity disorder (ADHD). Thus, in this study heterozygous *Snap25* knockout mice were investigated as a model of ADHD.

Heterozygous (+/-) *Snap25* knockout mice as well as their wild-type (+/+) littermates were reared under control conditions or underwent a Maternal Separation (MS) procedure. Starting at the age of 2 months, mice were tested for locomotor activity in a repeated long-term Open Field (OF) task, for attention deficits and impulsive behavior in the 5 Choice Serial Reaction Time Task (5CSRTT), for anxiety-like behavior in the Light-Dark Box (LDB) and for depression-like behavior in the Porsolt Forced Swim Test (FST). The brains of these mice were subsequently tested for the expression of several ADHD related genes in a quantitative Real-Time PCR (qRT-PCR) study. Another group of female mice (+/+; +/-) underwent a one hour OF test after oral administration of 45 mg/kg Methylphenidate (MPH) or placebo.

To find an optimized dosage for this MPH challenge, a pilot study was performed. Wild-type C57BL/6 mice were tested in a long-term OF with several dosages of MPH both intraperitoneally (i.p.) and orally. The brains of these animals were afterwards investigated for neurotransmitter concentrations. In this pilot study the dosages of MPH that were similarly behaviorally effective without causing symptoms of overdosing were 7.5-15 mg/kg intraperitoneally and 30-60 mg/kg orally. However, even though it was possible to find intraperitoneal and oral doses that correlate behaviorally, the neurochemistry was mostly different.

In the study on *Snap25*-deficient mice, unstressed controls showed a hyperactive phenotype in the second of two long-term OF sessions (60 min) spaced three weeks apart. Considering all groups, there was a significant interaction of stress and genotype in the second session, with animals subjected to MS being overall hyperactive with no genotype differences. In the training phase of the 5CSRTT only effects of stress were found, with MS animals finding and consuming fewer rewards. In the single test trial, several genotype

effects became apparent, with tendencies for the number of correct nose pokes and the number of rewards eaten, and a significant effect for the number of rewards eaten directly after the correct response. In all of these variables +/- mice performed worse than their wild-type littermates. In the LDB +/- mice entered the lit compartment of the arena earlier than the controls, thus showing attenuated anxiety-like behavior. Regarding depressive-like behavior in the FST, male +/- mice spent significantly less time struggling than male +/+ mice. In the gene expression study, +/- mice had lower expression levels of *Maoa* and *Comt*, and higher expression levels of *Nos1* than wild-types. Finally, the locomotor activity response to MPH was exaggerated in +/- mice as compared to controls.

Heterozygous Snap25 knockout mice show some of the behavioral characteristics of ADHD, as for example a mild hyperactivity in a familiar environment, difficulties in the correct execution of a given task and even some behavior that can be interpreted as delay aversion. Additionally, expression levels of three ADHD related genes were changed in these animals. Although the exaggerated locomotor activity response to MPH is not to be expected of an ADHD model, the difference in the response between +/+ and +/- mice nonetheless implicates a potential dysfunction of the brain dopaminergic system.

1 Introduction

1.1 Animal models of psychiatric disorders

1.1.1 What is an animal model?

There are hardly any scientific definitions to be found of what constitutes an animal model, although the subject is much discussed and opinions are manifold. In 1984, William McKinney, who contributed much to the theoretical background of what today is perceived as good scientific practice in animal research, put it like this: “Animal models represent experimental preparations developed in one species for the purpose of studying phenomena occurring in another species” (McKinney, 1984). As simple as this definition sounds, it entails more than is initially obvious and still holds true today.

1.1.2 Criteria for good animal models

In 1969, McKinney and Bunney argued for the importance of finding an animal model of depression (McKinney & Bunney, 1969). In this paper, they also proposed set of criteria for animal models of human mental disorders in general, namely that the model should resemble the condition it models in its etiology, biochemistry, symptomatology and treatment. 15 years later, several possible animal models of depression had been published and Paul Willner reviewed them in relation to three sets of validating criteria that were based on the criteria proposed by McKinney and Bunney. According to Willner, a perfect animal model should fulfill 3 forms of validity. Predictive validity is assessed by whether a model correctly identifies pharmacological treatment with a comparable clinical potency and without making errors of omission or commission. Face validity is assessed by whether the model resembles the disorder in a number of respects. Finally, construct validity is assessed by whether both the behavior in the model and the features of the disorder can be unambiguously interpreted, and are homologous and whether the feature being modelled stands in an established empirical and theoretical relationship to the disorder (Willner, 1984). Although 30 years have passed since these criteria were proposed, every animal researcher in the world is aware of their importance today.

1.1.3 Genetic mouse models of psychiatric disorders

There are several ways to come by an animal model of a (psychiatric) disorder. One is to pharmacologically induce a certain phenotype, for example by injecting an animal with an

agonist or antagonist to a specific receptor in the brain which has been previously associated with a certain disorder (Hashmi et al., 2014). Another is to screen a population of animals for a specific phenotype, then to selectively breed the top and the bottom percentiles of this phenotype and to continue this for some generations (Carroll et al., 2008). However, the most common type nowadays is the genetic animal (or mouse) model, whose genome has been randomly (by chemical mutagenesis) or, more frequently, specifically altered for genes that have been associated with psychiatric disorders. This can either be done by adding another gene, thus making the animal transgenic, through microinjection into the male pronucleus of a fertilized mouse (McKnight et al., 1983), or by specifically inactivating a gene by targeting it through homologous recombination and thus producing a knockout mouse (Osada & Maeda, 1998).

1.2 *SNAP25* and its relevance for psychiatric disorders

1.2.1 Neurobiology of SNAP25

Snap25 was first discovered as a neuron-specific mRNA in the mouse brain and found to be predominately localized in nerve terminals (Branks & Wilson, 1986). The human *SNAP25* gene was first cloned in 1994 and found to be highly and specifically expressed in the adult brain (Zhao et al., 1994). SNAP stands for "synaptosomal associated protein" and the 25 for its atomic mass of 25 kDa. It codes for a 206 amino acid long SNARE protein (soluble NSF attachment protein receptor where NSF stands for N-ethyl-maleimide-sensitive fusion protein) and as such has been implicated in most intracellular membrane trafficking events studied so far (Chen & Scheller, 2001). Together with syntaxin and the vesicle-associated membrane protein (VAMP, also called synaptobrevin) it was one of the first SNARE proteins discovered. Chen and Scheller's (2001) model of exocytosis (Figure 1) states that after the dissociation of n-Sec1 from syntaxin (possibly mediated by Rab proteins), the binding of the three neuronal SNAREs syntaxin, VAMP and SNAP25 (localized at the presynaptic plasma membrane of neurons) can occur. Syntaxin, VAMP and SNAP25 are helical proteins and together form a heterotrimer, arranged in parallel (Sutton et al., 1998; Figure 2). Full zipping of the coiled-coil complex is triggered by Ca^{2+}, which results in membrane fusion and release of vesicle contents into the synaptic cleft. After the fusion event, the SNARE complex is dissociated and recycled (Figure 1).

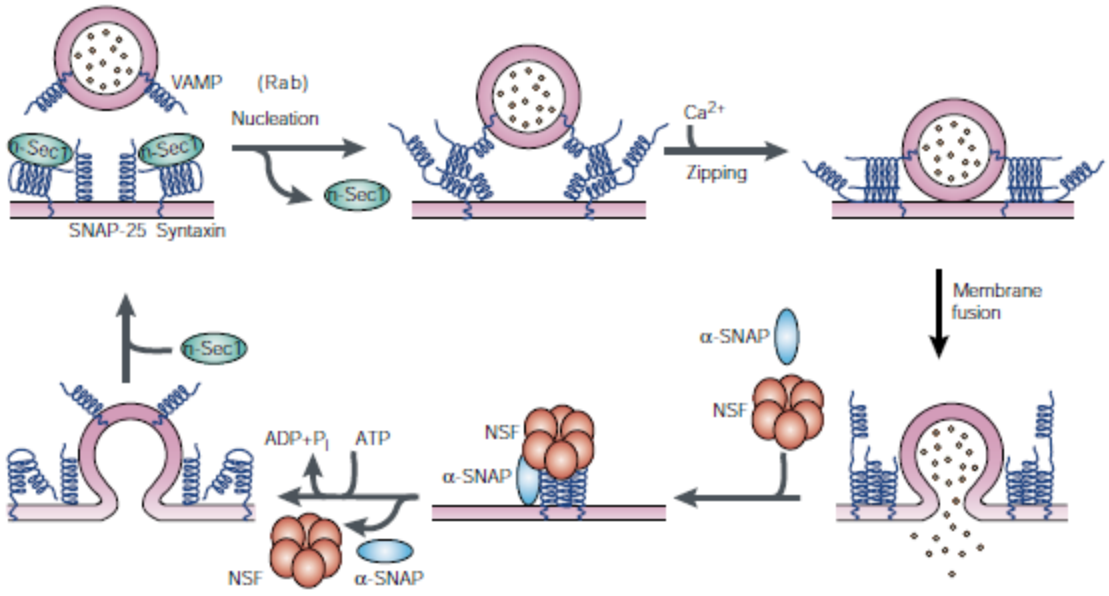

Figure 1: Molecular model of vesicle exocytosis (Chen & Scheller, 2001)

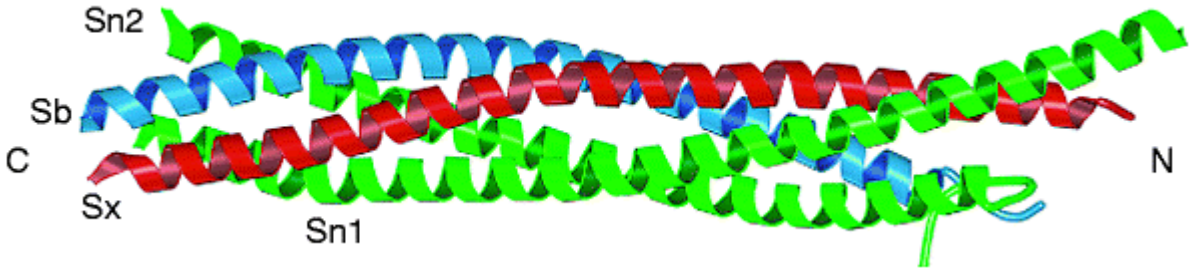

Figure 2: Backbone ribbon drawing of the SNARE complex; blue: VAMP; red: syntaxin; green: SNAP25b. From Sutton, Fasshauer, Jahn & Brunger, 1998.

There are two isoforms of SNAP25 which result from alternative splicing between the two exons 5a and 5b (Bark & Wilson, 1994). SNAP25a is found in earlier developmental stages, whereas SNAP25b is dominant in the adult brain (Bark et al., 1995). The two isoforms differ by nine amino acids, two of which alter the relative positioning of clustered cysteine residues that are sites for posttranslational fatty acetylation implicated in membrane anchoring (Bark et al., 1995;.Andersson et al., 2000). Membrane anchoring of SNAP25 is needed for the exocytosis functionality of the SNARE complex, which is why SNAP25b is the isoform that acts here.

1.2.2 Association of *SNAP25* with psychiatric disorders

Taking into account the important role of the SNARE proteins in neurotransmission, it is not surprising that the *SNAP25* gene, or rather a number of single nucleotide polymorphisms (SNPs) within the *SNAP25* gene, have repeatedly been associated with psychiatric disorders. Among others, schizophrenia (Lochman et al., 2013), Tourette syndrome (Gunther et al., 2012) and antisocial personality disorder (Basoglu et al., 2011) have been discussed to be connected to changes in *SNAP25*. Most prominently though, attention deficit hyperactivity disorder (ADHD) can be found linked to *SNAP25* in literature. Though there are studies that were not able to replicate such results (see for example Renner et al., 2008 in a German sample) or found only weak effects (Mill et al., 2005 in a sample from the UK) there are numerous published that verify a connection between *SNAP25* and the disorder. For example, positive association has been found in an Irish sample (Brophy et al., 2002), a Latin American sample (Gálvez et al., 2014), a Turkish (Pazvantoğlu et al., 2013) and a Canadian (Barr et al., 2000) sample. A study published in 2013 conducted on an Australian post mortem sample even found a reduced expression of *SNAP25* in the frontal cortex of ADHD patients in addition to a significant haplotype (Hawi et al., 2013). To concentrate as many results as possible, a computational analysis of multiple data sources using a new ADHD genetic database was conducted in 2012 to prioritize candidate genes for ADHD (Chang et al., 2012). The result of this study was a list of 16 prioritized genes, among which was *SNAP25.* Something similar had been concluded 6 years earlier in a review evaluating 8 candidate genes for ADHD and accepting 7 (including *SNAP25*) as valid (Faraone & Khan, 2006).

1.2.3 The coloboma mouse

The coloboma mutant mouse (or *Cm*/+ mouse) is a radiation mutant with a heterozygous mutation on mouse chromosome 2, encompassing *Snap25* (Hess et al., 1994). When homozygous, this mutation is embryonically lethal. *Cm*/+ mice display a hyperactive phenotype (Hess et al., 1992) that can be rescued with a genetic complementation of *Snap25*, but also pharmacologically with medium doses of amphetamine, but not MPH (Hess et al., 1996). In addition to hyperactivity, *Cm*/+ mice exhibit alterations in neuronal plasticity and impaired long-term potentiation (Steffensen et al., 1996), as well as marked deficits in Ca^{2+} dependent dopamine release in the dorsal striatum, implying the nigrostriatal dopamine pathway which regulates motor activity (Wilson, 2000). The transgenic rescue of

Snap25 not only restores activity levels, but also dopamine-modulated synaptic transmission (Steffensen et al., 1999).

1.2.4 The *Snap25* knockout mouse

In contrast to the coloboma mutant, the *Snap25* heterozygous knockout mouse only lacks *Snap25*, which was accomplished by replacing exons 5a and 5b through homologous recombination. Although this alteration is also embryonically lethal when homozygous, is has been shown that *Snap25* is not required for nerve growth, but rather is essential for evoked synaptic transmission (Washbourne et al., 2002). The *Snap25* heterozygous knockout mice have recently been investigated as a model of epilepsy (Corradini et al., 2014) and as a model of altered dopamine signaling, making it a potential model for both schizophrenia (Oliver & Davies, 2009) and ADHD (Baca et al., 2013).

1.3 Attention deficit/hyperactivity disorder (ADHD)

1.3.1 What is ADHD?

ADHD is one of the most common childhood disorders with a prevalence of 3-5%. About half of the affected children show persistent symptoms into adulthood (Renner et al., 2008). The *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition (DSM IV), defines hyperactivity/impulsivity and inattention as the major symptomatic dimensions of the disorder. Out of a list of symptoms for both dimensions, 6 have to be met in order to justify a diagnosis. Also, several symptoms must have been present prior to the age of 12 years. In addition to this central symptomatology, ADHD has been shown to be comorbid with several other psychiatric disorders. In children, mood disorders, anxiety disorders, oppositional defiant disorder and conduct disorder are the most common (Lycett et al., 2014), whereas in adults the most likely comorbidities are major depressive disorder, social phobia and substance abuse (Rucklidge et al., 2014). Since emotional lability can be seen in some of these comorbid disorders, for the longest time emotional lability was seen as a consequence of ADHD. Today, it has become clearer that emotional dysregulation may play a causal role in ADHD symptomatology (Villemonteix et al., 2014).

1.3.2 Neurobiology and treatment of ADHD

Imaging studies have strongly implicated frontostriatal dysfunctions in patients suffering from ADHD. Additionally, several other regions seem to be involved, as for example

the anterior cingulum, the prefrontal cortex, the orbitofrontal cortex, the caudate nucleus, the thalamus, the amygdala and the cerebellum (Kasparek et al., 2013). On a molecular level, the systems implicated are just as manifold. Studies have shown the dopaminergic, the nor-/adrenergic, the serotonergic and the cholinergic system to be involved in ADHD (Cortese, 2012). These systems also reflect the genes that are discussed as candidate genes for the disorder, as ADHD has a very high heritability of around 76% (Faraone & Mick, 2010). On the monoaminergic level, the genes coding for monoamine oxidase A (*MAOA*), catechol-O-methyl transferase (*COMT*), dopamine receptor 4 (*DRD4*), the dopamine transporter (*SLC6A3, DAT*), the serotonin transporter (*SLC6A4, 5HTT*), tryptophan hydroxylase 2 (*TPH2*) and several serotonin receptors, among others, have been shown to be associated with ADHD. But there are also candidate genes outside of this group, for example the neuronal nitric oxide synthase (*NOS1*) and, of course, *SNAP25* (Banaschewski et al., 2010).

Even though so many different systems are implicated, the dopaminergic system is probably the one that is most discussed in the etiology of ADHD. One reason for this is that the most commonly prescribed treatment today is pharmacotherapy with one of two psychostimulants. One is Methylphenidate (MPH), a dopamine/noradrenalin reuptake inhibitor (Heal & Pierce, 2006), and the other d-Amphetamine, a full agonist of trace amine-associated receptor 1, which, when activated, inhibits the function of the dopamine-, the norepinephrine- and the serotonin-transporter (Lewin et al., 2011). Additionally, the selective norepinephrine reuptake inhibitor Atomoxetine is often successfully used, as well as several new drugs that are up to now only available in the US. PET and SPECT studies about the dopaminergic system and its involvement with ADHD have found reduced dopamine transporter availability in patients, but are often controversial (Bolea-Alamañac et al., 2014). It is not yet fully understood if ADHD is a hyper-dopaminergic disorder, a hypo-dopaminergic disorder, or both (Ohno, 2003).

Apart from neurobiological and genetic factors, environmental influences have been found to be connected to the etiology of ADHD. Moreover, as with many psychiatric disorders, it has been hypothesized that the interplay of genetic and environmental factors, so called gene-by-environment interactions (G x E), cause the disorder and not only one or the other. In ADHD, the most commonly mentioned environmental influences are fetal exposure to smoking or alcohol, exposure to toxins (lead and mercury), pregnancy and

delivery complications and psychosocial adversity such as maltreatment or emotional trauma (Banerjee et al., 2007).

1.4 Aim of this thesis

The aim of this thesis was to evaluate heterozygous *Snap25* knockout mice with and without stressful experience as a model for ADHD. The main focus thereby lay on altered behavior as measured in a number of behavioral tests. The review of literature on *SNAP25* allocates plausible construct validity to this model, as does the fact that human and mouse *SNAP25* share 95.1% identity at the DNA and 100% identity at the protein level according to the NCBI HomoloGene Database.

Additionally, the normalization of the behavior was tried to accomplish with a pharmacological intervention (MPH) to ensure predictive validity. For this end, a pilot study was performed to determine a good dose both given orally and injected intraperitoneally (i.p.), measuring both locomotor activity and neurotransmitter concentrations in various brain regions 100 minutes after the drug application. This was necessary because, first of all, i.p. doses for MPH in mice in literature range from 1 mg/kg to 75 mg/kg (Fernández et al., 2008; Koda et al., 2010; Salahpour et al., 2008; Shuster et al., 1982; Tilley & Gu, 2008; Yan et al., 2010) with little inclination as to what constitutes an adequate dose. Secondly, MPH in humans is given orally, thus it was tried to establish a non-stressful way of oral application in mice through voluntary consumption of a sweet cereal flake infused with MPH.

A second pilot study was performed to assess the feasibility of the COGITAT holeboard test in mice as a measure of attention. The test was rejected for the *Snap25* heterozygous knockout mice project, due to its inability to measure impulsive tendencies. It was substituted with the better established 5CSRTT (Carli et al., 1983).

It was tried to ensure face validity by choosing transferable paradigms and behavioral tests. As a stressful environmental factor, MS was used, which is a time-tested method proven to alter brain activity, behavior and gene expression (Nishi et al., 2013), in this case aimed to model early-life adversities. In addition to the 5CSRTT, which is a reinforced learning paradigm initially developed to understand ADHD-like attentional and impulsive deficits (Robbins, 2002), a long-term, repeated OF test was used to study locomotor activity, in summary covering all three of the core symptoms of ADHD. The OF is a straightforward

exploration task of a rectangular or round arena (Walsh & Cummins, 1976). For this experiment, the focus was on locomotor activity.

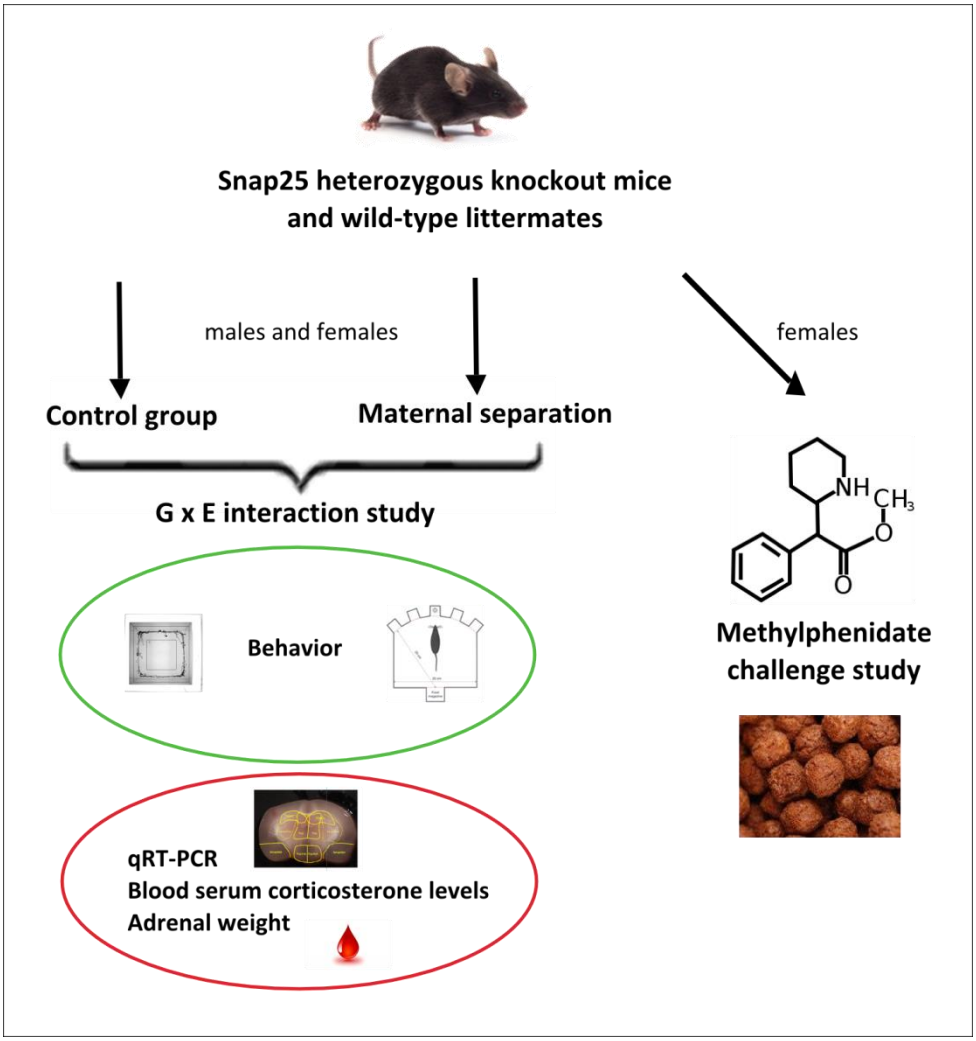

Figure 3: Schematic of the *Snap25* study

In addition to testing for core symptoms, different behavioral tests were employed to assess the emotionality aspects of the disorder. The LDB, a tool to study anxiety-like behavior, measures the conflict between the tendency to explore and the initial tendency to avoid the unfamiliar (Bourin & Hascoët, 2003) in a box consisting of an enclosed, dark, safe compartment and a larger, brightly lit, more exposed one. To assess depression-like behavior, the FST was used (Porsolt et al., 1977). This test is based on the observation that when placed in a cylinder containing water, rodents rapidly become immobile after unsuccessful attempts to escape. Antidepressants decrease the duration of immobility which is used as the main predictor of antidepressant-like behavior (Castagné et al., 2009). Finally,

though unsuccessfully, it was tried to assess aggressive behavior with the resident intruder paradigm (RI), aimed to test for territorial aggression (Miczek et al., 2001). After the behavioral tests, mice were sacrificed and their brains examined for gene expression. Genes of interest for this qRT-PCR study were, in addition to *Snap25* itself to check for actual expression levels as a post-hoc manipulation check, other candidate genes for ADHD to check for interactions with other genes. COMT is an enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine to catecholamines, including dopamine and norepinephrine. This process is one of the major inactivation pathways for these neurotransmitters and thus very important in several diseases in humans (Jiménez-Jiménez et al., 2014), including ADHD. Most commonly, the valine/methionine polymorphism in exon IV (rs4680) is discussed, though not conclusively, as many studies were not able to replicate the initial results (Caylak, 2012). MAOA is responsible for the breakdown of the monoamines 5HT, NA and DA. In particular, the 4 and 5 repeat alleles of a 30-bp tandem repeat in the promoter region is often found to be associated with ADHD (Faraone & Mick, 2010), although the gene's location on the X chromosome makes it susceptible for sexually dysmorphic effects (Biederman et al., 2008). DRD2 represents the main autoreceptor of the dopaminergic system, but is also critical for postsynaptic transmission (Lindgren et al., 2003). The TaqIA1 allele has been associated with ADHD, though also not conclusively (Faraone & Mick, 2010). NOS1 is an enzyme predominantly responsible for nitric oxide (NO) production in the nervous system, where the gaseous neurotransmitter acts as a biological mediator (Zhou & Zhu, 2009). A highly polymorphic dinucleotide repeat in the promoter region of the alternative exon 1f of *NOS1* (*NOS1* ex1f-VNTR) affects brain functioning in schizophrenia (Reif et al., 2006) and is also associated with a number of impulsive behaviors and ADHD (Reif et al., 2009). Also, a SNP within *NOS1* has been connected to quantitative traits in childhood ADHD in a genome-wide study (Franke et al., 2009). In addition to taking brains to assess expression levels of the above mentioned genes, blood and adrenals were also taken. Adrenals were weighed as a measure for stress (David et al., 2013) and blood plasma was tested for corticosterone levels.

2 Methods

2.1 MPH study

2.1.1 Animals

77 male C57BL/6N mice (7 per group, 11 groups), age-range 6-8 weeks, were purchased from Charles River (Sulzfeld, Germany). They were single housed (in Type II Makrolon cages) and allowed to habituate to the laboratory for a minimum of 2 weeks before testing under controlled temperature (21.6 °C±0.1 °C) and humidity (50.4%±0.5%) conditions, under a 12/12h light–dark cycle (lights on at 7AM and lights off at 7PM). Animals had unrestricted access to food and water. Each mouse was randomly assigned to one of the application-form and dosage groups (7 per group, see Table 1) and put through activity testing with drug application within 8 weeks of arriving at the laboratory. Mice were sacrificed immediately after the conclusion of the activity testing (within 15 minutes after being taken out of the OF), and brains were taken.

2.1.2 Drugs and application

MPH (Sigma Aldrich Cat. No. M2892) was dissolved either in physiological saline solution or water and then immediately taken for intraperitoneal (i.p.) or oral drug administration, respectively. Pure saline solution and water were used for the respective 0 mg/kg control conditions. For i.p. administration, MPH was diluted in such a way that mice had to be injected with 10 µl per gram of bodyweight to achieve the targeted dosage. For oral administration, MPH was diluted such that 1 µl per gram of bodyweight had to be put on a flake of chocolate flavored cereal (Crownfield Choco Moons; Lidl, Germany) to achieve the targeted dosage. Mice from the oral group were familiarized with the cereal for 5 consecutive days before testing to eliminate novelty effects.

Table 1: Groups in the MPH dose-response study

oral (in H_2O)	0 mg/kg	10 mg/kg	30 mg/kg	60 mg/kg	90 mg/kg	
intraperitoneal (in NaCl)	0 mg/kg	1 mg/kg	7,5 mg/kg	15 mg/kg	30 mg/kg	45 mg/kg

Drugs were administered after baseline activity testing. Animals were taken out of the OF and either directly injected with the required dosage (i.p. group), or placed into the homecage to be presented with the infused cereal flake (oral group). The different dosages for both the oral and the i.p. groups can be found in Table 1. All mice from the oral group consumed their cereal flake within 5 minutes after being presented with it and were then immediately put back into the OF, the same as the i.p. group after injection.

2.1.3 Activity testing

All activity testing was conducted during the first 4 hours of the light phase. The Open Field used for activity testing consisted of a quadratic black opaque PERSPEX XT box (50×50×40 cm, semi-permeable to infrared light, TSE Systems, Inc., Bad Homburg, Germany). The apparatus was illuminated by infrared LEDs from below. Activity monitoring was conducted using an infrared sensitive CCD camera and the computer-based video-tracking software VideoMot 2 (TSE Systems, Bad Homburg, Germany).

Mice first underwent 30 minutes of baseline activity testing, after which drugs were administered as described above, and were then put back into the Open Field for another 90 minutes to test for activity under the influence of MPH. The parameter recorded was the distance travelled in both the baseline and the testing phase.

2.1.4 Brain dissection

Brains were frozen in ice-cold Isopentane (2-Methylbutane, AppliChem GmbH, Darmstadt, Germany) and stored at -20 °C until dissection. Dissection was done on a plate cooler at -10°C. Regions were dissected by slicing the brains into 6 sections and then excising the desired areas (Figure 4). Regions taken were the frontal cortex (prefrontal cortex and motor cortex), the striatum including the accumbens nucleus, the hippocampus, and the amygdala. After dissection, the different regions were put in 1.5 ml Eppendorf tubes and stored at -20 °C until HPLC analysis. The frontal cortex and the striatum went into HPLC as primary regions of interest and were weighed on precision scales before further processing.

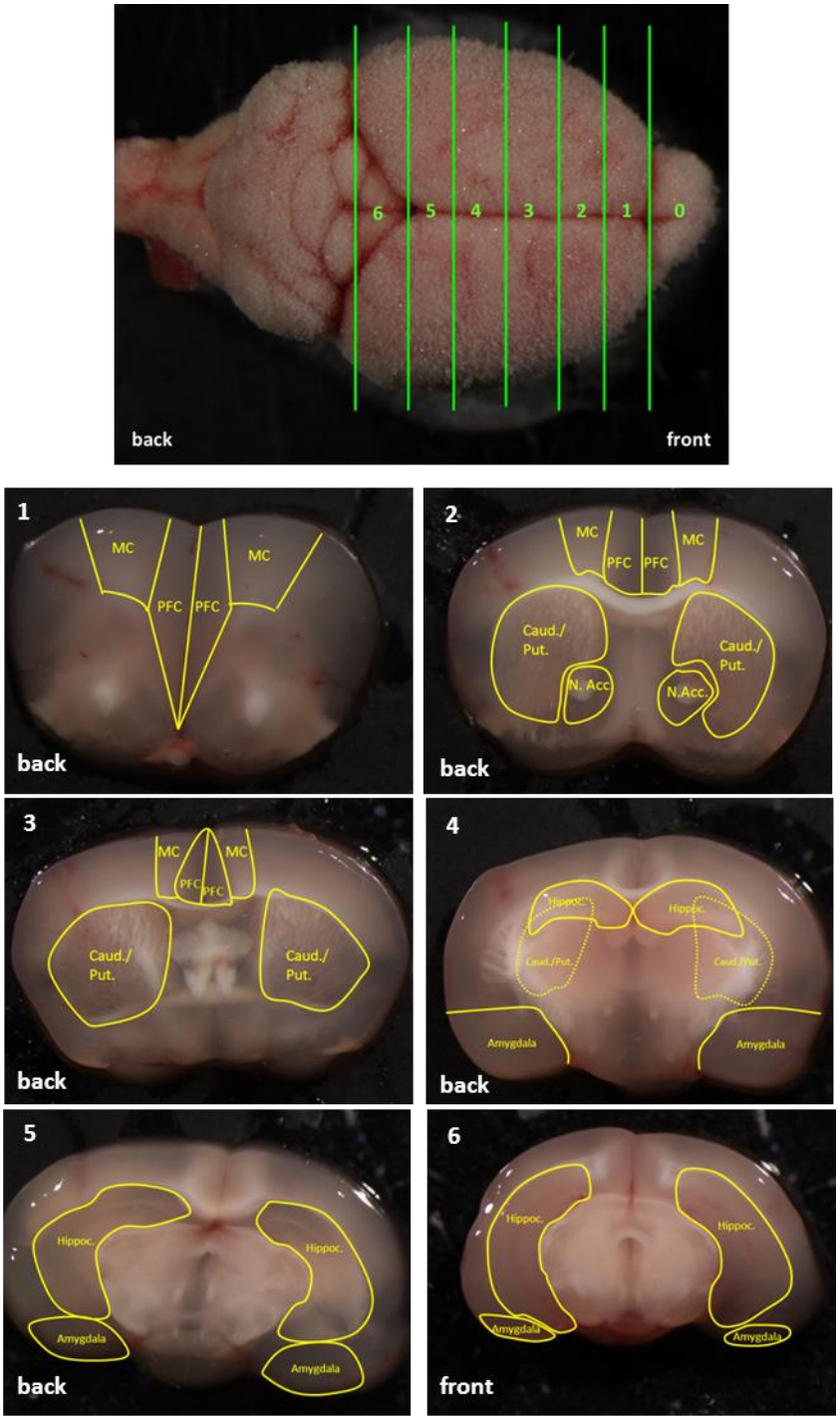

Figure 4: Dissected brain regions for the MPH dose-response study. The upmost picture shows the section planes that are specified in the upper left corners of the 6 pictures below. In the lower left corners, the view from front or back is specified. MC: motor cortex; PFC: prefrontal cortex; Caud./Put.: striatum (caudate nucleus and putamen); N.Acc.: accumbens nucleus; Hippoc.: hippocampus; Amygdala: amygdala region;

2.1.5 HPLC

The tissue was diluted 1:20 with buffer containing H_3PO_4 (150 mM) and DTPA [Bis-(2-aminoethyl)-amine-N,N,N',N'',N''-penta-acetic acid; 500 µM] and sonicated on ice under Argon atmosphere. The homogenate was centrifuged (20 min; 4-8 °C; 19000 rpm) and the

supernatant transferred into Eppendorf-caps and stored at -20 °C until analysis. Prior to analysis, the thawed homogenate was filtered via a microcentrifugal filter (membrane of regenerated cellulose; pore size 0,2 µm; amchro GmbH, Hattersheim, Germany). For analysis, the supernatant without further treatment was injected into the HPLC-system, the injection volume per sample being 50 µl. The analysis of neurotransmitters and their metabolites was performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) with electrochemical detection (model 1640; BioRad, Munich, Germany) according to a previously described method (Riederer & Burger, 2009). If saturation of the electrochemical detection system was reached during measurement, the injection volume was reduced or the sample was diluted before reinjection. The neurotransmitter and metabolites measured were 5HT, DA, NA, 3-Methoxy-4-hydroxyphenylglycol (MHPG), 3,4-Dihydroxyphenylacetic acid (DOPAC), 5-Hydroxyindoleacetic acid (5HIAA), and Homovanillic acid (HVA). The parameter recorded was nanograms of neurotransmitter or metabolite per gram of brain tissue. For final analysis, the three neurotransmitters (DA, 5HT, NA), as well as their respective turnovers metabolic turnovers ((HVA + DOPAC) / DA; 5HIAA / 5HT; MHPG / NA) were taken into account (Okada et al., 2013). Tissue preparation was done by Esin Candemir and HPLC analysis was done by Florian Proft.

2.1.6 Statistical analysis

Analysis was done separately for the i.p. and the oral groups, both for the behavioral and the neurotransmitter data. For the behavioral data, both the 30 minutes of baseline activity and the 90 minutes of experimental activity data were broken down into intervals of 2 minutes and then evaluated in a Split-Plot ANOVA with “interval” as the within factor and “dosage group” as the between factor. For the neurotransmitter analysis, 6 one-way ANOVAS were performed for every brain region, with the three neurotransmitters (5HT, NA, DA) and their metabolites in relation to them (5HIAA/5HT, MHPG/NA, (HVA + DOPAC)/DA) as dependent variables. Dosage group served as independent variable. Post-hoc Scheffé tests were performed for dosage group in each analysis.

2.2 Pilot Study COGITAT Holeboard System

2.2.1 Animals

30 male C57BL/6J mice, age-range 8-10 weeks, were purchased from Charles River (Sulzfeld, Germany). They were group housed in groups of 5 upon arrival (in Type III Makrolon cages) and were allowed to habituate to the laboratory for a minimum of 2 weeks before testing under controlled temperature (21±0.1 °C) and humidity (55±0.5%) conditions, under a 12/12h light–dark cycle (lights on at 6 AM and lights off at 6 PM). Animals had unrestricted access to food during habituation and to water throughout the experiment. Seven days prior to behavioral testing, mice were single housed (in Type II Makrolon cages), weighed and put on a restricted diet of 2-3.5 g (plus 2 sugar pellets to get familiarized with the reward) of chow per day (depending on the initial body weight), resulting in a weight reduction of no more than between 10 and 15%. This feeding protocol was maintained throughout the behavioral testing period.

2.2.2 The COGITAT Holeboard

During the test period, mice explored a modified COGITAT hole board (Cognitron GmbH, Göttingen, Germany; size 660×670 mm, inner surface; Figure 5) bordered by a clear plexiglas boundary (height 270 mm) giving access to distal spatial cues. The board contained an array of 5 × 5 holes (diameter, 35 mm; distance apart center to center, 127 mm), each consisting of a cylindrical tube closed off at its lower end by an adjustable feeding plate (50 mm below the upper surface) with a cavity into which a sugar pellet (0.045 g; Bio-Serv, Frenchtown, NJ) fits exactly. The colors of the feeding plate and of the food pellet were a perfect match. The ground below the feeding plate and the cylindrical tubes was covered with vanilla odor (Dr. Oetker™ Pudding powder, Vanilla flavor) to prevent the animals from working out the distribution of the pellets by using olfactory stimuli. In one experimental run, five of the cylinders were baited with one pellet each. A trial was automatically ended after 240 s. During this time, the animals had the opportunity of finding and eating the 5 food pellets, recognizing the spatial pattern in which the pellets were presented. The system uses an infrared system to record different aspects of activity: dips of the head at the upper level of the tubes (upper light beam [ulb] 10 mm beneath the upper surface) were equivalent to inspections; exploration deep into the hole (lower light beam [llb], 20 mm above the level of the pellet), were scored as visits; collections of the pellets by eating them

(detected by an infrared light barrier at the level of the pellet). There are manifold, partially inter-dependent parameters that can be recorded automatically and simultaneously. Since the COGITAT test was in the end not chosen for the G x E study, only two variables from the automatic output were selected for presentation here (Table 2) in order to give an impression. Additional surveillance with a video camera (VideoMot2, TSE Systems, Bad Homburg, Germany) offered the possibility of recording not only the correct path and speed of the animals, but also their general level of activity. This variable is also presented here (Table 2). Further results can be found in the already published manuscript (Post et al., 2011).

Table 2: Variables from the COGITAT Holeboard study discussed

Parameter	Definition/Explanation
Activity: Total distance travelled	The total distance (in cm) that each animal travelled per trial, as recorded by the VideoMot system
Acquisition: Pellets eaten	The number of pellets eaten in one session
Errors: Working memory errors, total	The percentage of the sum of inspections and visits to previously baited holes in relation to the total

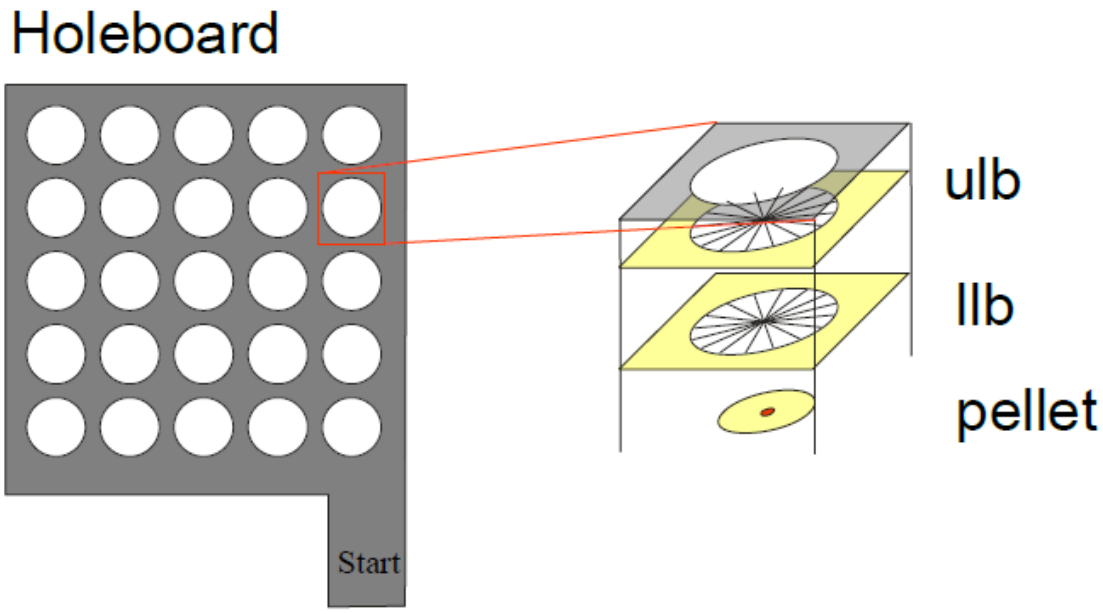

Figure 5: Schematic drawing of the COGITAT Holeboard system; ulb: upper light beam; llb: lower light beam;

2.2.3 Drugs and application

The effects of the non-selective muscarinic antagonist scopolamine and the acetyl-cholinesterase inhibitor metrifonate on learning and memory in contrast to a sodium chloride control group were investigated. Scopolamine hydrochloride, known for its memory

impairing effects (Platel & Porsolt, 1982), was dissolved in saline and administered at 0.05 ml/10 g of bodyweight in a concentration of 0.1 mg/kg subcutaneously. Metrifonate, a known spatial memory enhancer (Ikonen et al., 1999), was dissolved in saline and administered at 0.1 ml/10 g in a concentration of 50 mg/kg i.p. Each drug was administered 30 min prior to the daily session. Control animals received a saline injection at a volume of 0.05 ml/10 g i.p.. Mice were trained for 5 consecutive days and went through 6 trials per day.

2.2.4 Test procedure

Experiments were carried out between 8 AM and 4 PM. Five holes of the COGITAT system were serially baited in an L-shaped pattern with food pellets not visible to the animals while moving. Each daily session consisted of six 240 s trials with an inter-trial interval of 30 min. Animals were tested in a random order. A trial was completed as soon as the animal had collected and eaten all of the pellets within the allotted time span of 240 s or when the time span had elapsed, whichever came first. Spatial cues available for the animals inside the holeboard enclosure were the entrance with the starting box, the four corners of the enclosure, the upper edges of the 25 holes, and the sidewalls for orientation. Outside the enclosure, distal cues visible through the transparent Plexiglas walls were a wall, a window, a black curtain and a rack with the cages of the remaining experimental animals. In the center above the Holeboard the video camera (VideoMot2, TSE Systems, Bad Homburg, Germany) was attached. Each animal was subjected to six trials per day.

2.2.5 Statistical analysis

For statistical evaluation a repeated measures analysis of variance (ANOVA), with Greenhouse-Geisser adjustments (groups and trials as factors), was used for each variable. Scheffé tests served as post-hoc-analyses of between group differences. The results are displayed as means ± SEMs of the individual trials of the corresponding experimental periods.

2.3 G x E study

2.3.1 Animals

38 wild-type (21 males, 17 females) and 38 heterozygous (16 males, 22 females) Snap25 knockout animals were used in the G x E behavioral part of this study. For the non-

stressed control group, animals were bred in the breeding area of the facility and then transferred to the behavioral lab, where they were allowed to habituate for 2 weeks before the beginning of the behavioral testing (this group consisted of 24 wild-type animals (12male, 12 female) and 24 heterozygous animals (12 male, 12 female)). The MS group was bred inside the behavioral lab where the early-life stress procedure was carried out for 21 days, starting directly after birth. After the procedure, at an age of about 25 days, animals were weaned and allowed to stay in the behavioral lab until they reached testing age. All animals were single housed during testing, starting upon arrival in the behavioral lab for the control group and after weaning for the MS group. During breeding and group housing, mice were housed in Type III Makrolon cages and in Type II during single housing. One week after the conclusion of the behavioral experiments, mice were sacrificed. Blood was taken from the from the neck stump (into heparinized blood collection tubes), kept on ice and subsequently centrifuged at 3000 g for 5 min at 4 °C. The supernatant containing the plasma was then removed and stored at -20 °C until corticosterone analysis at Maastricht University (see 2.3.5). Brains were taken, frozen in ice-cold Isopentane and stored at -20 °C until dissection. Additionally, adrenals were taken, frozen on dry ice and also stored at -20 °C until further analysis.

For the MPH challenge OF study, another 32 female *Snap25* knockout mice (16 wild-types, 16 heterozygous) were used. They were housed in groups of four (in Type III Makrolon cages) and allowed to habituate to the lab for 2 weeks before testing.

All animals were between 8 and 12 weeks of age when testing began and lived under controlled temperature (21.3 ± 0.1 °C) and humidity (50.8 ± 0.5%) conditions, under a 12/12h light-dark cycle (lights on at 7 AM and lights off at 7 PM) with unrestricted access to food (except during the 5CSRTT, see 2.3.3.3.2) and water.

2.3.2 Early-life stress

Table 3: Temperature and humidity conditions during the 21-day maternal separation procedure

PND	Temperature	Humidity
1-7	35 ± 5 °C	70 ± 5%
8-14	30 ± 5 °C	60 ± 5%
15-21	25 ± 5 °C	50 ± 5%

The early-life stress MS paradigm started on post-natal day (PND) 1 (PND 0 being the day of birth) and consisted of 3-hour separation sessions on each day from PND1 to PND 21. Separation took place in the mornings between 9 and 12. Litters were removed from their homecages and put in a Type II cage (with woodchip bedding, cellulose sheets, and an egg carton) that they were assigned to for the 21 days of the procedure. Cages were then covered with a dampened cloth and heated from above with infrared lamps. Temperature and humidity conditions were maintained at certain levels for the different weeks in the procedure, starting with high-temperature/high-humidity conditions (mimicking the situation in the nest) and slowly working towards normal lab conditions (see Table 3, from PND 15 to 21, the dampened cloth was omitted).

2.3.3 Behavioral testing

2.3.3.1 Long-Term Open Field (OF)

The OF consisted of a quadratic black opaque PERSPEX XT box (50×50×40 cm, semi-permeable to infrared light, TSE Systems, Inc., Bad Homburg, Germany). The apparatus was illuminated by infrared LEDs from below. Activity monitoring was conducted using an infrared sensitive CCD camera and the computer-based video-tracking software VideoMot 2 (TSE Systems, Bad Homburg, Germany). Mice were individually placed against a predetermined retaining wall and behavior was registered for 60 min. Three weeks later, the procedure was repeated to test for activity in a more familiar environment. The main parameter taken was the distance travelled. After each mouse, the arena was thoroughly cleaned with disinfectant.

2.3.3.2 Light-Dark Box (LDB)

The rectangular-shaped LDB consisted of a transparent Perspex ‘light’ compartment (40x40x27 cm) and a black opaque ‘dark’ compartment (40x20x27 cm). The dark chamber contained a small opening at floor level (5x5 cm) and was covered by a removable lid, resulting in an almost complete absence of illumination in its interior (0-10 lux). The light compartment was uncovered and brightly illuminated (Illumination level of the light compartment around 250 lux). Mice were placed into the dark compartment and allowed to

freely explore the chamber for 5 min. Parameters recorded were transitions between compartments, time spent in the light and time spent in the dark compartment. After the test, the chamber was thoroughly cleaned with disinfectant.

2.3.3.3 Modified 5-Choice Serial-Reaction-Time-Task (5CSRTT)

2.3.3.3.1 Apparatus

The apparatus used for the experiment was the 5-hole box from TSE Systems (Bad Homburg, Germany; Figure 6). Dustless precision pellets (20 mg, also TSE Systems Inc., Bad Homburg, Germany) served as rewards and were delivered directly into the respective target hole via pellet dispensers. The house light was generally not illuminated during the testing except for time-out periods. The protocol used was adapted from Steckler, Sauvage, & Holsboer (2000).

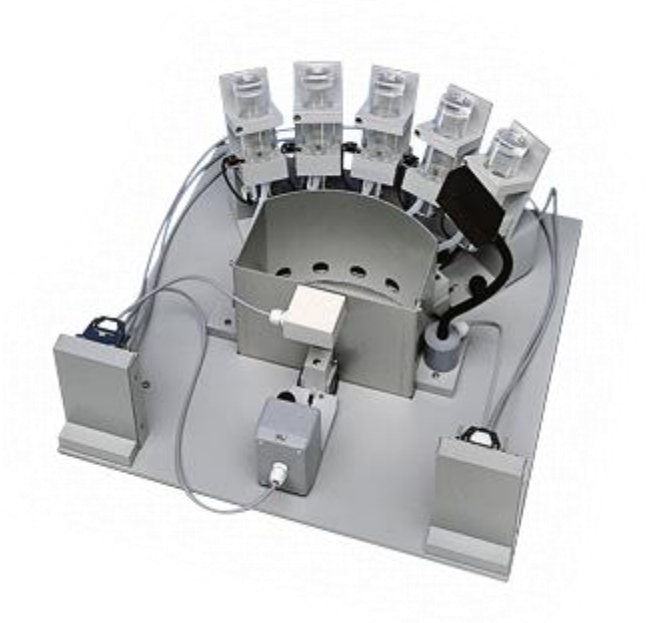

Figure 6: 5 Hole Box (TSE Systems)

2.3.3.3.2 Food restriction protocol

Depending on their initial weight, mice were given between 2.5 and 3 g of mouse chow every day after testing, losing a maximum of 10-15% of their initial body weight. Weight was checked 2-3 times a week. In addition to the regular mouse chow, every mouse was given 2 to 3 of the reward pellets every day in the week before the experiment started to get accustomed to the taste.

2.3.3.3.3 Habituation phase

Mice were placed into the test arena with house light and target lights off and 1 pellet lying in each of the 5 target holes. They were allowed to explore the arena and eat the reward pellets for a maximum of 5 minutes per session. *Time to explore all 5 holes*, *Time to eat all 5 pellets* (300 s when all holes were not explored / all pellets were not eaten) and *Number of pellets eaten per trial* were recorded. If all 5 pellets were eaten before 5 minutes had passed, the trial was stopped. Each mouse underwent 9 habituation sessions over a period of 5 days. For details on all sessions see Table 4. After that, all female mice had an average of at least 2 eaten pellets per session, whereas male mice did not. In fact, half of the male mice had not eaten a single pellet during habituation phase. This bad performance was consistent and did not improve at all for the males all through the different phases of the experiment, which made it necessary to exclude them from the statistics due to obvious motivation difficulties.

2.3.3.3.4 Autoshaping 1 phase

In this phase, mice were again placed into the dark test arena. For each trial, 1 of the target lights was illuminated and a pellet was directly delivered into the hole. The trial ended when the mouse made a nose-poke into the hole (the target light was turned off as a consequence) and was succeeded by a 10 second inter-trial interval. A session ended after 10 trials or 10 minutes, whichever came first. Variables recorded were *Time to finish*, *Number of correct nose-pokes,* the total *Number of nose-pokes* and *Number of pellets eaten*. Mice underwent 5 daily Autoshaping 1 sessions before moving on to Autoshaping 2.

2.3.3.3.5 Autoshaping 2 phase

The Autoshaping 2 phase was very similar to the Autoshaping 1 phase, with the exception that pellets were only delivered into the target hole after a mouse made the correct nose-poke. Additionally to the variables from Autoshaping 1, the *Number of pellets eaten correctly* (i.e. directly after making the nose-poke into the correct hole) was recorded, since mice diverged immensely regarding that behavior. After 4 daily Autoshaping 2 sessions, mice moved on to the experimental phase.

2.3.3.3.6 Experimental phase with 20 s stimuli

The experimental phase was similar to the Autoshaping 2 phase with the exception that stimuli lasted only for 20 s and incorrect nose-pokes (into non-illuminated holes) were

penalized with a 10 s time-out with house light on. After the 20 s stimulus a 5 s hold interval was introduced, in which the mouse could still respond and be rewarded, but the target light was not on. A false nose-poke was penalized with a 10 s time-out with house light on. The *Number of premature nose-pokes* (nose-pokes during inter-trial intervals) was additionally taken as a variable. 10 sessions were performed over a period of 7 days.

2.3.3.3.7 Test Trial with 9 s stimuli

After the last experimental 20 s stimuli phase, one single test trial session was done with every mouse to check for performance under faster circumstances. Each trial was 9 seconds long, with a 1 s hold interval, followed by 8 seconds of inter-trial interval. A false nose-poke was penalized with a 5 s time-out with house light on.

Table 4: Phases of the modified 5-choice-serial-reaction-time-task

Phase	Stimulus duration	Hold	Inter-trial interval	Timeout	Number of trials	Number of sessions	Time
Habituation	----	----	----	----	----	9	300 s
Autoshaping 1	till nose-poke occurs	----	10 s	----	10	5	300 s
Autoshaping 2	till nose-poke occurs	----	10 s	----	10	4	300 s
Experimental	20 s	5 s	8 s	10 s	10	10	600 s
Test Trial	9 s	1	8 s	5 s	15	1	300 s

2.3.3.4 Forced-Swim Test (FST)

The FST was performed in a 2 l glass beaker, filled with water (26 ± 2 °C) to the 1600ml mark. The mice were picked up by their tail and individually placed in the beaker. Behavior was recorded for 5 min. After that, the mouse was taken out of the beaker and returned to its home cage. Mobility and immobility were recorded as well as the latency to the first immobility. The water was changed between animals.

2.3.3.5 Resident-Intruder Paradigm (RI)

The RI test was exclusively performed with male mice. Cages were changed 5 days before the test, so that the homecage of an animal (the resident) sufficiently smelled like the respective animal and thus could be considered “home territory”. Everything except bedding

and mouse was removed from the cage, a slightly smaller male wild-type *Snap25* (the intruder) mouse was also placed into the cage and behavior of the resident was recorded for 10 min. Since not a single mouse in the experiment behaved in any way aggressive towards the intruder mouse during this test there were no variables recorded.

2.3.3.6 Methylphenidate Challenge Open Field

The repeated long-term OF was performed in two 60 minute sessions, spaced three weeks apart, the same way as explained in 2.3.3.1. Before the second session, half of the wild-type mice and half of the heterozygous mice (8 each) were given 45 mg/kg MPH orally on a chocolate flavored cereal flake (see 2.1.2). The main parameter taken was the distance travelled. After each mouse, the arena was thoroughly cleaned with disinfectant.

2.3.4 Brain dissection

Brains were dissected as described in 2.1.4. The frontal cortex, the striatum and the hippocampus were taken as regions of interest. Regions were collected in Eppendorf Safe-Lock Biopur 1.5 ml tubes (sterile, free of Pyrogen, RNase, DNA and ATP; Eppendorf AG, Hamburg, Germany) and stored at -20 °C until RNA extraction for the qRT-PCR.

2.3.5 Corticosterone assays and adrenal weights

The corticosterone analysis from blood was done by Daniel van den Hove at Maastricht University in the Netherlands using a radioimmunoassay previously described (van den Hove et al., 2006). To assess a further measure for stress, adrenals were weighed on precision scales.

2.3.6 Quantitative real-time PCR

RNA isolation, purification and removal of potential remaining genomic DNA from mouse brain tissue was performed by Theresia Töpner using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), substituting the lysis buffer for PeqGold RNA Pure (PEQLAB, Erlangen, Germany). RNA concentration and quality were determined using the automated electrophoresis system Experion™ (Biorad, Munich, Germany) as described in the corresponding manual. 3 samples were excluded due to an RQI (RNA quality indicator; according to the Experion™ system) value of less than 7.

Table 5: Self-designed primer pairs for reference genes and genes of interest used for quantitative real-time PCR; Genes of interest are highlighted in grey;

Gene	Primer	Sequence 5' – 3'
Sdha	MmSDHA-F	GGACAGGCCACTCACTCTTAC
	MmSDHA-R	CACAGTGCAATGACACCACG
Pgk	MmPGK-F	TCGCTTTCCAACAAGCTGAC
	MmPGK-R	TTGATGCTTGGAACAGCAGC
Tbp	MmTBP-F	ACCTTATGCTCAGGGCTTGG
	MmTBP-R	TGCCGTAAGGCATCATTGGA
B2m	MmB2M-F	ACTGACCGGCCTGTATGCTA
	MmB2M-R	CAATGTGAGGCGGGTGGAA
Tfrc	MmTFRC-F	TCCGCTCGTGGAGACTACTT
	MmTFRC-R	ACATAGGGCGACAGGAAGTG
Hprt	MmHPRT-F	TGCTGACCTGCTGGATTACA
	MmHPRT-R	TTTATGTCCCCCGTTGACTGA
Snap25	Mm_Snap25-F	ATCAGTGGTGGCTTCATCCG
	Mm_Snap25-R	CATATGGCGGAGGTTTCCGA
Drd2	Mm_Drd2-F	ATGCCCTGGGTCGTCTATCT
	Mm_Drd2-R	TACCTGTCGATGCTGATGGC
Maoa	Mm_Maoa-F	TCGGGAGAATTTTACCCAAACCA
	Mm_Maoa-R	AACTCTATCCCGGGCTTCCA
Comt	Mm_Comt-F	ACCGCTACCTTCCAGACACA
	Mm_Comt-R	GCCAGGAAGTCAGGGGTTC

2 µg of total RNA were reversely transcribed into complementary DNA using the iScript™ cDNA synthesis kit (Biorad, Munich, Germany). After the reverse transcription reaction the cDNA was diluted 1:5 with 1x TE buffer.

For quantitative real-time PCR, the SYBR® Select Master Mix (Life Technologies GmbH, Darmstadt, Germany) and either Quantitec (QIAGEN, Hilden Germany) primer assays (only in the case of *Nos1: Mm_Nos1_2_SG*) or self-designed primers (for *Snap25*, *Mao-a*, *Drd2* and *Comt;* see Table 5) were used. In a Pilot study to find suitable reference genes *Sdha* (Succinate Dehydrogenase Complex, Subunit A), *Pgkh* (phosphoglycerate kinase), *Tbp* (TATA box binding protein), *B2m* (beta-2-microglobulin), *Tfrc* (transferrin receptor) and *Hprt* (hypoxanthine phosphoribosyltransferase) were tested. All primers for this Pilot study were self-designed (see Table 5). Finally, *Sdha* and *Pgk* were selected as the most stable reference genes to go into the analysis. Each 10 µl reaction volume contained 5 µl 1 x SYBR® Select Master Mix, 1x Quantitec primers or 500 nM of the oligonucleotide primer and 1 µl of the diluted cDNA. PCR and fluorescence measurements were run in the CFX384™ Real-Time PCR detection system (Biorad, Munich, Germany). The reaction conditions can be found in Table 6.

Table 6: Quantitative real-time PCR protocol

Step	Temperature	Time	Repeats
1	50 °C	2 min	1
2	95 °C	2 min	1
3	95 °C	15 s	40 cycles
4	60 °C	1 min	
5	95 °C	10 s	1
6	65–95 °C	5 s	60 x 0.5 °C steps

qRT-PCR was also done by Theresia Töpner, using 96-well plates. Samples were tested in duplicates and every 96 well plate contained 4 inter-run calibrator wells and one negative H_2O control well.

Data analysis and normalization was done by Lena Weißflog. PCR efficiencies were determined based on raw data using the software tool LinReg. Baseline correction of the threshold cycle (Ct) values was performed with the CFX Manager™ software (Biorad, Munich, Germany). This software also calculates relative quantities (Q values), which were normalized based on the relative quantities of the two considered reference genes.

2.3.7 Statistical analysis

Every analysis for the G x E study was first done with a 3-way ANOVA with sex (male/female), stress (control/maternal separation) and genotype (wild-type/heterozygous) as factors. In the event of significant sex effects, data was split for sexes and two 2-way ANOVAs (genotype and stress as factors) were calculated. When there was no significant sex effect, data analysis was redone with a 2-way ANOVA (genotype and stress as factors). For a significant genotype x stress interaction, 4 Bonferroni-Holm adjusted t-tests were done (wild-type Control vs wild-type MS / heterozygous Control vs. heterozygous MS / wild-type Control vs. heterozygous Control / wild-type MS vs heterozygous MS).

For the 5-Choice-Serial-Reaction-Time-Task, only the female data was taken into account, since males were unable to learn the task adequately enough to go into the test trial. Thus, only genotype and stress remained as between factors. For all stages of the experiment except for the test trial, the number of the trial served as within factor. For the within factor and all its interactions, the Greenhouse-Geisser correction was used in the event of a violation of sphericity.

3 Results

3.1 MPH study

3.1.1 Activity

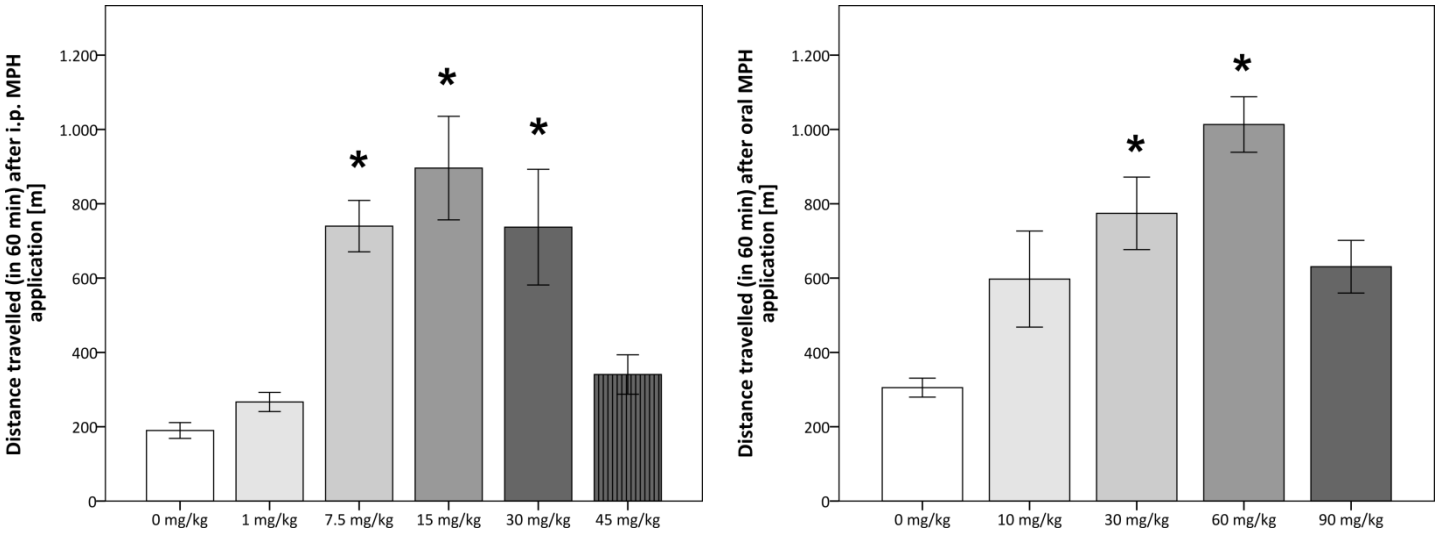

Figure 7: Behavioral results (total distance travelled without baseline) from the ip (left) and oral (right) MPH groups; * signify statistically significant (p<0.05) differences as compared to the control group (0 mg/kg)

Both the injected and the oral MPH animals show the expected elevated activity with higher doses of MPH (see Figure 7). In addition, also for both the ip and oral group, activity declines for the highest doses that could be construed as overdosing. The ANOVA results show that the change in activity with MPH is highly significant for both administration methods (see Table 7).

Table 7: ANOVA results for total distance travelled both during baseline and after drug administration for the different MPH dosage groups

Dependent variable	Effect	F	Significance
Distance travelled baseline	Different dosage groups intraperitoneal $F_{(5;36)}$	0.581	p = 0.714
	Different dosage groups oral $F_{(4;30)}$	2.216	p = 0.091
Distance travelled after MPH	Different dosage groups intraperitoneal $F_{(5;36)}$	10.123	***p < 0.001***
	Different dosage groups oral $F_{(4;30)}$	8.952	***p < 0.001***

3.1.2 HPLC

3.1.2.1 ANOVA results

Table 8 and Table 9 summarize the results from the oral and i.p. ANOVAs of the dosage effects for dopamine, serotonin, norepinephrine and their metabolite quotients both in the hippocampus and the striatum. Significant effects were followed up with Scheffé post-hoc tests of every dose compared to the respective control condition (0 mg/kg), these results can be found in 3.1.2.2. to 3.1.2.7..

Table 8: ANOVA results for the i.p. dosage effects of MPH on neurotransmitter concentrations in the frontal cortex and the striatum

Intraperitoneal	Dependent variable	$F_{(5;36)}$	Significance
Frontal cortex	Dopamine	1.380	p = 0.255
	(HVA + DOPAC) / DA	6.326	***p < 0.001***
	Serotonin	6.192	***p < 0.001***
	5HIAA/5HT	14.073	***p < 0.001***
	Norepinephrine	2.316	p = 0.064
	MHPG/NA	2.550	***p = 0.045***
Striatum	Dopamine	4.463	***p < 0.001***
	(HVA + DOPAC) / DA	10.155	***p < 0.001***
	Serotonin	6.121	***p < 0.001***
	5HIAA/5HT	28.314	***p < 0.001***
	Norepinephrine	2.296	p = 0.066
	MHPG/NA	1.501	p = 0.214

Table 9: ANOVA results for the oral dosage effects of MPH on neurotransmitter concentrations in the frontal cortex and the striatum

Oral	Dependent variable	$F_{(4;30)}$	Significance
Frontal cortex	Dopamine	1.534	p = 0.218
	(HVA + DOPAC) / DA	7.906	***p < 0.001***
	Serotonin	2.959	***p = 0.036***
	5HIAA/5HT	1.689	p = 0.178
	Norepinephrine	1.252	p = 0.311
	MHPG/NA	0.343	p = 0.847
Striatum	Dopamine	3.711	***p = 0.014***
	(HVA + DOPAC) / DA	4.100	***p = 0.009***
	Serotonin	1.038	p = 0.404
	5HIAA/5HT	0.483	p = 0.748
	Norepinephrine	1.090	p = 0.379
	MHPG/NA	0.381	p = 0.820

3.1.2.2 Dopamine in the frontal cortex

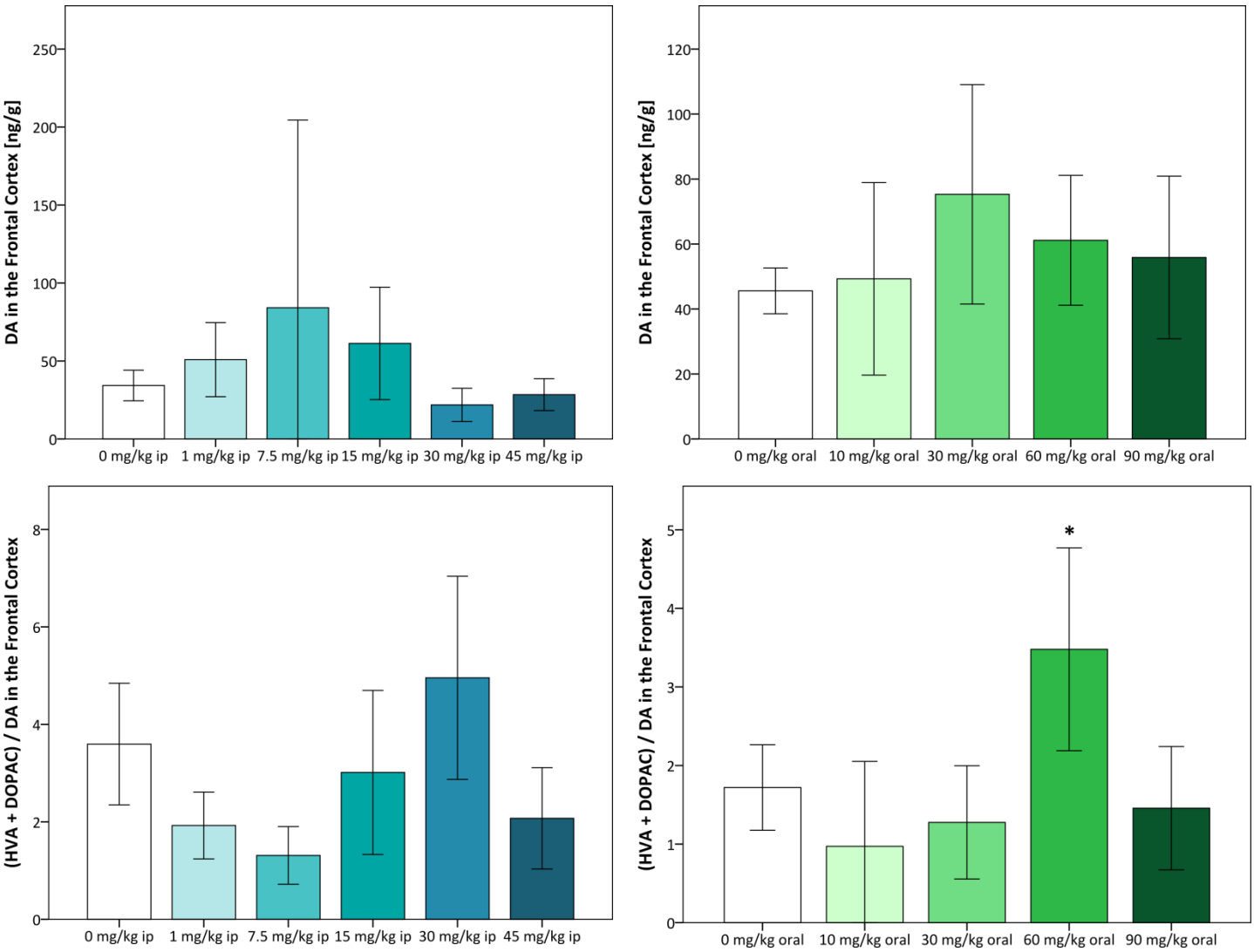

Figure 8: Dopamine concentrations and the respective metabolite quotients for the different i.p. and oral doses in the frontal cortex; ** : p<0.01, * : p<0.05, # : p<0.1;

Dopamine concentration in the frontal cortex peeks at 7.5 mg/kg and 30 mg/kg for i.p. and oral administration, respectively, but not significantly, even though all of the main effects for dopamine and its metabolite quotient are significant in the ANOVA. At higher doses, the dopamine levels decline again for both forms of administration. The 60 mg/kg oral dose is the only one in which the metabolite quotient significantly deviates from the control condition. Direction-wise the two graphs are again very similar, although the absolute levels are rather different.

3.1.2.3 Dopamine in the striatum

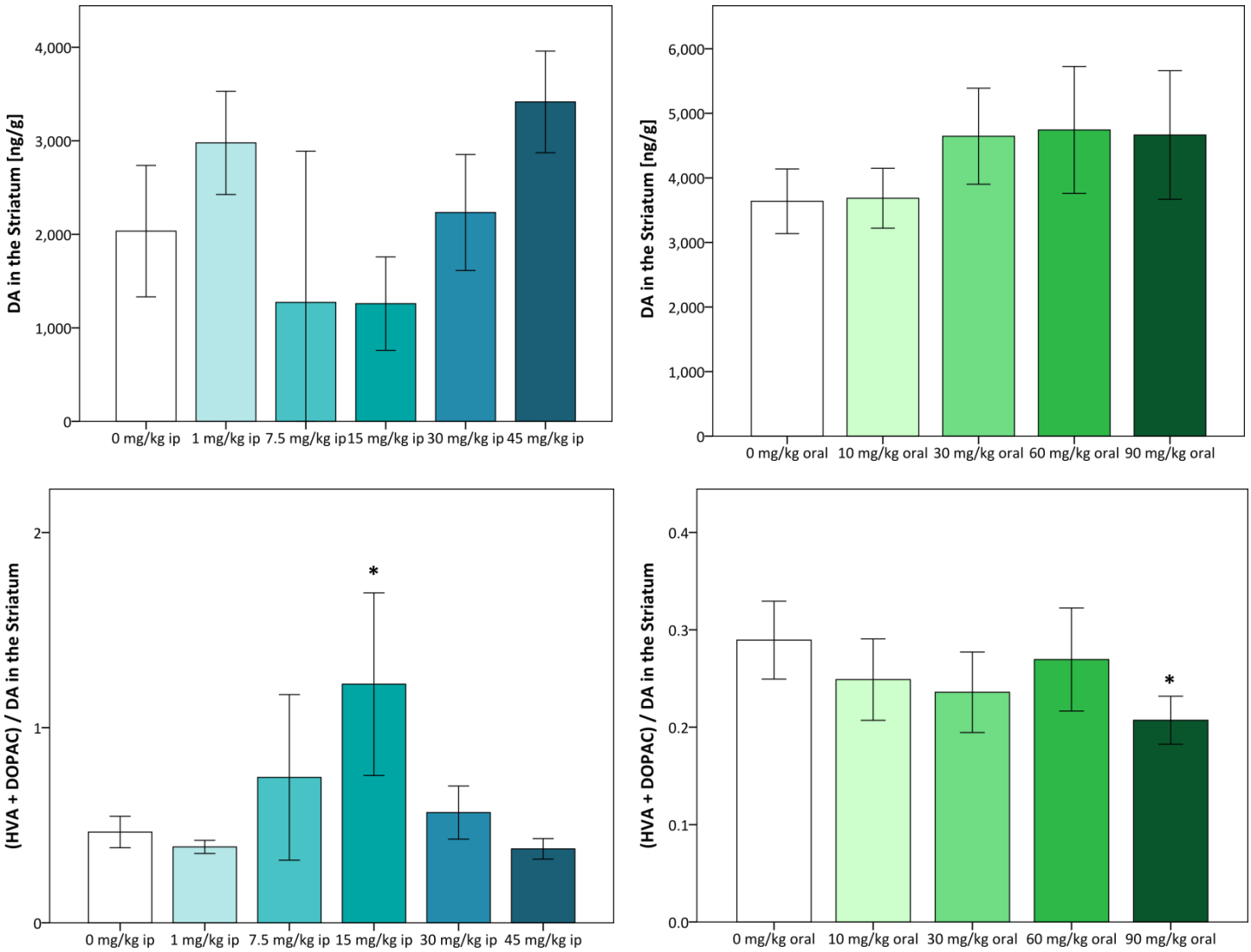

Figure 9: Dopamine concentrations and the respective metabolite quotients for the different i.p. and oral doses in the striatum; ** : p<0.01, * : p<0.05, # : p<0.1;

Though all dopamine/striatum related effects in the ANOVA are significant, there are no significant post-hoc effects. Especially in the oral condition no directionality is visible. The same seems to be true for the oral quotient, although higher doses (90 mg/kg) significantly lower the ratio from metabolites to neurotransmitter. For the i.p. quotient, a dose of 15 mg/kg significantly increases the ratio. Overall, absolute levels vary considerately between oral and i.p. administration.

3.1.2.4 Serotonin in the frontal cortex

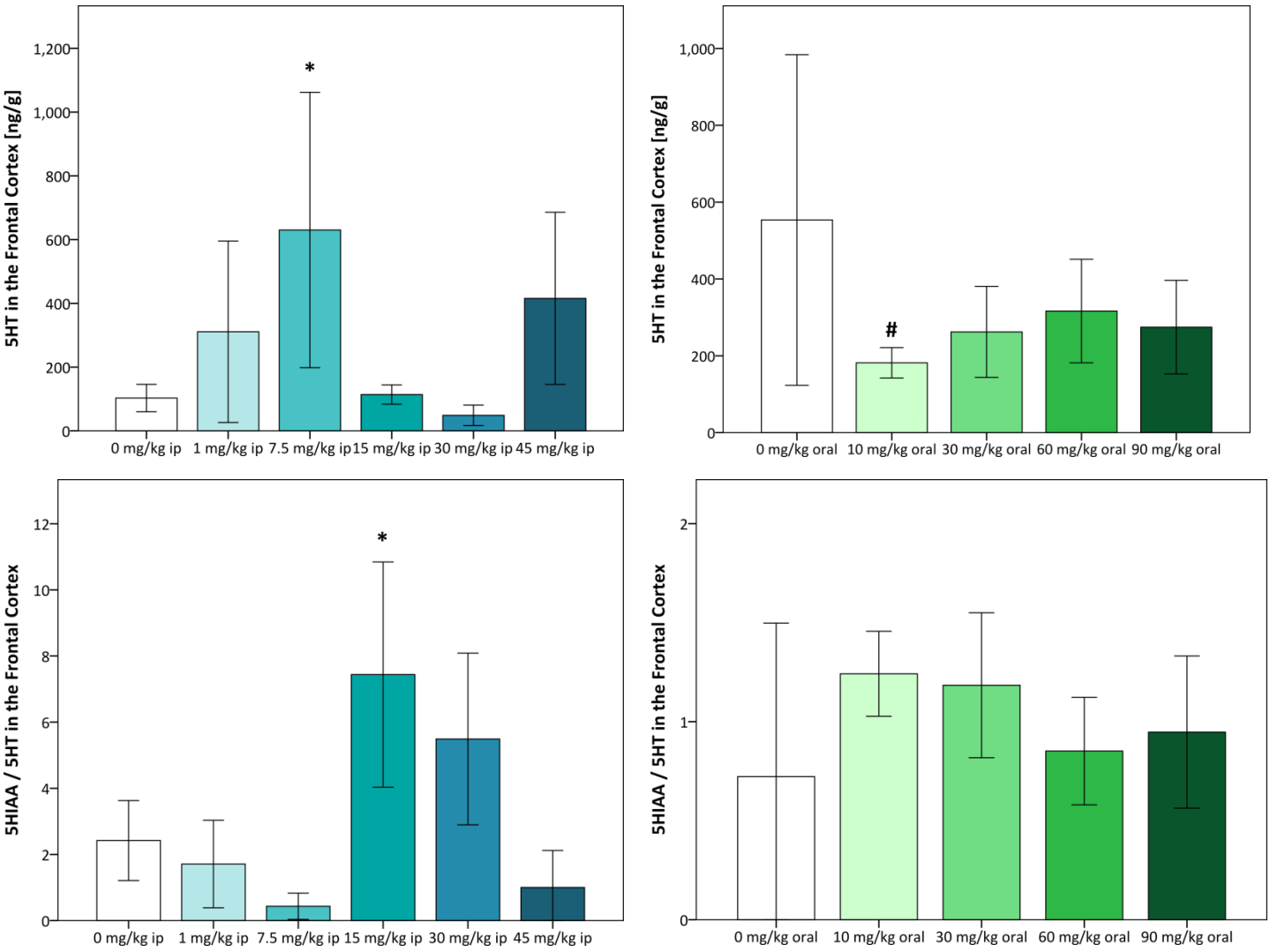

Figure 10: Serotonin concentrations and the respective metabolite quotients for the different i.p. and oral doses in the frontal cortex; ** : p<0.01, * : p<0.05, # : p<0.1;

Absolute levels for serotonin and metabolite quotient in the frontal cortex are very different between oral and i.p. administration. Also the directions of the graphs are quite divergent. For the i.p. group, serotonin levels go up with smaller doses whereas in the oral group they go down. Concerning the metabolite quotient, the oral group does not reach significance on the whole in the ANOVA. In contrast, medium concentrations of i.p. MPH significantly enhance the quotient.

3.1.2.5 Serotonin in the striatum

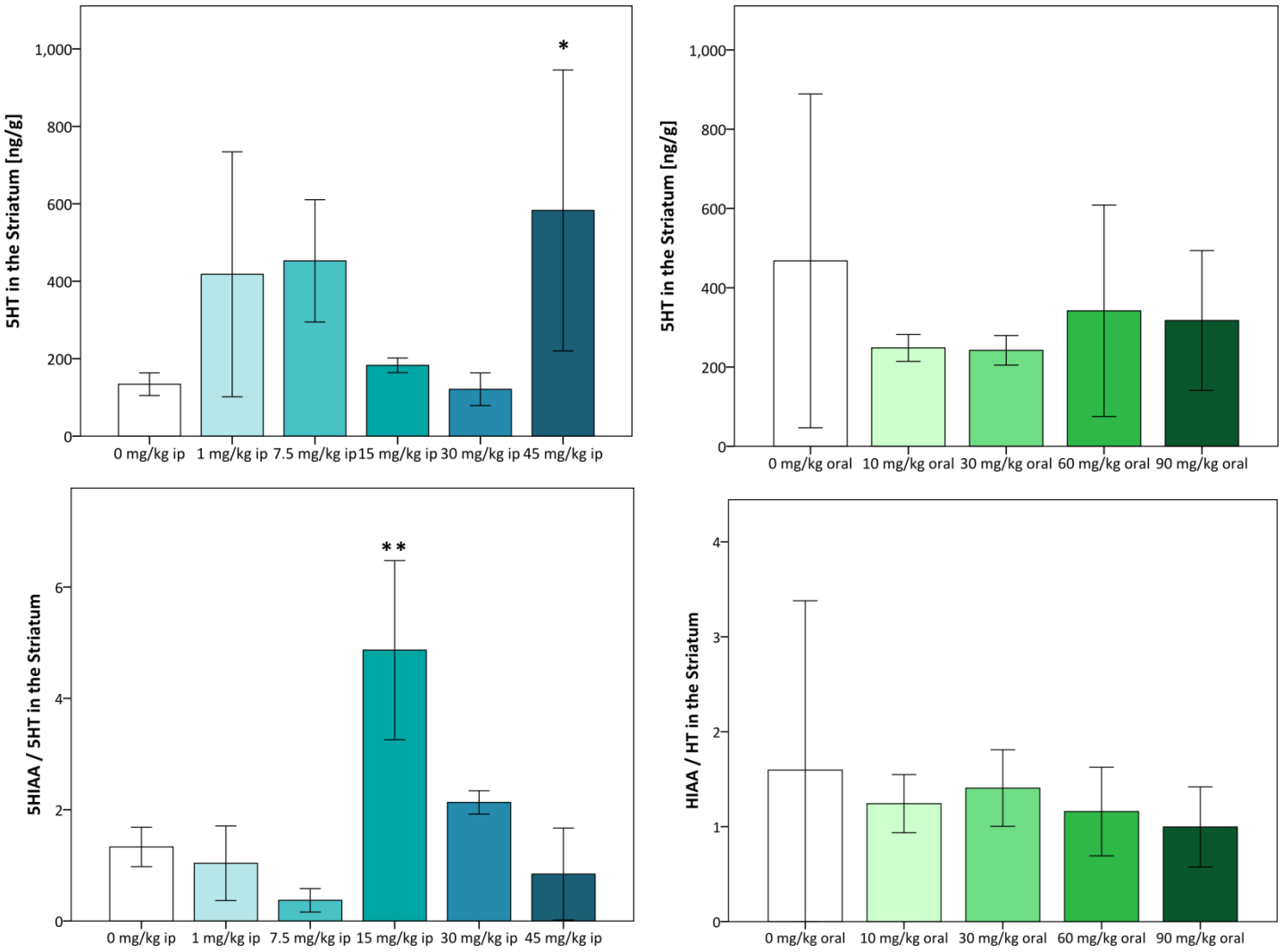

Figure 11: Serotonin concentrations and the respective metabolite quotients for the different i.p. and oral doses in the striatum; ** : p<0.01, * : p<0.05, # : p<0.1;

Regarding serotonin levels and its metabolite quotient in the striatum, oral MPH administration does not have a statistically significant effect in the ANOVA. In contrast, i.p. MPH administration alters striatal serotonin levels and, most significantly, its metabolite quotient at a dose of 15 mg/kg. Again, basal serotonin levels are very dissimilar between the i.p. and the oral group.

3.1.2.6 Norepinephrine in the frontal cortex

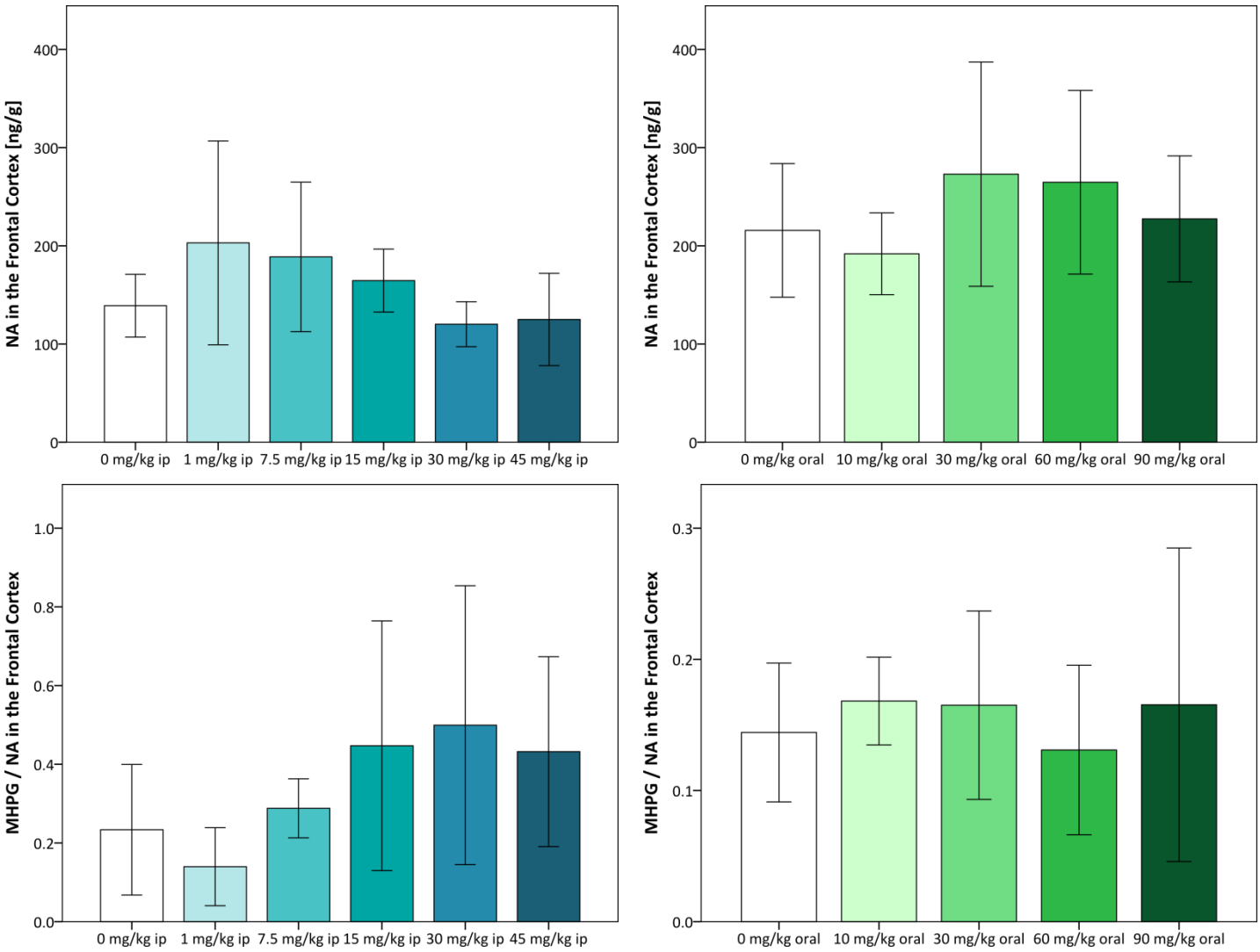

Figure 12: Norepinephrine concentrations and the respective metabolite quotients for the different i.p. and oral doses in the frontal cortex; ** : p<0.01, * : p<0.05, # : p<0.1;

Only in the i.p. group norepinephrine levels reach marginal significance in the ANOVA, though no differences occur in the post-hoc tests between dosages. Overall, neither i.p. nor oral MPH seems to massively affect norepinephrine levels or the metabolite/norepinephrine quotient in the frontal cortex. Absolute levels between the oral and the i.p. condition don't differ drastically.

3.1.2.7 Norepinephrine in the striatum

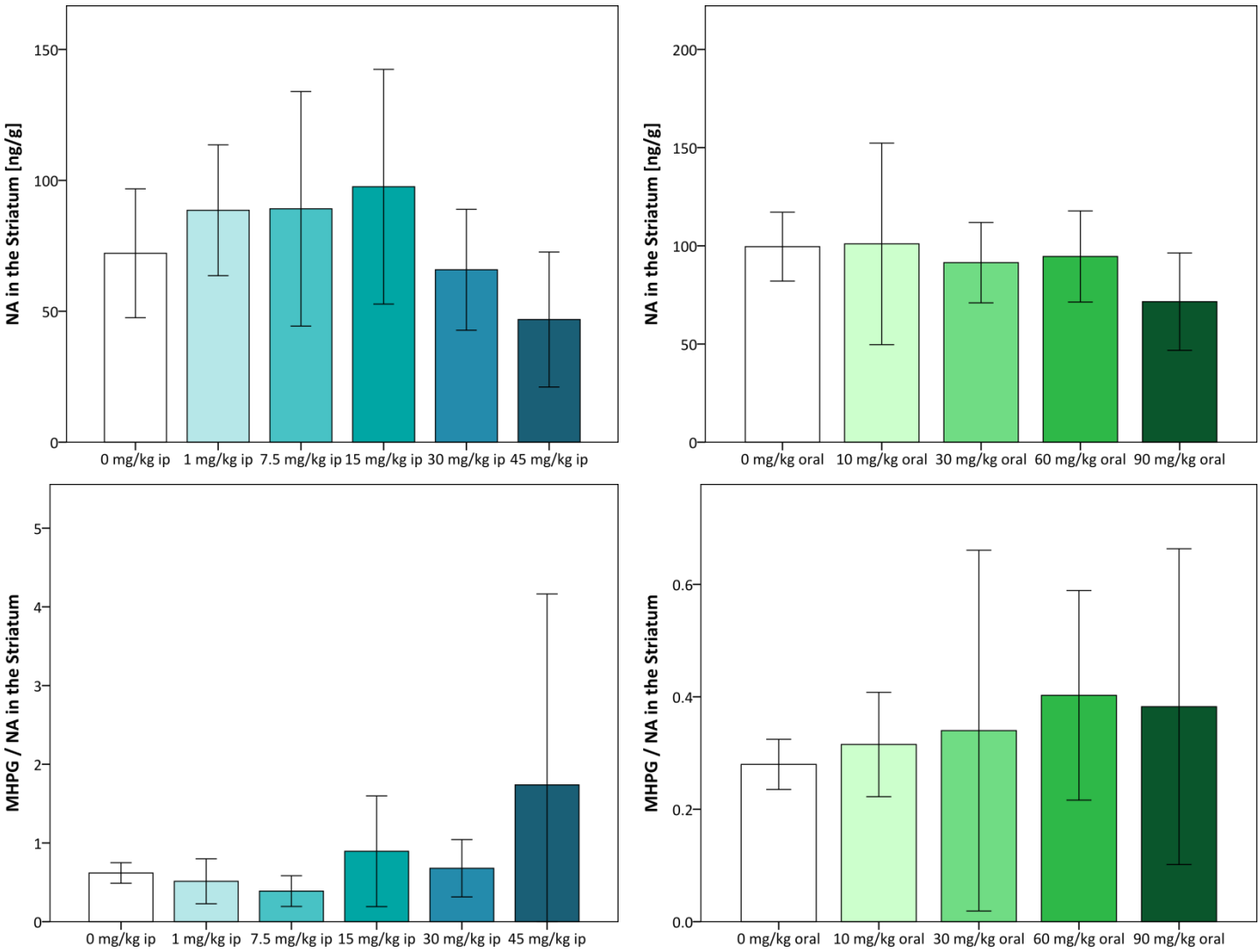

Figure 13: Norepinephrine concentrations and the respective metabolite quotients for the different i.p. and oral doses in the striatum; ** : p<0.01, * : p<0.05, # : p<0.1;

The same as in the frontal cortex, norepinephrine only reaches marginal significance in the i.p. condition in the striatum, again with no significant post-hoc results. Also similar to the results from the frontal cortex, MPH administration at the dosages tested does not seem to greatly influence norepinephrine levels and the metabolite quotient on the whole in the striatum.

3.2 Pilot Study COGITAT Holeboard System

Table 10: Results from the discussed variables of the COGITAT Holeboard task; >/< signify significant results (p<0.05) from the Scheffé post hoc test after a significant main effect in the ANOVA; M: metrifonate / S: scopolamine / V: vehicle

Parameter	
Total distance travelled	n.s.
Pellets eaten	M > V > S
Working memory errors, total	M,V < S

Table 10 summarizes the ANOVA results from the three variables discussed. As it was to be expected, there were no statistically significant differences between the different treatment groups when it came to locomotion, although the scopolamine treated group numerically travelled longer distances than the other two in the beginning (Figure 14).

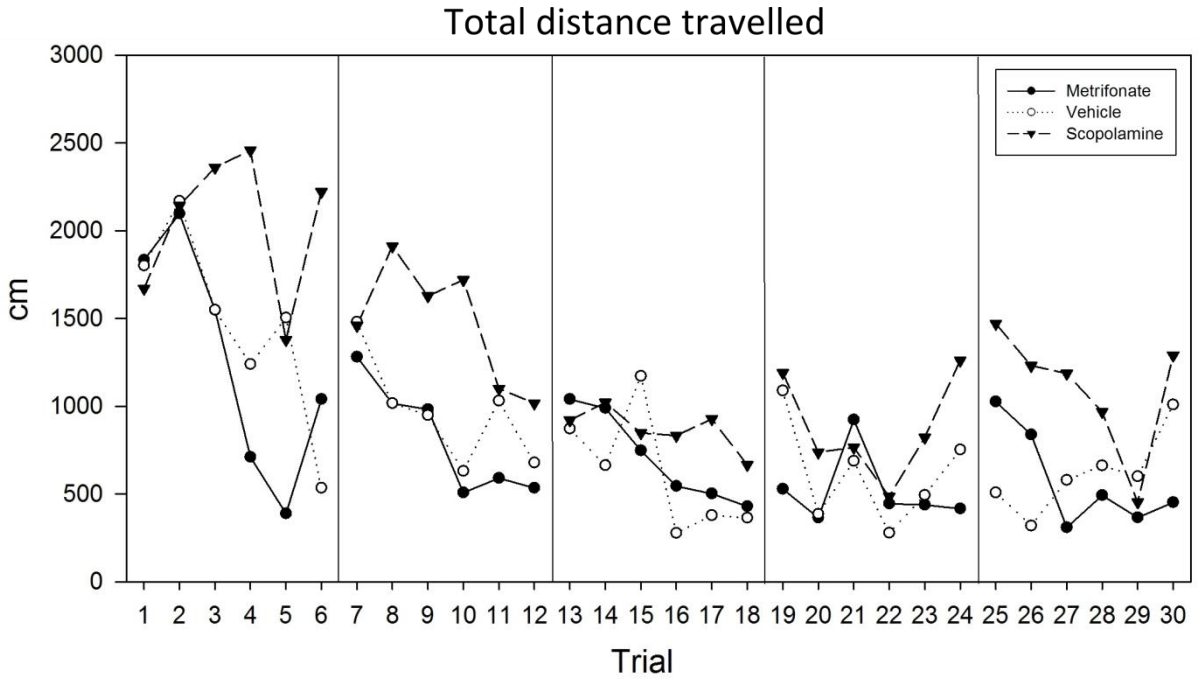

Figure 14: Total distance travelled for the different groups (Vehicle, Scopolamine, Metrifonate) over the 30 trials of the COGITAT Holeboard test

The variable most successful in separating between groups was the acquisition variable “pellets eaten”. Metrifonate treated animals find and eat significantly more reward pellets than the vehicle control group, whereas scopolamine treated animals find and eat significantly less pellets than the vehicle group (Table 10). However, this effect seems numerically most pronounced in the first 6 trials of the experiment (see Figure 15).

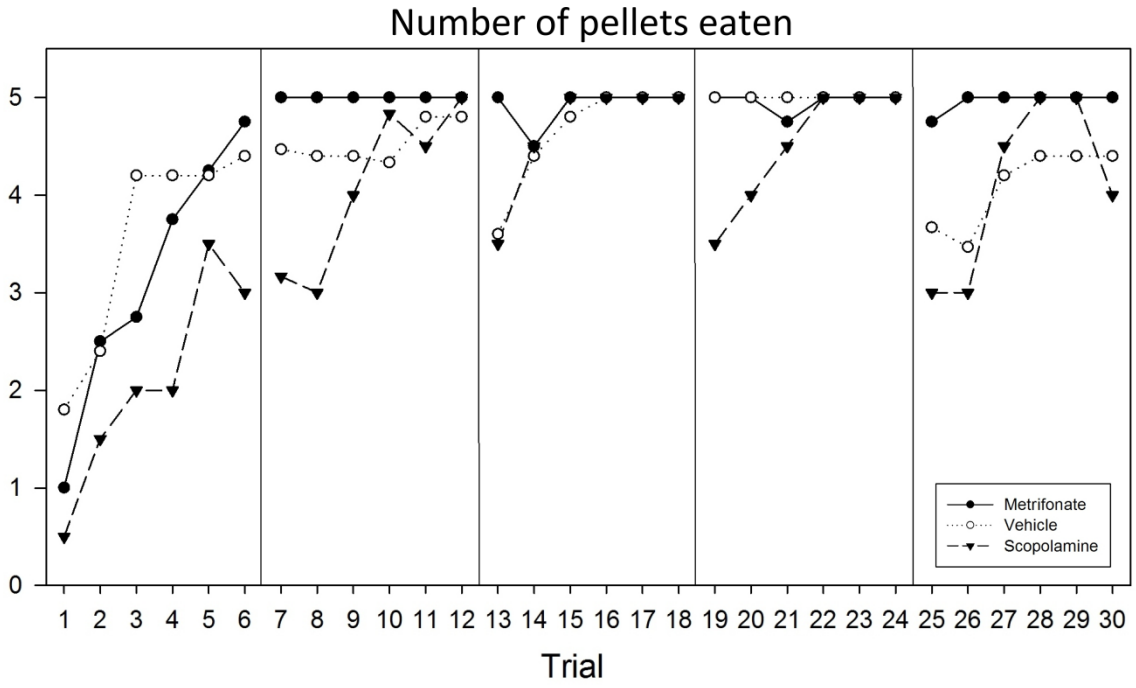

Figure 15: Number of pellets eaten for the different groups (Vehicle, Scopolamine, Metrifonate) over the 30 trials of the COGITAT Holeboard test

When it comes to working memory errors, the vehicle and the metrifonate group did not differ from each other, but both groups of animals made significantly fewer errors than the scopolamine treated group (Figure 16; Table 10).

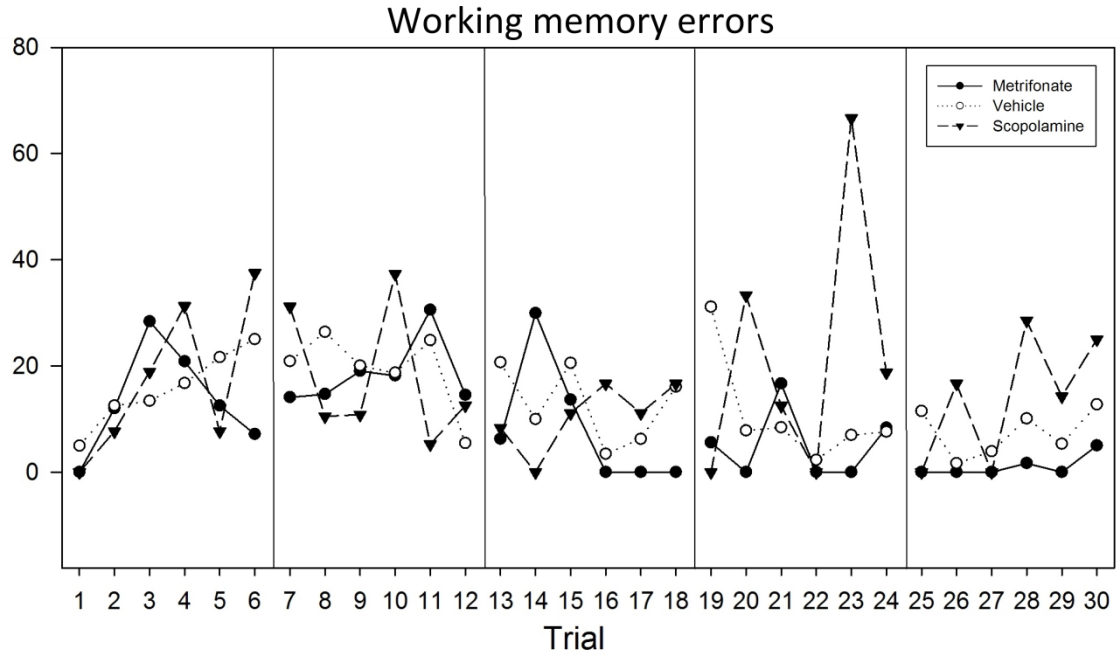

Figure 16: Working memory errors for the different groups (Vehicle, Scopolamine, Metrifonate) over the 30 trials of the COGITAT Holeboard test

3.3 G x E study

3.3.1 Behavior

3.3.1.1 Long-Term Open Field

Table 11 summarizes the ANOVA results for both the first and the second Open Field test. The environmental manipulation does not reach the significance level for either of the two sessions. But whereas in OF1 there is no difference between the two Genotypes, in OF2 the main effect Genotype is at least marginally significant (p<0.1). Interestingly, in both OF1 and OF2 the G x E interaction is significant.

Table 11: ANOVA results for the total distance travelled in Open Field 1 and 2

	Effect	$F_{(1;72)}$	Significance
Distance travelled OF1	Genotype	0.156	p = 0.694
	Environment	1.841	p = 0.179
	G x E	5.384	***p = 0.023***
Distance travelled OF2	Genotype	3.815	p = 0.055
	Environment	0.660	p = 0.419
	G x E	4.594	***p = 0.036***

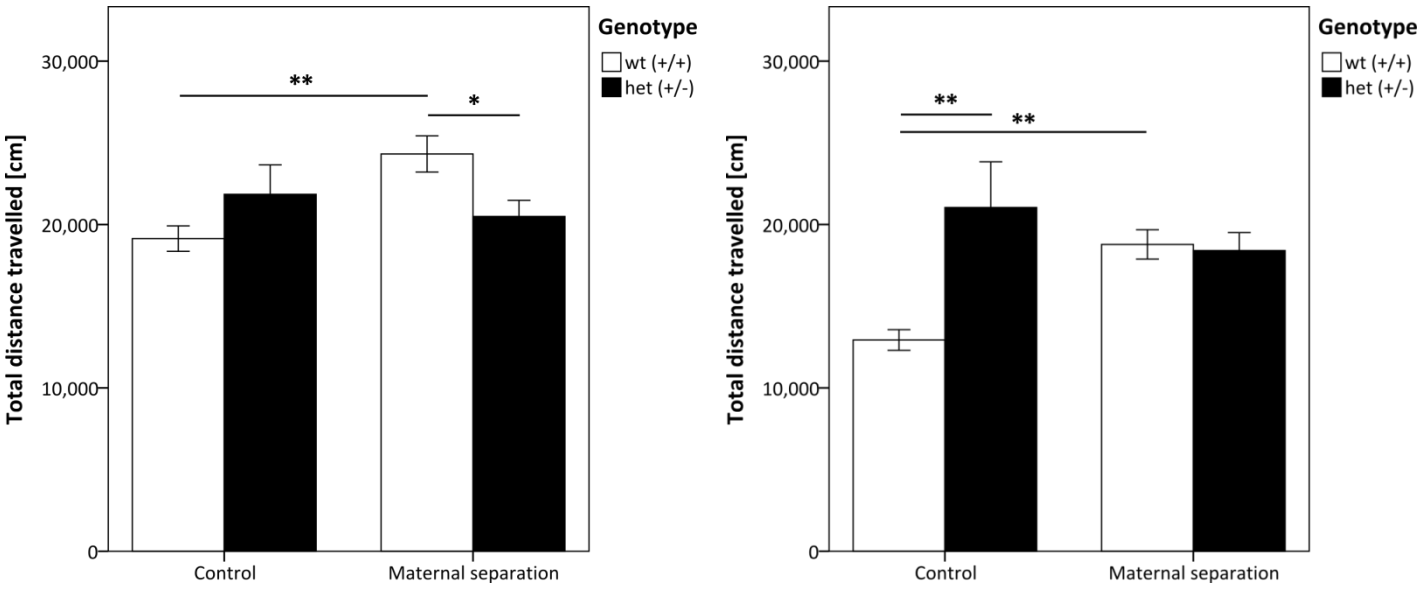

Figure 17: Distance travelled in the first (OF1, left) and second (OF2 right) one-hour Open Field test; Data are presented as means +/- SEM; **: p<0.01 / *: p<0.05 / #: p<0.1

OF1: The Bonferroni-Holm adjusted post-hoc t-tests reveal that though wild-type mice were significantly more active when they were subjected to MS, heterozygous mice remain on the same activity level. Heterozygous stressed mice are also significantly less active than stressed wild-type mice (Figure 17).

OF2: In the control group, heterozygous mice are more active than wild-type mice, but not in the MS group. As in OF1, for the wild-types the early-life stress has an enhancing effect on activity, but there is no difference for the heterozygous animal.

3.3.1.2 Light-Dark-Box

Table 12 summarizes the ANOVA results for the two most important measurements from the LDB. Time spent in the lit compartment did not reach significance for either the main effects or the interaction, but the latency to enter the lit compartment is significantly different between the two environment conditions and even marginally significant for the interaction of genotype and environment.

Table 12: ANOVA results for the Light-Dark-Box

	Effect	$F_{(1;72)}$	Significance
Time lit zone	Genotype	0.315	p = 0.577
	Environment	1.750	p = 0.190
	G x E	1.243	p = 0.269
Latency to lit zone	Genotype	0.039	p = 0.844
	Environment	14.394	***p < 0.001***
	G x E	2.849	p = 0.096

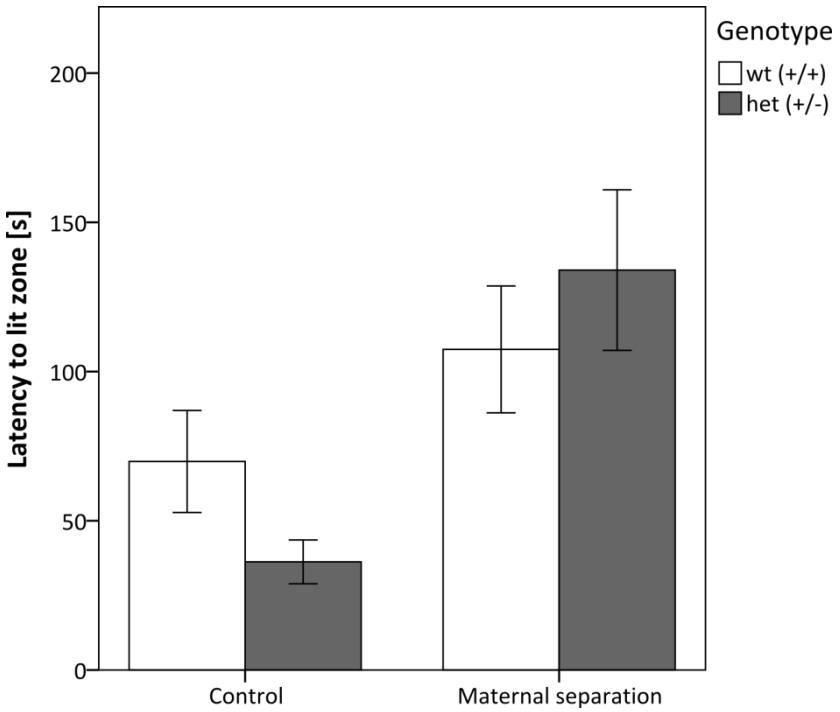

Figure 18: The latency to enter the lit zone of the Light-Dark Box

Figure 18 shows that independent from the genotype animals from the MS group take longer to enter the lit compartment. Even though the post-hoc tests don't reach significance, the marginally significant effect for the interaction seems to stem from the numerical difference between the heterozygous animals in the two environmental groups.

3.3.1.3 Modified 5-Choice-Serial-Reaction-Time-Task

3.3.1.3.1 Habituation

The ANOVA results from selected parameters of the habituation phase of the 5CSRTT can be found in Table 13. Heterozygous animals are marginally faster to inspect all 5 holes than the wild-types (means not shown), and although the trial effect almost reaches significance (meaning the time on the whole goes down over trials) this is overshadowed by a strong effect for trial x environment which shows that although the time to inspect all holes goes down over trials for the control group, it remains static for the MS group (means not shown). The same is true for the trial x environment effect for the total number of nose-pokes, but with the control group making more and more nose-pokes over trial while the MS group stagnates.

Table 13 : ANOVA results for the Habituation phase of the 5CSRTT

		Effect	F	Significance
Time to inspect all holes	between $F_{(1;35)}$	Genotype	3.247	p = 0.080
		Environment	0.284	p = 0.597
		G x E	0.001	p = 0.982
	within $F_{(4.892;171.234)}$	Trial	2.247	p = 0.053
		Trial x G	0.830	p = 0.528
		Trial x E	6.111	**p < 0.001**
		Trial x G x E	0.795	p = 0.552
Number of nose-pokes	between $F_{(1;35)}$	Genotype	1.797	p = 0.189
		Environment	0.001	p = 0.973
		G x E	3.117	p = 0.086
	within $F_{(3.553;124.363)}$	Trial	1.436	p = 0.230
		Trial x G	0.694	p = 0.581
		Trial x E	4.805	***p = 0.002***
		Trial x G x E	0.959	p = 0.425
Number of pellets eaten	between $F_{(1;35)}$	Genotype	0.983	p = 0.328
		Environment	2.644	p = 0.113
		G x E	0.285	p = 0.597
	within $F_{(4.746;166.096)}$	Trial	6.745	***p < 0.001***
		Trial x G	0.754	p = 0.578
		Trial x E	13.238	***p < 0.001***
		Trial x G x E	1.357	p = 0.245

Exemplary for the many significant trial x environment interactions in the habituation phase, mean data for the parameter “pellets eaten” are shown here (Figure 19). The left side shows the control groups, for which the number of rewards consumed increases over time, as it can be expected when learning a new task. For the maternal separation groups (right side), the number of pellets eaten remains the same or even goes down a little.

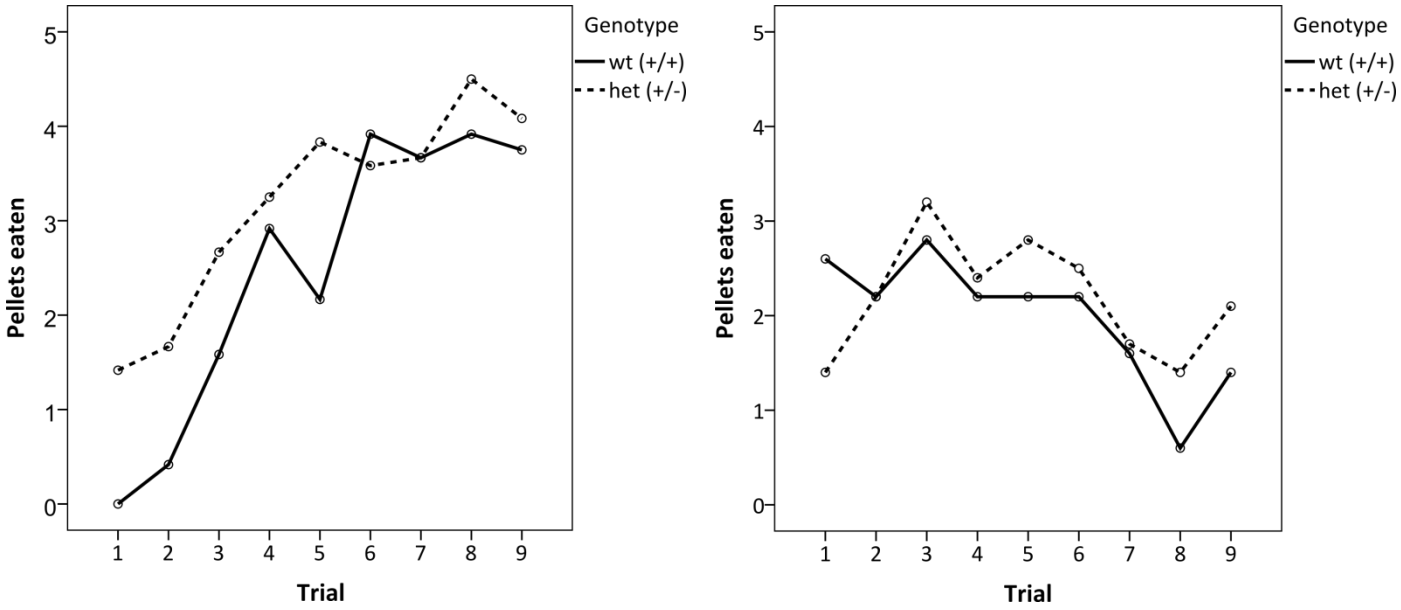

Figure 19: Pellets eaten for the 9 sessions of the habituation phase; Control group on the left, MS group on the right;

3.3.1.3.2 Autoshaping 1

The same as in the habituation phase, in Autoshaping 1 the predominant effects are the trial x environment interactions, which overshadow the simple main effects for trial and environment (see Table 14). For example, the number of total nose-pokes goes down over time for the control groups (as it is to be expected when the task is accurately learned and less nose-pokes are required to reach the goal), but up for the MS groups (means not shown). As an example, the parameter “number of pellets eaten” is shown in Figure 20. Here the main effect environment can be seen (Control groups eat more rewards than the MS groups), but also the trial x environment interaction (Control groups eat a constant of around 9 pellets per session, whereas MS groups start at around 3 and work their way up to around 7 over sessions.

Table 14: ANOVA results for the Autoshaping 1 phase of the 5CSRTT

		Effect	F	Significance
Time to finish	between $F_{(1;30)}$	Genotype	0.796	p = 0.379
		Environment	16.072	***p < 0.001***
		G x E	0.069	p = 0.795
	within $F_{(3.058;\ 91.727)}$	Trial	22.537	***p < 0.001***
		Trial x G	0.999	p = 0.398
		Trial x E	2.642	p = 0.053
		Trial x G x E	1.329	p = 0.270
Number of nose-pokes	between $F_{(1;30)}$	Genotype	0.013	p = 0.910
		Environment	13.469	***p = 0.001***
		G x E	0.732	p = 0.399
	within $F_{(2.927;87.807)}$	Trial	0.612	p = 0.605
		Trial x G	1.174	p = 0.324
		Trial x E	4.755	***p = 0.004***
		Trial x G x E	0.790	p = 0.500
Number of correct nose-pokes	between $F_{(1;30)}$	Genotype	0.279	p = 0.601
		Environment	20.287	***p < 0.001***
		G x E	0.507	p = 0.482
	within $F_{(3.453;103.583)}$	Trial	11.331	***p < 0.001***
		Trial x G	0.491	p = 0.715
		Trial x E	5.668	***p = 0.001***
		Trial x G x E	0.510	p = 0.702
Number of pellets eaten	between $F_{(1;30)}$	Genotype	0.343	p = 0.562
		Environment	34.599	***p < 0.001***
		G x E	0.385	p = 0.540
	within $F_{(2.921;87.618)}$	Trial	18.780	***p < 0.001***
		Trial x G	0.483	p = 0.690
		Trial x E	9.272	***p < 0.001***
		Trial x G x E	0.337	p = 0.794

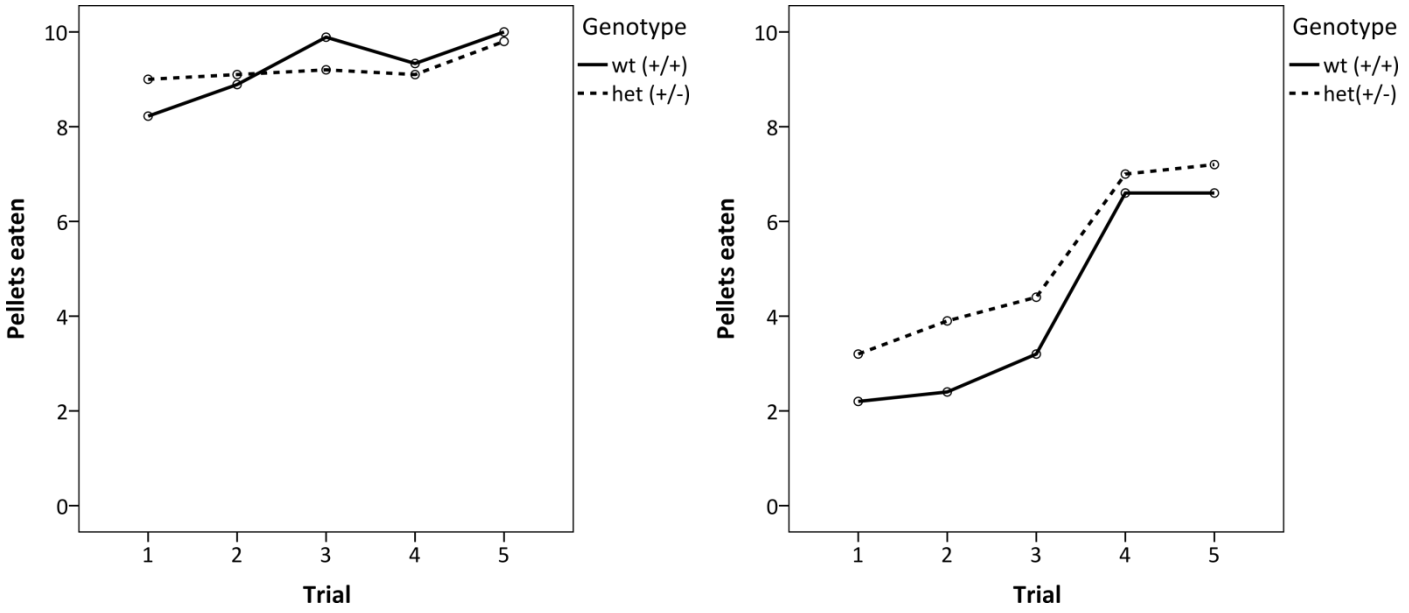

Figure 20: Pellets eaten for the 5 sessions of the Autoshaping 1 phase; Control group: left; MS group: right;

3.3.1.3.3 Autoshaping 2

In the Autoshaping 2 phase, the predominant effect was the main effect for trial, which can be found in all of the parameters in Table 15. Interestingly, the new parameter measured (pellets eaten correctly) was the only one to show a strong genotype effect, that is to say the wild-types ate more pellets directly after the correct response than the heterozygous animals, both for the MS and the control group (Figure 21).

Table 15: ANOVA results for the Autoshaping 2 phase of the 5CSRTT

		Effect	F	Significance
Time to finish	between $F_{(1;30)}$	Genotype	0.648	p = 0.427
		Environment	0.482	p = 0.493
		G x E	0.211	p = 0.650
	within $F_{(2.240;67.214)}$	Trial	3.136	***p = 0.044***
		Trial x G	0.351	p = 0.729
		Trial x E	0.498	p = 0.631
		Trial x G x E	1.401	p = 0.253
Number of nose-pokes	between $F_{(1;30)}$	Genotype	0.789	p = 0.381
		Environment	0.430	p = 0.517
		G x E	2.615	p = 0.116
	within $F_{(2.748;82.443}$	Trial	9.412	***p < 0.001***
		Trial x G	0.707	p = 0.539
		Trial x E	1.559	p = 0.209
		Trial x G x E	1.459	p = 0.234
Number of correct nose-pokes	between $F_{(1;30)}$	Genotype	0.488	p = 0.490
		Environment	0.676	p = 0.417
		G x E	1.703	p = 0.202
	within $F_{(2.716;81.489)}$	Trial	3.299	***p = 0.028***
		Trial x G	0.282	p = 0.819
		Trial x E	1.292	p = 0.283
		Trial x G x E	0.614	p = 0.592
Number of pellets eaten	between $F_{(1;30)}$	Genotype	0.168	p = 0.685
		Environment	2.540	p = 0.121
		G x E	1.627	p = 0.212
	within $F_{(2.579;77.373)}$	Trial	2.670	p = 0.062
		Trial x G	0.483	p = 0.667
		Trial x E	1.191	p = 0.316
		Trial x G x E	0.650	p = 0.563
Number of pellets eaten correctly	between $F_{(1;30)}$	Genotype	8.620	***p = 0.006***
		Environment	0.542	p = 0.467
		G x E	1.086	p = 0.306
	within $F_{(2.506;75.195)}$	Trial	5.122	***p = 0.005***
		Trial x G	0.948	p = 0.409
		Trial x E	0.951	p = 0.408
		Trial x G x E	0.942	p = 0.412

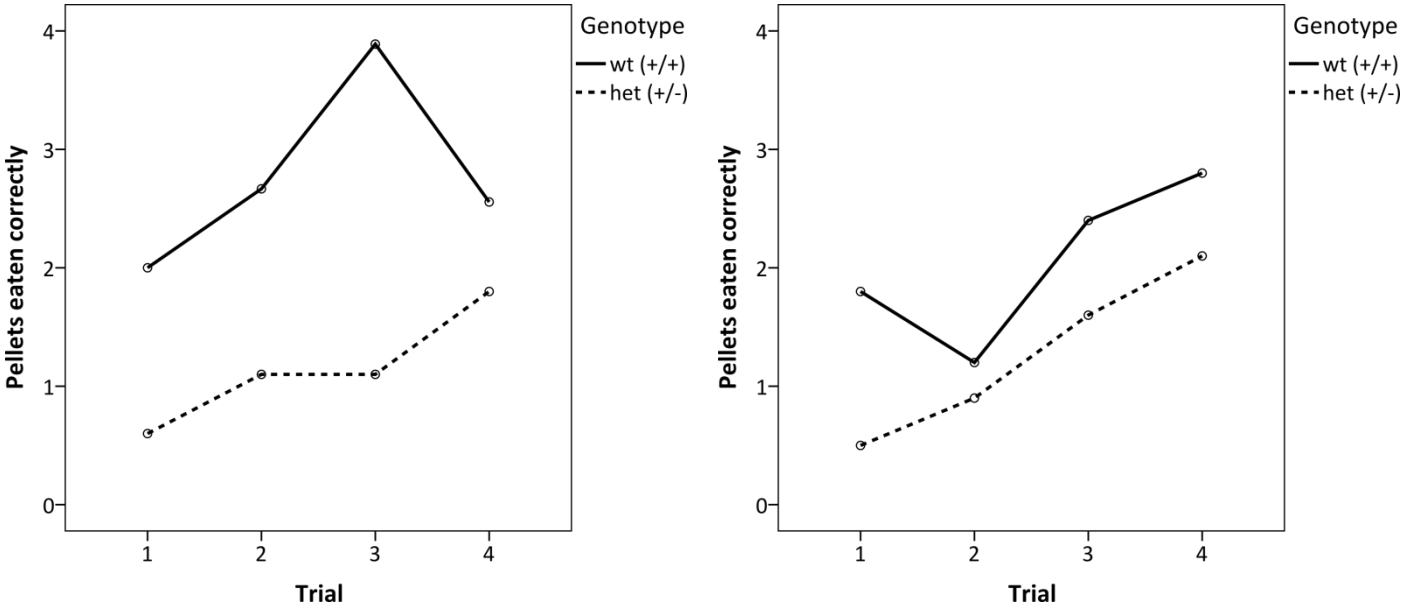

Figure 21: Pellets eaten correctly for the 4 sessions of the Autoshaping 2 phase; Control group on the left, MS group on the right;

3.3.1.3.4 Experimental phase with 20 s stimuli

In the experimental phase, environmental effects were the most common (Table 16). MS animals made less overall nose-pokes, and also less premature and correct nose-pokes than the control group. Additionally, they ate fewer pellets and also failed more at eating them directly after the correct response (means not shown). The only genotype effect, albeit only marginally significant, could again be found in the new parameter "pellets eaten correctly", in such a way that, again, the heterozygous animals were prone to not directly eating their pellets after the correct nose-poke (Figure 22).

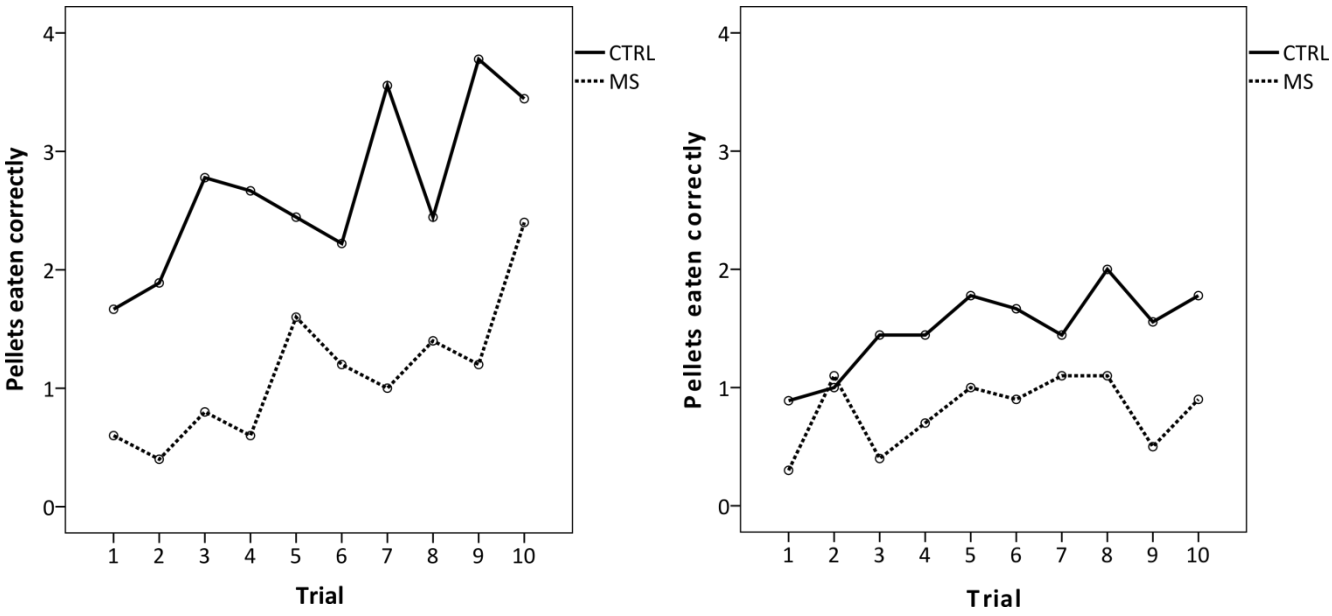

Figure 22: Pellets eaten correctly for the 10 sessions of the experimental 20s phase; wild-types on the left, heterozygous animals on the right;

Table 16: ANOVA results for the Ex20s phase of the 5CSRTT

		Effect	F	Significance
Time to finish	between $F_{(1;29)}$	Genotype	0.072	p = 0.791
		Environment	1.509	p = 0.229
		G x E	0.019	p = 0.893
	within $F_{(6.170;178.935)}$	Trial	6.827	***p < 0.001***
		Trial x G	0.387	p = 0.891
		Trial x E	1.192	p = 0.312
		Trial x G x E	1.000	p = 0.428
Number of nose-pokes	between $F_{(1;29)}$	Genotype	0.790	p = 0.381
		Environment	7.835	***p = 0.009***
		G x E	0.104	p = 0.749
	within $F_{(5.765;167.174)}$	Trial	7.094	***p < 0.001***
		Trial x G	0.730	p = 0.621
		Trial x E	1.957	p = 0.077
		Trial x G x E	0.669	p = 0.668
Number of correct nose-pokes	between $F_{(1;29)}$	Genotype	0.192	p = 0.665
		Environment	5.517	***p = 0.026***
		G x E	0.340	p = 0.564
	within $F_{(9;261)}$	Trial	2.808	***p = 0.004***
		Trial x G	1.902	p = 0.052
		Trial x E	1.029	p = 0.417
		Trial x G x E	0.455	p = 0.904
Number of premature nose-pokes	between $F_{(1;29)}$	Genotype	0.026	p = 0.874
		Environment	5.027	***p = 0.033***
		G x E	0.102	p = 0.752
	within $F_{(9;261)}$	Trial	4.495	***p < 0.001***
		Trial x G	0.467	p = 0.839
		Trial x E	2.200	***p = 0.022***
		Trial x G x E	0.726	p = 0.685
Number of pellets eaten	between $F_{(1;29)}$	Genotype	0.091	p = 0.765
		Environment	7.523	***p = 0.010***
		G x E	0.412	p = 0.526
	within $F_{(9;261)}$	Trial	3.071	***p = 0.002***
		Trial x G	2.124	***p = 0.028***
		Trial x E	1.059	p = 0.393
		Trial x G x E	0.570	p = 0.821
Number of pellets eaten correctly	between $F_{(1;29)}$	Genotype	3.293	p = 0.080
		Environment	7.445	***p = 0.011***
		G x E	1.092	p = 0.305
	within $F_{(6.482;187.990)}$	Trial	3.022	***p = 0.006***
		Trial x G	1.248	p = 0.281
		Trial x E	0.784	p = 0.593
		Trial x G x E	0.784	p = 0.592

3.3.1.3.5 Test Trial with 9 s stimuli

Rather different than in the other phases of the experiment, in the test trial the genotype effects were the most common (Table 17). The only significant environment effect found was for the time to finish the experiment, for which the MS animals took longer than the controls (means not shown). Significant and marginally significant effects for genotype included the heterozygous animals making fewer correct nose-pokes (Figure 23, although not fewer on the whole), ate fewer pellets and also fewer directly after the correct response (Figure 24).

Table 17: ANOVA results for the 9 s test trial of the 5CSRTT

	Effect	$F_{(1;29)}$	Significance
Time to finish	Genotype	0.592	p = 0.448
	Environment	4.392	***p = 0.045***
	G x E	0.226	p = 0.638
Number of nose-pokes	Genotype	1.354	p = 0.254
	Environment	0.916	p = 0.346
	G x E	2.383	p = 0.134
Number of correct nose-pokes	Genotype	4.133	p = 0.051
	Environment	2.565	p = 0.120
	G x E	0.650	p = 0.427
Percent correct nose-pokes	Genotype	4.508	***p = 0.041***
	Environment	0.114	p = 0.738
	G x E	1.800	p = 0.190
Number of premature nose-pokes	Genotype	0.801	p = 0.378
	Environment	0.146	p = 0.705
	G x E	2.951	p = 0.096
Number of pellets eaten	Genotype	3.743	p = 0.063
	Environment	2.566	p = 0.120
	G x E	0.701	p = 0.409
Number of pellets eaten correctly	Genotype	7.054	***p = 0.013***
	Environment	2.572	p = 0.120
	G x E	0.112	p = 0.740

Figure 25 depicts the number of premature nose-pokes, which is the only parameter with an – at least – marginally significant G x E interaction. Although not significant in the post-hoc test, this result seems to be based on the MS heterozygous animals, which, numerically, make fewer premature nose-pokes than all the other groups.

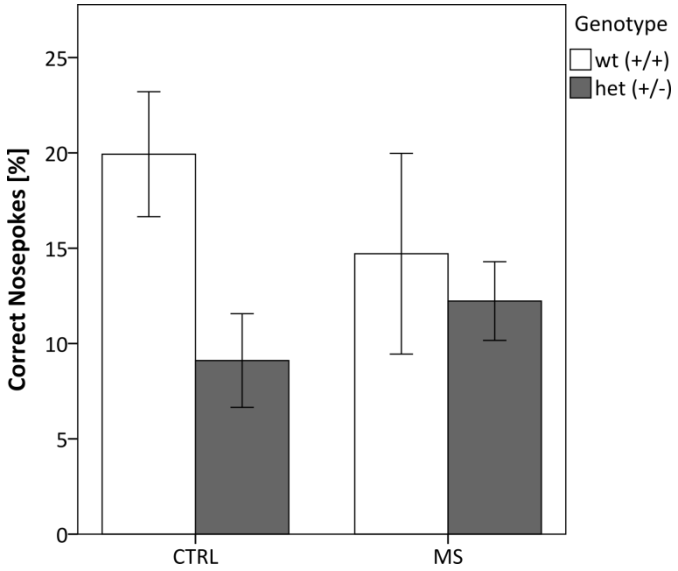

Figure 23: Percentage of correct nose-pokes in the test trial with 9 s stimuli

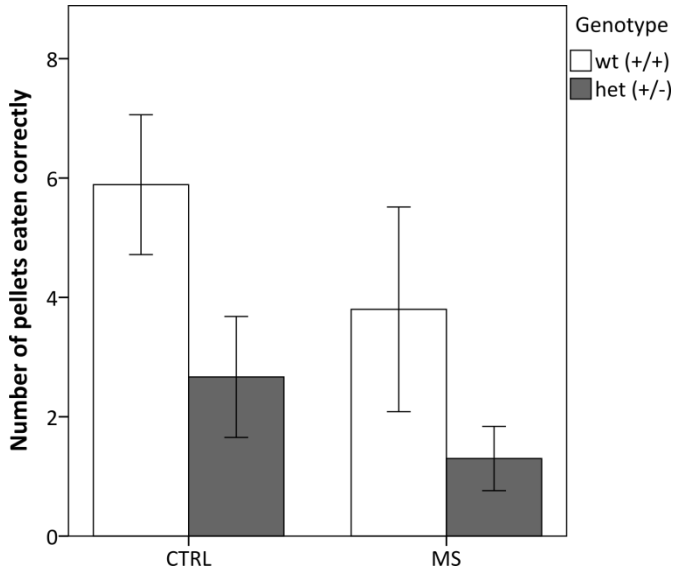

Figure 24: Number of pellets eaten correctly in the test trial with 9 s stimuli

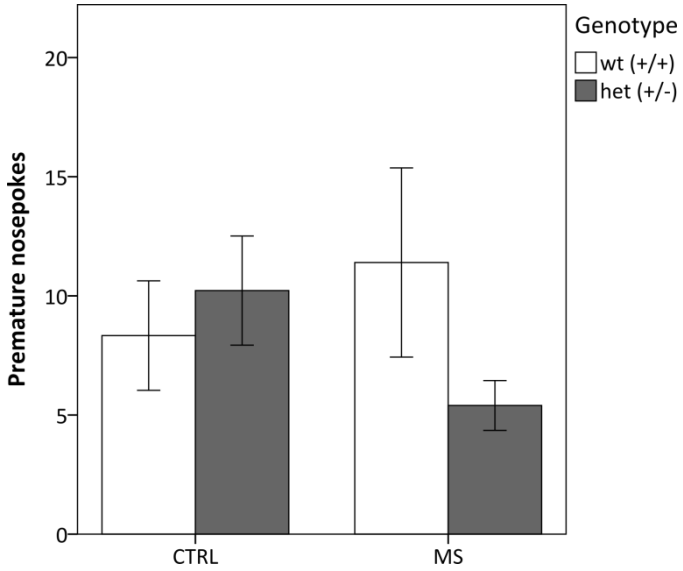

Figure 25: Number of premature nose-pokes in the test trial with 9 s stimuli

3.3.1.4 Forced-Swim-Test

Table 18 summarizes the ANOVA results for the Forced swim test, separately for males and females. The only significant result is the main effect for genotype in the males. As it can be seen in Figure 26 on the right side, heterozygous males spend significantly more time immobile independent from the stress group, arguing for elevated depression-like behavior. The same is not true for female mice.

Table 18: ANOVA results for immobility times in the Forced Swim Test

	Effect	$F_{males\ (1;26)/females\ (1;28)}$	Significance
Immobility time males	Genotype	11.459	***p = 0.002***
	Environment	0.752	p = 0.394
	G x E	0.652	p = 0.427
Immobility time females	Genotype	0.572	p = 0.456
	Environment	0.197	p = 0.661
	G x E	1.833	p = 0.187

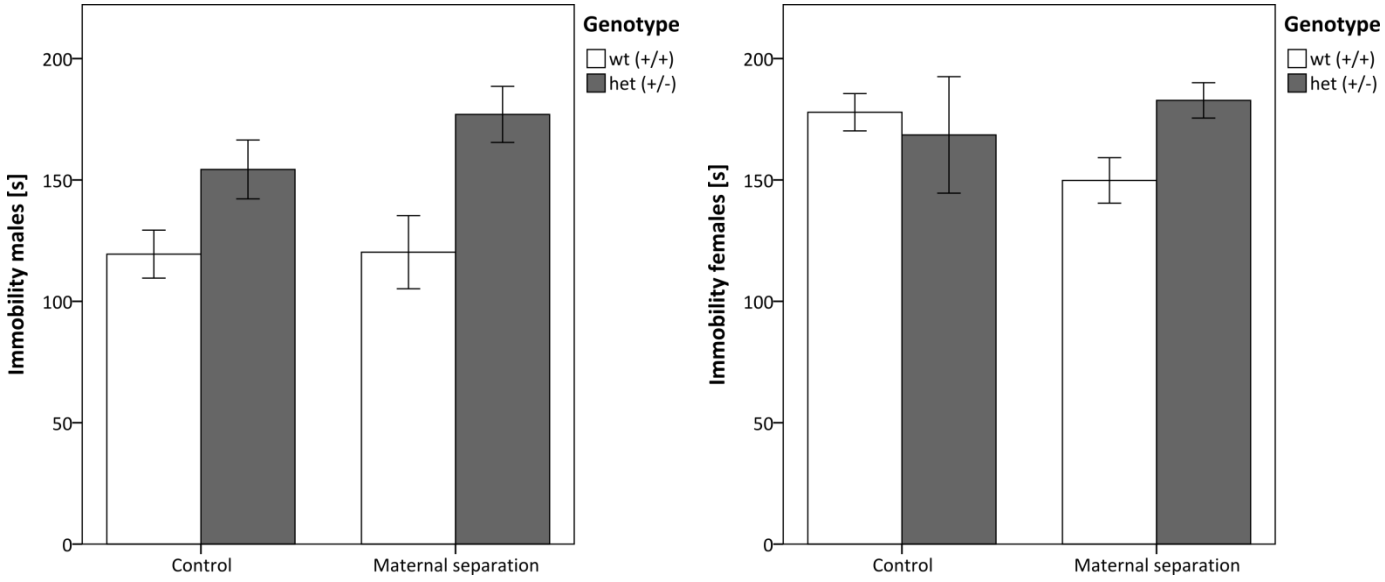

Figure 26: Time spent immobile in the Forced Swim Test (males on the right, females on the left)

3.3.1.5 Resident-Intruder-Paradigm

Since no mouse attacked another in the Resident-Intruder-Paradigm, data could not be evaluated due to the variation being equal to zero.

3.3.1.6 Methylphenidate Challenge Open Field

In the MPH challenge both the main effects and the interaction reached at least marginal significance (Table 19). The strongest effect was seen for the MPH treatment which enhanced activity overall, as it was to be expected. The marginal significance for the genotype by treatment interaction can numerically be explained from the more pronounced increase in activity after MPH administration for the heterozygous animals (Figure 27).

Table 19: ANOVA results for distance travelled in the Open Field after MPH challenge

	Effect	$F_{(1;27)}$	Significance
Distance travelled OF	Genotype	3.555	p = 0.070
	MPH	8.564	***p = 0.007***
	Genotype x MPH	3.109	p = 0.089

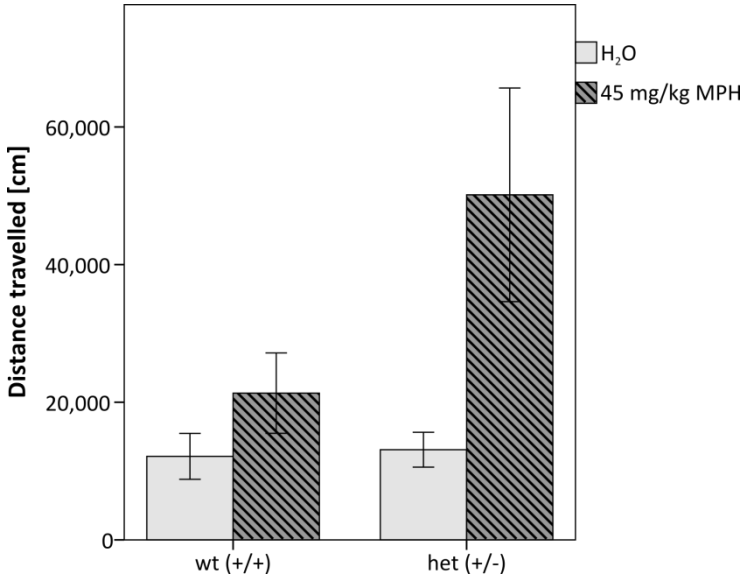

Figure 27: Distance travelled after the consumption of a cereal flake with 45 mg/kg MPH or water

3.3.2 Quantitative Real-Time Polymerase Chain Reaction

3.3.2.1 Snap25

Snap25 expression was significantly enhanced in all three analyzed brain regions (see Table 20 and Figure 28). Numerically, wild-types expressed almost twice the amount of *Snap25* mRNA as heterozygous animals.

Table 20: ANOVA results for *Snap25* expression

Snap25	**Effect**	**$F_{(1;70)}$**	**Significance**
Frontal cortex	Genotype	276.313	***p < 0.001***
	Environment	0.034	p = 0.855
	G x E	0.275	p = 0.602
Hippocampus	Genotype	330.673	***p < 0.001***
	Environment	0.074	p = 0.787
	G x E	0.596	p = 0.443
Striatum	Genotype	204.608	***p < 0.001***
	Environment	0.097	p = 0.756
	G x E	4.333	***p = 0.041***

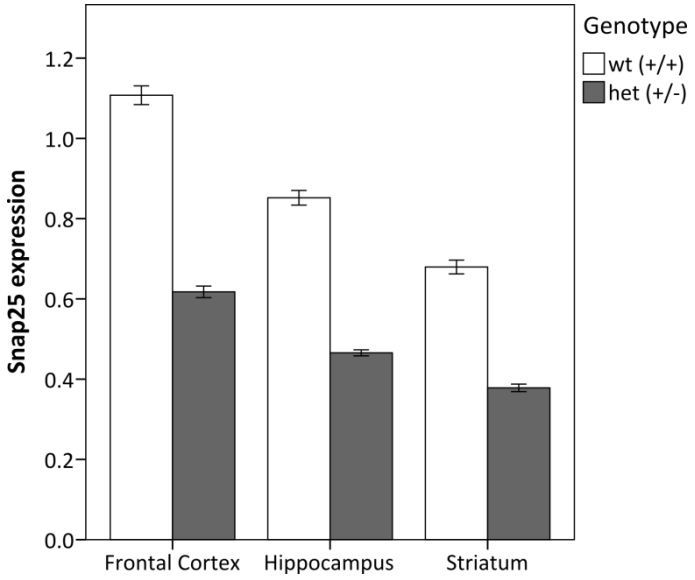

Figure 28: *Snap25* expression (independent of stress group) in the Frontal cortex, the Hippocampus and the Striatum of *Snap25* +/+ and +/- mice

3.3.2.2 Comt

Table 21: ANOVA results for *Comt* expression

Comt	Effect	$F_{(1;71)}$	Significance
Frontal cortex	Genotype	5.487	***p = 0.022***
	Environment	2.007	p = 0.161
	G x E	2.181	p = 0.144
Hippocampus	Genotype	0.632	p = 0.429
	Environment	4.432	***p = 0.039***
	G x E	0.155	p = 0.695
Striatum	Genotype	0.577	p = 0.450
	Environment	3.254	p = 0.075
	G x E	0.004	p = 0.949

Comt expression in the frontal cortex depended significantly on the genotype; heterozygous animals expressed less *Comt* mRNA than wild-types. In the hippocampus and the striatum, the main effect environment reached at least marginal significance. In both cases, stressed animals were prone to lower expression levels (Table 21, Figure 29).

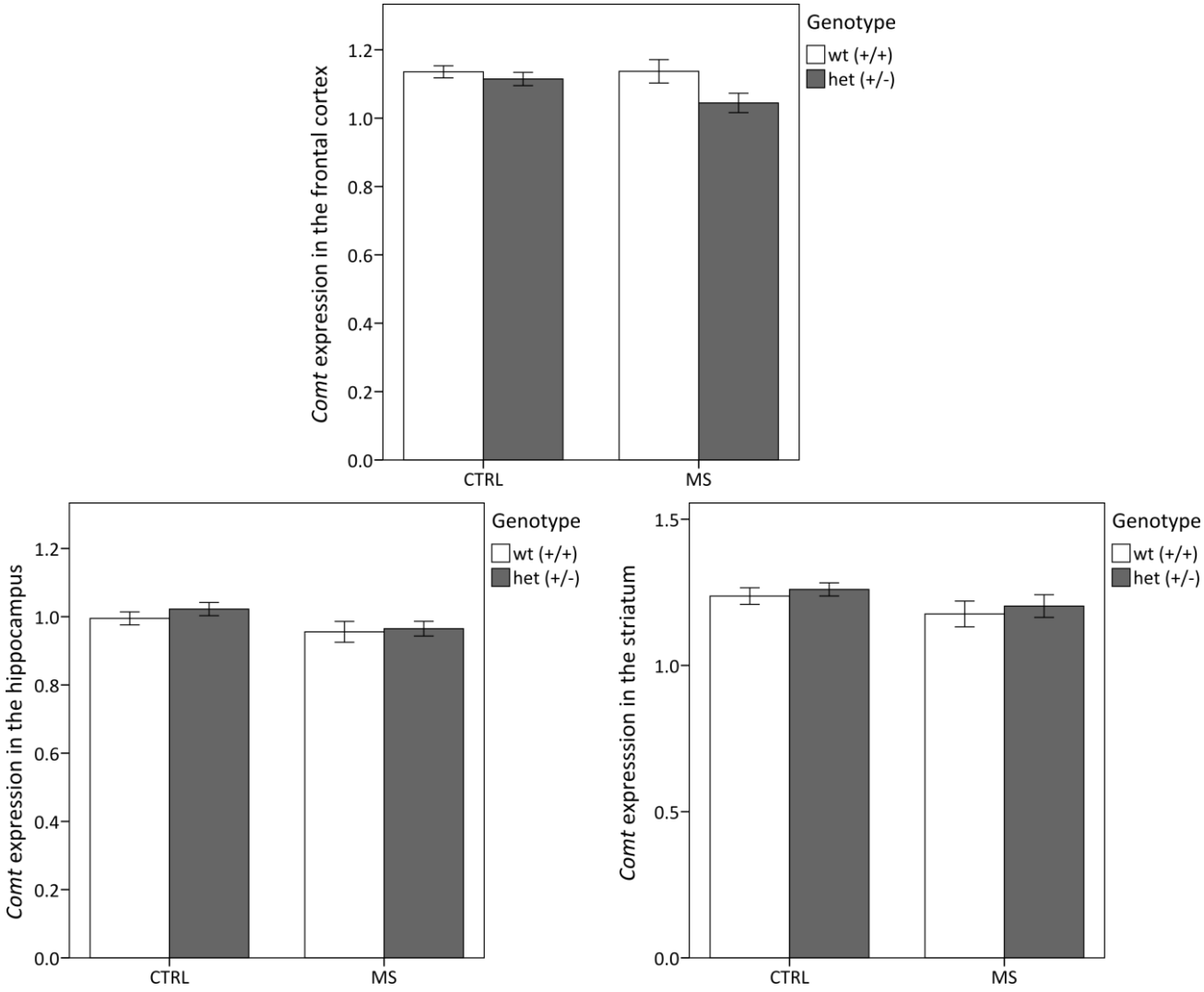

Figure 29: *Comt* expression in the Frontal cortex (top), the Hippocampus (bottom left) and the Striatum (bottom right) for both stress groups of Snap25 +/+ and +/- mice

3.3.2.3 Maoa

Table 22: ANOVA results for *Mao-a* expression

Maoa	**Effect**	**$F_{(1;71)}$**	**Significance**
Frontal cortex	Genotype	9.251	***p = 0.003***
	Environment	0.080	p = 0.777
	G x E	3.398	p = 0.069
Hippocampus	Genotype	1.999	p = 0.162
	Environment	1.075	p = 0.303
	G x E	0.157	p = 0.693
Striatum	Genotype	0.688	p = 0.410
	Environment	0.169	p = 0.682
	G x E	2.579	p = 0.113

Maoa expression only was significantly altered in the frontal cortex, where the main effect for genotype was the strongest (Table 22): wild-types had higher expression levels than heterozygous animals (Figure 30). The marginal significance for the G x E interaction is numerically based on the fact that wild-types and heterozygous animals differ more strongly for the control group than for the MS group.

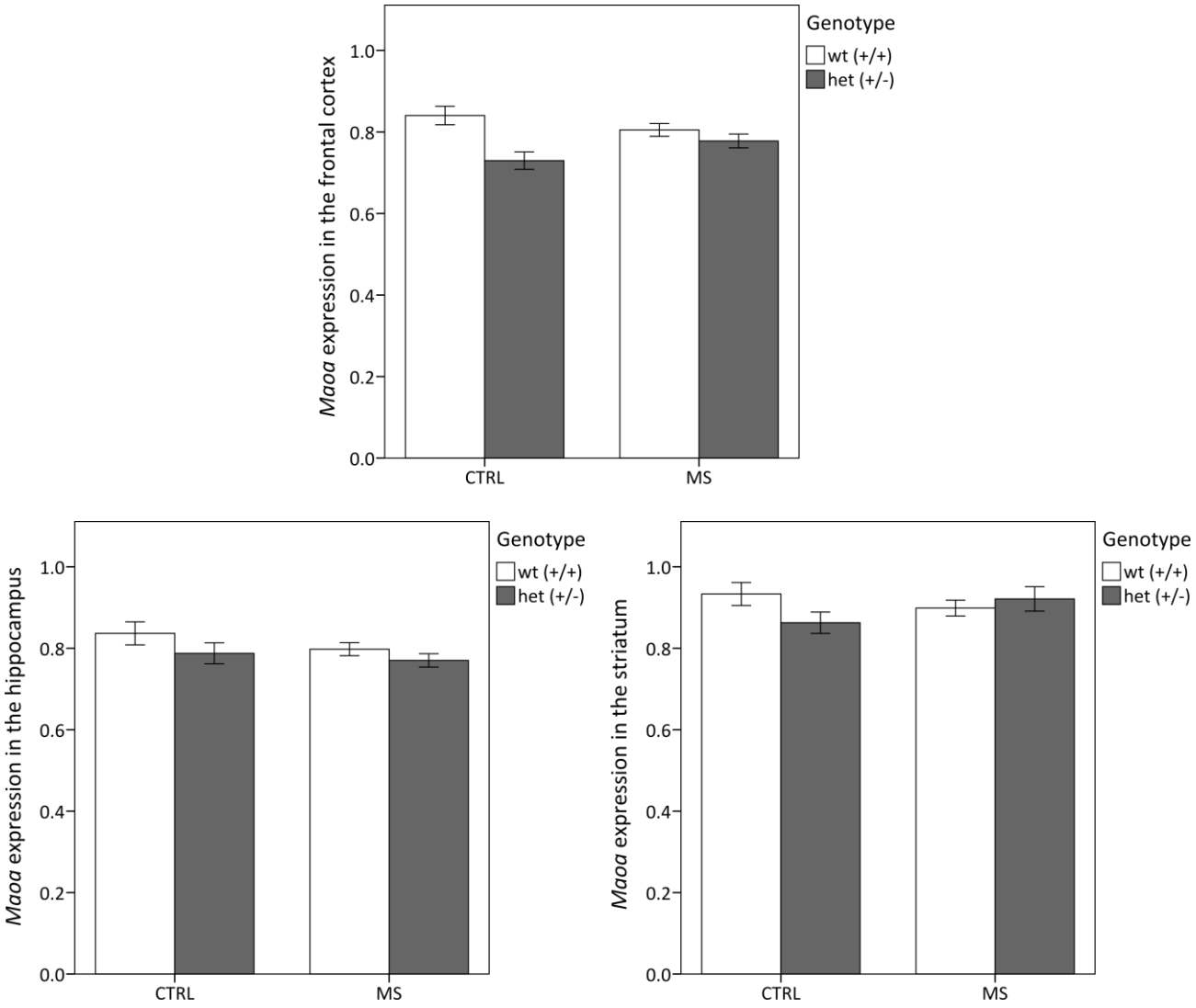

Figure 30: *Maoa* expression in the Frontal cortex (top), the Hippocampus (bottom left) and the Striatum (bottom right) for both stress groups of Snap25 +/+ and +/- mice

3.3.2.4 Drd2

Except for a significant main effect of environment in the striatum, there are no effects to be found on *Drd2* expression (Table 23). Stressed animals were lower in DRD2 mRNA expression than controls (Figure 31).

Table 23: ANOVA results for *Drd2* expression

Drd2	Effect	$F_{(1;71)}$	Significance
Frontal cortex	Genotype	0.654	p = 0.422
	Environment	2.209	p = 0.142
	G x E	0.094	p = 0.760
Hippocampus	Genotype	0.058	p = 0.811
	Environment	1.609	p = 0.209
	G x E	0.077	p = 0.783
Striatum	Genotype	1.154	p = 0.286
	Environment	5.170	***p = 0.026***
	G x E	2.024	p = 0.159

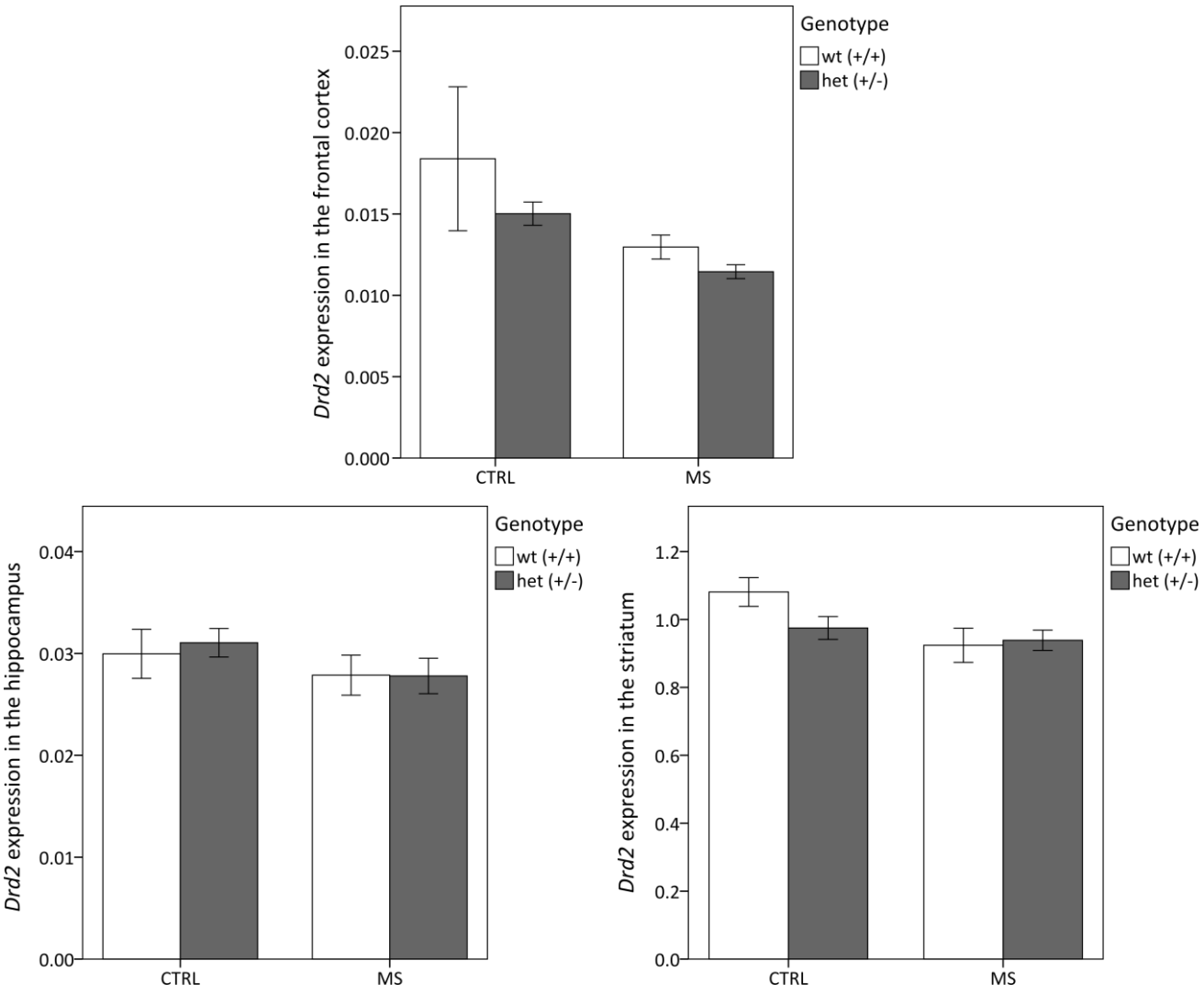

Figure 31: *Drd2* expression in the Frontal cortex (top), the Hippocampus (bottom left) and the Striatum (bottom right) for both stress groups of Snap25 +/+ and +/- mice

3.3.2.5 Nos1

Nos1 expression was strongly affected in all three examined brain regions (Table 24). All three showed strongly significant main effects for environment, for all of which it was true that animals from the control group had higher levels of expression than the stressed group. In the frontal cortex, heterozygous animals expressed more *Nos1* than wild-types, although this effect seems stronger in the non-stressed control group, probably due to the marginally significant G x E interaction (Figure 32). This interaction effect looks very similar to the significant one in the striatum, where heterozygous animals expressed significantly more *Nos1* than wild-types in the control condition, but went down in expression significantly when stressed, whereas wild-types stayed at the same level (Figure 32).

Table 24: ANOVA results for NOS1 expression

Nos1	**Effect**	**$F_{(1;71)}$**	**Significance**
Frontal cortex	Genotype	7.050	***p = 0.010***
	Environment	13.546	***p < 0.001***
	G x E	3.717	p = 0.058
Hippocampus	Genotype	0.142	p = 0.707
	Environment	8.776	***p = 0.004***
	G x E	0.709	p = 0.403
Striatum	Genotype	2.109	p = 0.151
	Environment	28.545	***p < 0.001***
	G x E	8.090	***p = 0.006***

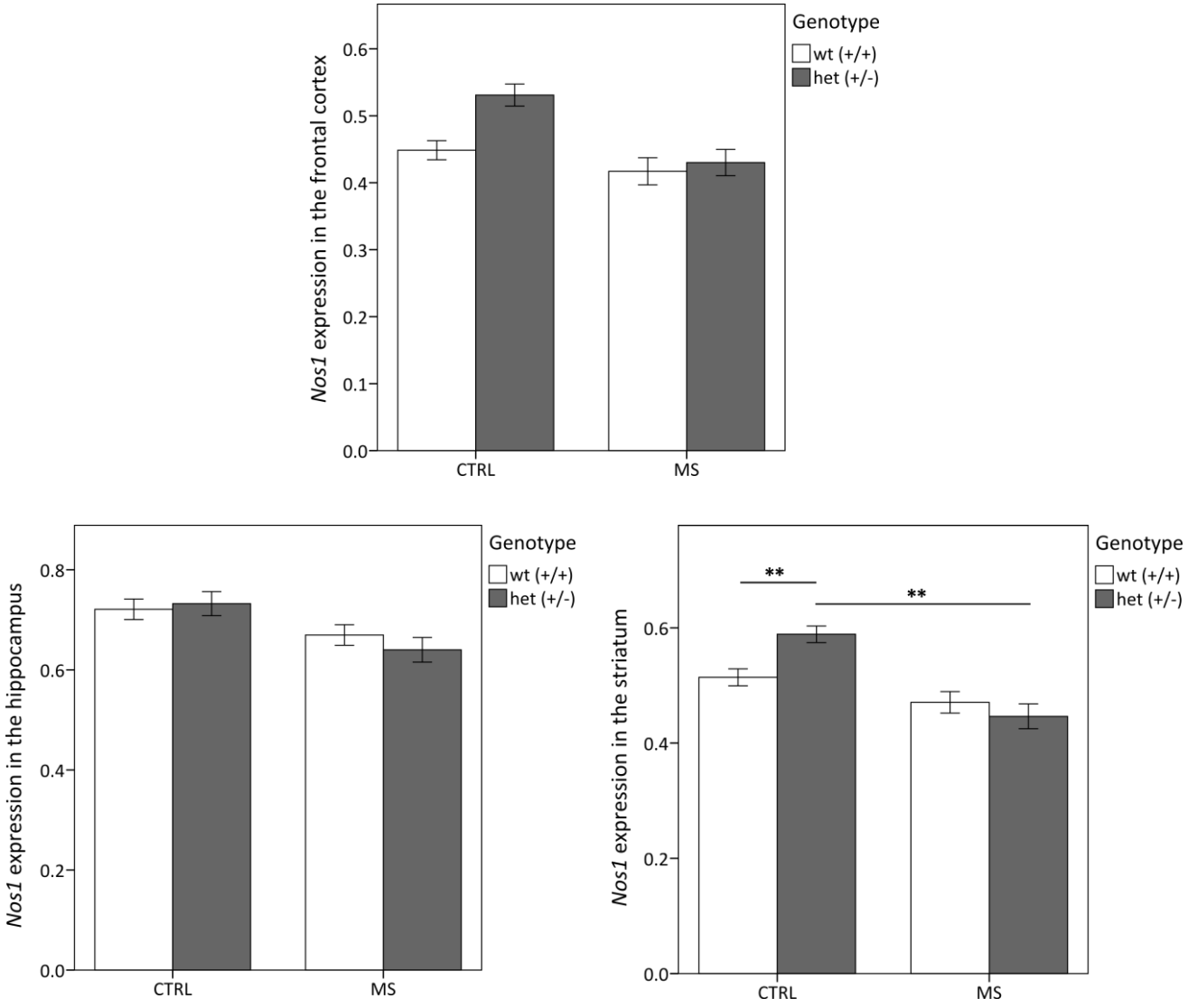

Figure 32: *Nos1* expression in the Frontal cortex (top), the Hippocampus (bottom left) and the Striatum (bottom right) for both stress groups of Snap25 +/+ and +/- mice

3.3.3 Corticosterone Analysis and adrenal weights

Heterozygous animals had significantly lower corticosterone plasma levels than wild-types (Table 25, Figure 33) independent from the stress group, arguing for a dysregulation of the HPA axis.

Table 25: ANOVA results for corticosterone levels in blood plasma

	Effect	$F_{(1;71)}$	Significance
Corticosterone in plasma	Genotype	3.967	***p = 0.050***
	Environment	0.043	p = 0.836
	G x E	0.063	p = 0.803

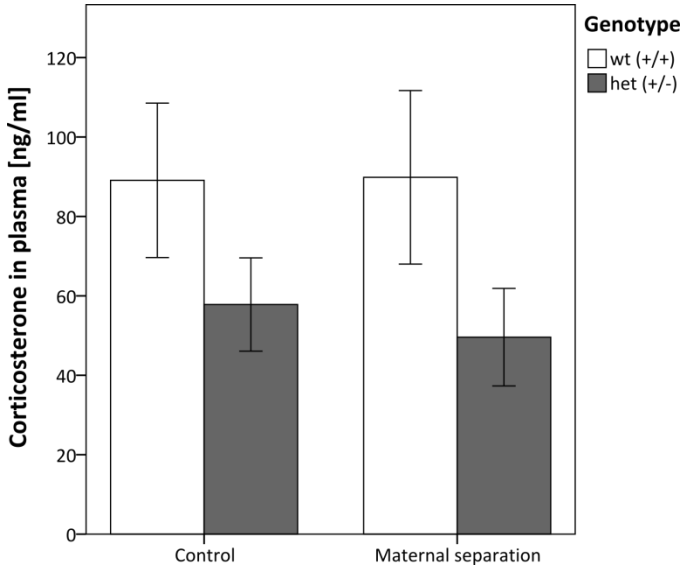

Figure 33: Post mortem corticosterone levels in blood plasma of all animals from the G x E study

The adrenal weights in males were significantly dependent on the environment conditions (Table 26); stressed animals had lower adrenal weights than un-stressed controls (Figure 34). In females the environment did not play a role, but heterozygous animals had lower adrenal weights than wild-types (Figure 34).

Table 26: ANOVA results for adrenal weights

	Effect	$F_{(1;34)}$	Significance
Adrenal weight males	Genotype	0.039	p = 0.844
	Environment	6.446	***p = 0.016***
	G x E	0.946	p = 0.338
Adrenal weight females	Genotype	12.594	***p = 0.001***
	Environment	2.422	p = 0.129
	G x E	0.665	p = 0.420

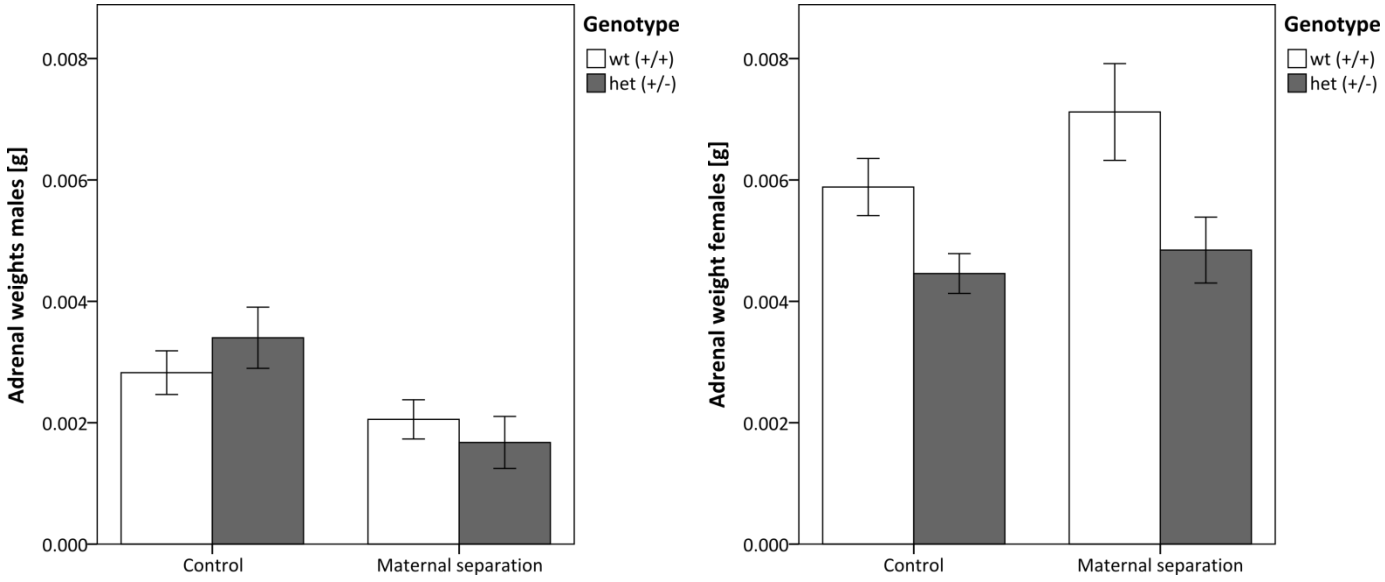

Figure 34: Post mortem adrenal weights of all animals from the G x E study; left: males; right: females

4 Discussion

4.1 Pilot study MPH

The MPH pilot study was performed to find an optimal dosage of MPH for potential mouse ADHD models in behavioral experiments both i.p. and oral. Although an effective dosage of MPH in patients with ADHD results in an enhanced focus and thus usually attenuates the symptom of hyperactivity, it acts as a stimulant in healthy subjects and is even known to lead to dependence in some cases (Kollins et al., 2001). Thus, in healthy control mice like the ones used in this pilot study, the expected effect of MPH was not decreased, but rather increased locomotor activity (Tilley & Gu, 2008). For both the i.p. and the oral group, MPH as expected significantly changed locomotor activity. In the former, the first significant dosage was 7.5 mg/kg and in the latter 15 mg/kg, whereas they peaked in activity at 30 mg/kg and 60 mg/kg, respectively. At doses higher than that, activity declined again for both i.p. and oral groups, making the two resulting graphs bell-shaped. This resembles graphs from studies researching critical dopamine levels and dopamine D1 receptor activation (Goldman-Rakic, 2000; Seamans & Yang, 2004), which concluded that both too little and too much dopamine availability can have adverse effects, and an optimal, intermediate dopamine level is to be aimed for when medicating patients with deficiencies in the dopaminergic system. Since MPH acts as a dopamine / noradrenalin reuptake inhibitor and as such increases dopamine levels in the synaptic cleft, the bell-shaped graphs found here are in line with these conclusions. On the behavioral level, the optimal dosage for mice should be one that significantly and stably modifies the contemplated behavior. Thus 7.5 mg/kg i.p. is here considered a good dose. The question of what is the best oral dose is a little more complicated to answer, since although the total distance travelled in the 15 mg/kg oral group is very much like in the 7.5 mg/kg i.p. group, the time response over 90 minutes is rather different (data not shown). In the 15 mg/kg oral group, activity after the first 30 minutes after drug application declines more than in the 7.5 mg/kg i.p. group and overall, the curve is flatter. In contrast, the graph for the 60 mg/kg oral group is not as flat and appears more stable, but exhibits some inconsistencies at the end of the 90 minutes with rather high activity peaks. Thus an intermediate dose of 45 mg/kg was chosen as the best oral dose and used in the *Snap25* study.

For the HPLC analysis, the two brain regions of interest chosen were the frontal cortex (encompassing the prefrontal and the motor cortex) and the striatum, since frontostriatal pathways are implied in ADHD (Kasparek et al., 2013) and both the motor cortex and the nigrostriatal dopamine system are associated with motor function (Wise, 2004). The neurotransmitters and metabolites investigated were DA, 5HT, NA and their metabolic turnovers, because MPH binds to the dopamine transporter, the norepinephrine transporter and the serotonin transporter in decreasing affinity (Gatley et al., 1996). Probably the most interesting result is DA in the frontal cortex where the curves are very similar for i.p. and oral, both in level and shape of the graphs. Numerically, they peak at 7.5 mg/kg i.p. and 30 mg/kg oral, which is in line with the first doses that were effective behaviorally. The metabolic turnovers peak a 30 mg/kg i.p. and 60 mg/kg oral and decline at the higher doses, thus a bell-shaped curve results. Considering the work of Goldman-Rakic (2000) and Seamans & Yang (2004), it appears as though the optimal concentration of dopamine in the synaptic cleft and thus the optimal binding to dopamine receptors in the frontal cortex is achieved somewhere in the range of 7.5-30 mg/kg i.p. and 30-60 mg/kg oral. In the striatum, where dopaminergic neurotransmission should also affect motor function, since the nigrostriatal pathway projects into the dorsal striatum (Wise, 2004), the picture is quite different. While for i.p. doses the DA concentration values numerically peak at 1 mg/kg and the metabolic turnovers significantly peak at 15 mg/kg and then decline, the different oral doses do not change DA concentrations at all. The metabolic turnover is only affected at the highest dose of 90 mg/kg, which might well be an artefact. Seemingly, oral application of MPH in mice does not influence DA concentrations in the striatum. Another issue to address here is the marked difference in DA concentration between the oral and the i.p. 0 mg/kg groups. These differences can also be found for 5HT and NA in the frontal cortex and the striatum. Since none of these groups actually received an effective substance, the reason for this is probably founded on the application method. It is well known today that laboratory environment can influence behavioral tests in rodents (Crabbe, 1999). This includes for example temperature, lighting, housing and weather (Stille et al., 1968) and of course painful and stressful events like injections (Drude et al., 2011). It has been one major aim of this pilot study to establish a relatively stress-free oral application method. This appears to be not only important in regard to behavior, but also to basal neurotransmitter levels. Serotonin concentrations in the frontal cortex and the striatum are very different for the i.p.

and the oral groups. Apart from the control group differences in both brain regions, 5HT concentration in the frontal cortex goes up for 7.5 mg/kg i.p. and then down again, whereas it goes down for 10 mg/kg oral and then slightly up again. A similar picture can be seen in the striatum with an i.p. peak of 45 mg/kg but no effects oral. This could be an artefact of the lower baseline in the i.p. groups but also be based on the application method itself as the bioavailability for i.p. and oral application MPH is very different (Gerasimov et al., 2000). Additionally, it is possible that the time point of brain removal at about 100 minutes after the drug application was either too early or too late for the oral group and not at an effective level. For a future study, microdialysis might be a better method to investigate this on-line. Norepinephrine concentrations do not differ for either brain region or application method.

4.2 Pilot study COGITAT

The pilot study COGITAT Holeboard was carried out to modify and validate the system that had previously been validated in rats (Heim et al., 2000) and to assess the feasibility of this set-up as a measure of ADHD-like (endo-)phenotypes. The COGITAT Holeboard was able to assess activity and learning measures in mice. In contrast to the Morris Water Maze (Morris, 1984) where learning occurs under pressure as mice are forced to find an escape from the water as quickly as possible, the COGITAT system is based on motivational parameters. On the other hand, in mice this means having to put them on a restrictive diet, which also can act as a stressor (Guarnieri et al., 2012), as mice that are not hungry are hard to motivate. Even though food restriction has been reported to enhance memory function initially, it can also impair consolidation (Talhati et al., 2014).

On the whole, cognitive enhancer treatment with metrifonate resulted in better performance, and also scopolamine treatment as means to disrupt memory mostly had the expected result. It was possible to simultaneously measure activity and reference / working memory with this set-up making up two of the three primary symptoms of ADHD. Unfortunately, the system is not equipped to measure any impulsive tendencies, which underlie more subtle mechanisms. Thus it was excluded from use in the *Snap25* Gene x Environment study.

4.3 G x E study

Maternal separation protocol

Maternal separation in rodents is an established model of early life stress and has been shown to affect neuronal activity, memory and gene expression (Nishi et al., 2013) even though behavioral effects are not consistent and very strain dependent (Millstein & Holmes, 2007). There are various protocols to be found in literature; here it was chosen to separate the pups from their mothers at a regular time during the light phase for 3 hours per day for the first 21 days of life. Pup mortality was even lower than the usual 32% that is found in laboratory C57BL/6 mice (Weber et al., 2013).

Activity

When only considering the non-stressed animals, heterozygous mice are not more active than wild-types in the first long-term OF, but are so in the second. This is not because they cover more distance in the second than in the first, but because they remain on the same level as in the first, although the situation is a familiar one. The same is not true for the wild-types, whose activity level goes down in the second OF session. This appears to be normal behavior, as it can also be seen in other studies doing repeated OF tests (see for expample Pan et al., 2008). Heterozygous animals do not show this decline in activity in a more familiar environment. In both sessions, the G x E interaction is significant, which apparently results from the fact that both MS stress and a heterozygous *Snap25* deletion have enhancing effects on activity. This has previously been shown for the former in wild-type C57BL/6 mice (Carlyle et al., 2012), and for the latter in combination with nicotine (Baca et al., 2013). In contrast to the study by Baca and colleagues (2013), environmental adversity in this study does not add to the enhanced activity effect of MS in heterozygous *Snap25* animals. On the contrary, in the first OF session MS heterozygous mice are significantly less active than MS wild-types. In the second session, all animals that either underwent MS or were heterozygous for *Snap25* or both were similarly more active than control wild-types. Thus, it appears as tough in a somewhat familiar environment, both reduced levels of Snap25 and early life stress produce a slightly hyperactive phenotype in mice, but this effect does not intensify when both prerequisites are met simultaneously.

Learning / Attention / Impulsivity

What kinds of effects were predominant in the 5CSRTT largely relied on the phase of the experiment. During the introduction to the task, namely in the habituation phase and the first Autoshaping phase, successfully acquiring the reward was based mainly on the animals' exploration skills. The strongest effects found in both phases were environment effects and trial x environment interactions, no genotype effects became apparent. Unlike mice from the control group, MS animals did not increase the number of pellets they ate over sessions, but remained static or even declined. There are studies showing difficulties in the execution of learning-tasks in animals that were subjected to stressors both prenatally (Bustamante et al., 2010) and postnatally (Spinelli et al., 2013; Wang et al., 2014). Still, it is not clear if in this case the problem is a matter of cognitive skills or motivation, especially when taking into account the results from the next phase of the experiment: In the Autoshaping phase 2, environmental effects disappeared and only trial effects were apparent. The one crucial thing that changed in this phase was that the animals needed to actively trigger the reward. In contrast to the first two phases, this was equally easy or challenging for all groups when only the normal parameters were taken into account. But as many heterozygous *Snap25* knockout mice were easy to identify on the 5CSRTT videos due to their increased activity (a parameter unfortunately not measured by the system), a means was found to include this nervous-looking behavior. The measure "number of pellets eaten correctly", meaning pellets eaten directly after the correct nose-poke, was found adequate to map this agitated state: heterozygous animals significantly more often did not eat their reward directly after the correct response, but rather explored some more in the second it took for the reward pellet to fall into the hole. Since, statistically speaking, heterozygous mice made no more mistakes than the wild-types during this phase, random responses could not have caused this effect. It rather appears that it is a distinct feature of this genotype to be too active or too unwilling to wait for this short period of time. It has been known for some time that children with ADHD prefer small immediate rewards over larger delayed ones. This inability to wait has been termed "delay aversion" (Sonuga-Barke et al., 1992) and been proven to be an important feature of ADHD with neurophysiological correlates over the years (Wilbertz et al., 2013). Though the 5CSRTT is not adequate to completely uncover the complexity of delay aversion, it seems legitimate to speculate on a potential delay

aversion phenotype in *Snap25* heterozygous knockout mice based on the new parameter "pellets eaten correctly". In the experimental phase with 20 s stimuli, this parameter again reaches marginal significance. As in the other phases, there are some effects of environment identifying stressed mice as having the inferior learning skills. Interestingly, in the last test trial with 9 s stimuli, the most common effect found was not the environment, as in the other phases, but the genotype. The shorter stimuli appear to be able to uncover more about the heterozygous mice, namely that they made less correct nose-pokes, ate less pellets and less pellets correctly as in the two preceding phases. Shorter and thus more rapidly changing stimuli require more attentional resources when trying to react correctly to as many as possible, thus heterozygous mice showed inadequacy in this regard as well. When it comes to the most often used impulsivity measure in this test, the "number of premature nose-pokes", hardly any effects were found in the different phases of the experiment. Only in the last test trial a marginally significant gene x environment interaction was uncovered, in which the heterozygous mice did not differ from wild-types under control conditions, but when they were stressed. In contrast to the other found effects, the stress caused them to make less premature nose-pokes, which is the opposite one would expect from an ADHD model.

Depression related behavior

Regarding depression-like behavior in the forced-swim test, remarkable sex-differences were found. Females on the whole had higher levels of immobility time than males, independent from genotype or stress-group. This has been previously shown and is probably a result of estrogen in the brain and its impact on hippocampal nitric oxide levels (Hu et al., 2012). More interestingly, when sexes were regarded separately, female mice did not show any effect of genotype or environment, but male heterozygous mice spent significantly more time immobile than male wild-types, and thus showed more depression-like behavior, no matter if they had been stressed in early life or not. However, it should not be disregarded that the forced-swim test was the second to last test in a relatively long series. As it has become clear that even normal laboratory routines are stressful for mice (Drude et al., 2011), it could be said that at the point in time of the testing, all mice were under the influence of chronic stress which could have masked an early-life stress effect.

Anxiety-like behavior

In the Light-Dark-Box, no effects were found for the parameters "time in lit compartment" and "transitions between compartments". The only significant effect could be found for environment in "latency to enter lit compartment". Animals that had been subjected to early-life stress took significantly longer to enter the lit compartment for the first time and thus showed more anxiety-like behavior than non-stressed animals. This effect has previously been reported for different kinds of stressors (Kitaoka et al., 2013; Sarro et al., 2014) and is therefore not surprising. More interestingly, the gene x environment interaction reaches marginal significance. Although not significant in the post-hoc tests, it seems that heterozygous mice under control conditions were faster to enter the lit compartment than the wild-types and reacted more strongly to the stressor through even longer latency times. As this is similar to what is sometimes observed in humans with certain susceptibility genotypes (Caspi et al., 2003), it should be kept in mind.

Aggression

Aggression testing was unsuccessful because of the apparent low aggression levels in the mice tested, but also because of flaws in experimental design. Mice were single housed for a long period of time, mainly to make food restriction protocols easier in the 5CSRTT. Though single housing can sometimes be beneficial for aggression tests, it is detrimental when it is done for longer periods and can also lead to abnormal or even pathological forms of aggression (Miczek et al., 2001). Ideally, males should be housed with a female to display territorial aggression. Moreover, the test should be done repeatedly in order for aggressive behavior to stabilize (Newman et al., 2012).

MPH challenge

When testing a potential ADHD mouse model in an activity test with MPH, the expected result is that hyperactivity goes down and approximates a normal control level (see for example Zhu et al., 2014). This is sometimes called "paradoxical effect", as MPH is a psychostimulant and in healthy controls leads to higher blood pressure and heart rate (Tomasi et al., 2011). However, apart from activity patterns, patients and controls react quite similarly to the drug (Rapoport & Inoff-Germain, 2002). In this study, the effect found was quite reversed. MPH administration significantly increased activity on the whole and the

marginal significant effect for the gene x MPH interaction is a result of the even higher increase in activity for the heterozygous mice as compared to the wild-types. Nonetheless, this difference is probably based on altered dopaminergic and noradrenergic signaling in the *Snap25* heterozygous knockout mice, which is implied in ADHD, even if the directionality is wrong.

Gene expression

Genes investigated in the qRT-PCR - in addition to *Snap25* itself to ascertain that genetic manipulation was effective - all have been previously associated with ADHD. *Snap25* expression was significantly decreased in all investigated brain regions in all heterozygous animals, independent from environment and gender, showing almost perfect dose-effect expression levels of about 50 % of wild-type expression. *Comt* and *Maoa* had similar expression patterns in the frontal cortex with significantly lower mRNA levels for the heterozygous animals. Since both Maoa and Comt are enzymes which are, among others, responsible for breaking down dopamine and norepinephrine, and frontostriatal pathways are implicated in ADHD (Banaschewski et al., 2010), these results are in line with a potential ADHD model. Then again, both the *Maoa* and the *Comt* knockout mouse display hypoactive behaviors, the former in the form of hypo-locomotion (Bortolato et al., 2009) and the latter in the form of decreased rearing behavior (Babovic et al., 2007), which is both not true for the *Snap25* knockout mice in this experiment. Unfortunately, to date there are no studies on the effects of *Maoa* and *Comt* knockout on *Snap25* expression. Seemingly, these three genes influence each other in a more complex fashion with interesting effects on locomotive behavior. The only dopamine receptor investigated was *Drd2*, the dopamine autoreceptor. Its expression was only dependent on the environment and not on the genotype. This environmental effect has previously been described in rats (Li et al., 2013), also with decreased expression rates in early-life stress animals. However, the most striking effects found in the qRT-PCR analysis were for *Nos1* expression with G x E interactions in both the frontal cortex and the striatum. In both brain regions, *Nos1* expression was higher for the control heterozygous animals than for the wild-type controls, but went down with MS for both genotypes to the same level. This multifaceted gene has been implicated in a great number of psychiatric (Franke et al., 2009; Reif et al., 2006; Reif et al., 2009) and neurological (Chabrier et al., 1999) disorders and its product's substrate has countless

modes of action. This study found that *Nos1* expression is both influenced by environmental stress and *Snap25* levels in the striatum and the frontal cortex. It is therefore likely that *Nos1* levels are also causally involved in the behaviors seen here.

Stress parameters

Corticosterone levels did not differ between male and female mice on the whole, although this has often been described (see for example Coleman et al., 1998) but levels of heterozygous mice were approximately 30 % lower than those of wild-types, independent from stress group. Although this was not expected as altered corticosterone levels in stressed animals have been reported before (Roque et al., 2014), again the duration of the experimental series and the stress experienced through behavioral testing might be the reason for this lacking environmental effect. Lower corticosterone levels in heterozygous mice indicate a dysfunction of the hypothalamus – pituitary – adrenal (HPA) axis as a result of the genetic modification. Adrenals were weighed as a further measure of this complex circuitry and here the expected gender difference was found. Not only were female adrenals around twice as heavy as male adrenals, but the effects within these groups were rather different, when being looked at separately. Where for male mice differences were not based on genotype, but only on environment, for females it was the other way around: the adrenals of heterozygous females were much smaller than those of wild-type females. When comparing this with the corticosterone levels in serum, the question arises whether the non-existent genotype difference there has its seeds in a gender bias, as most of the heterozygous animals from the MS group were females due to a breeding disequilibrium.

5 Conclusion

Two things were tried to accomplish in this thesis: Firstly, to evaluate different doses and different application methods of MPH on a behavioral and neurochemical level; Secondly, to thoroughly investigate heterozygous Snap25 knockout mice as a potential model for ADHD.

Findings from the MPH pilot study suggest that even though it is possible to find i.p. and oral doses that correlate behaviorally in mice, the neurochemistry is mostly different. The questions arises which application method models application in humans better,

because even though a model might behaviorally fit with the human condition, the neurochemistry involved could be quite different.

The G x E *Snap25* study was able to uncover behavioral deficits in the heterozygous mice. In addition to a mild hyperactivity in a familiar environment, the mice showed elevated depression-like behavior and attenuated anxiety-like behavior. Moreover, some parameters from the 5CSRTT hint at an attentional inadequacy and some aspects of delay aversion. The stress parameters measured uncovered an imbalance in the HPA axis, which usually accompanies psychiatric disease, and qRT-PCR found expression changes in 3 genes that have been associated with ADHD. All these findings are in line with an ADHD model. However, MPH application had an effect that was contrary to the expected one but still elucidated deficits in systems tightly linked to the disorder. In conclusion, the heterozygous knockout of *Snap25* in mice does not lead to full occurrence of ADHD-like symptoms, but nonetheless results in an endophenotype of increased activity and irritability which, considered together with the changes in gene expression, constitutes another step towards the understanding of not only ADHD, but also other psychiatric disorders.

6 Appendix

6.1 References

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6.3 List of tables

6.4 List of figures

6.6 List of abbreviations

+/-	Heterozygous knockout
+/+	Wild-type
5CSRTT	5-Choice-Serial-Reaction-Time-Task
5HIAA	5-Hydroxyindoleacetic acid
5HT / 5HTT	Serotonin (-transporter)
ADHD	Attention deficit/hyperactivity disorder
ATP	Adenosine triphosphate
B2m	Beta-2-microglobulin
bp	Base pair
cDNA	Complementary DNA
Cm/+	Heterozygous coloboma mutant mouse
Comt	Catechol-O-methyl transferase
DA / DAT	Dopamine (-transporter)
DNA	Deoxyribonucleic acid
DOPAC	3,4-Dihydroxyphenylacetic acid
Drd2	Dopamine receptor 2
Drd4	Dopamine receptor 4
FST	Porsolt Forced-Swim Test
G x E	Gene-by-environment interaction
HPLC	High Performance Liquid Chromatography
Hprt	Hypoxanthine phosphoribosyltransferase
HVA	Homovanillic acid
i.p.	Intraperitoneal
LDB	Light-Dark Box
Maoa	Monoamine oxidase A
MHPG	3-Methoxy-4-hydroxyphenylglycol

MPH	Methylphenidate ; (±)-Methyl α-Phenyl-α-(2-piperidyl)acetate hydrochloride
MS	Maternal Separation
NA / NAT	Norepinephrine (-transporter)
NO	Nitric oxide
Nos1 / nNos	Nitric oxide synthase 1 (also: neuronal Nos)
n-Sec1	Neuronal syntaxin-binding protein
OF	Open Field
Pgkh	Phosphoglycerate kinase
PND	Post-Natal Day
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Rab	Ras-related in brain
RI	Resident Intruder paradigm
RNA	Ribonucleic acid
RNase	Ribonuclease
Sdha	Succinate Dehydrogenase Complex, Subunit A
Snap25	Synaptosomal-Associated Protein of 25 kDa
SNARE protein	Soluble NSF attachment protein receptor, where NSF stands for N-ethyl-maleimide-sensitive fusion protein
SNP	Single nucleotide polymorphism
Tbp	TATA box binding protein
Tfrc	Transferrin receptor
Tph2	Tryptophan hydroxylase 2
VAMP	Vesicle-associated membrane protein
VNTR	Variable number tandem repeat

6.7 CV

Name: Antonia Margareta Post

Date of birth: 30.05.1979

Place of birth: Augsburg, Germany

Education:

07/1998	Abitur (diploma from German secondary school qualifying for university admission); Maria-Theresia-Gymnasium Augsburg, Germany
11/1998 – 05/1999	Study of Medical Informatics at the Fachhochschule Heilbronn, Germany / Ruprecht-Karls-Universität Heidelberg, Germany
11/1999 – 02/2004	Study of Biochemistry at the Universität Bayreuth, Germany (Intermediate Diploma in March 2002)
04/2004 – 04/2009	Study of Psychology at the Julius-Maximilians-Universität Würzburg, Germany; Diploma in April 2009
07/2009 – 06/2012	Member of the GK Emotions (RTG 1253/1), PhD student in Prof. Klaus-Peter Lesch's group at the University Hospital of Würzburg, Department of Psychiatry, Psychosomatics and Psychotherapy (Molecular Psychiatry)
07/2012 – 06/2014	Research associate in Prof. Andreas Reif's group at the University Hospital of Würzburg, Department of Psychiatry, Psychosomatics and Psychotherapy (Psychiatric Neurobiology)

Würzburg, _____ _____

Date Antonia Post

6.8 Publications

Post, A.M., Weyers, P., Holzer, P., Painsipp, E., Pauli, P., Wultsch, T., Reif, A., Lesch, K.-P., 2011. Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models. Behav. Brain Res. 218, 99–105.

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Affidavit

I hereby confirm that my thesis entitled **"*Snap25* heterozygous knockout mice as a potential model for attention deficit/hyperactivity disorder (ADHD)"** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **„Heterozygote *Snap25* Knockout-Mäuse als potentielles Modell für Aufmerksamkeitsdefizit- / Hyperaktivitätssyndrom (ADHS)"** eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, …… ……..

Datum Unterschrift