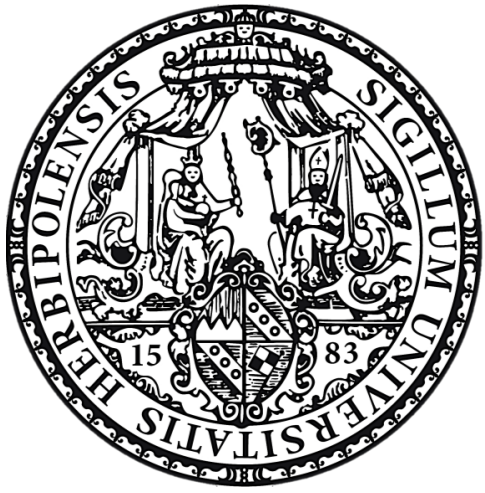

Gene x Environment Interactions in *Cdh13*-deficient Mice: CDH13 as a Factor for Adaptation to the Environment

Gen x Umwelt-Interaktionen in *Cdh13*-defizienten Mäusen: CDH13 als ein Faktor für Umweltanpassung

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Submitted by:

Dominik Pascal Kiser

from

Detmold

Würzburg, 2019

Submitted on: ……

Office stamp

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Supervisor (Fourth): Dr. Daniel vanden Hove

Date of Public Defence: **……**

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Abstract

Neurodevelopmental disorders, including attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) are disorders of mostly unknown etiopathogenesis, for which both genetic and environmental influences are expected to contribute to the phenotype observed in patients. Changes at all levels of brain function, from network connectivity between brain areas, over neuronal survival, synaptic connectivity and axonal growth, down to molecular changes and epigenetic modifications are suspected to play a key roles in these diseases, resulting in life-long behavioural changes.

Genome-wide association as well as copy-number variation studies have linked cadherin-13 (*CDH13*) as a novel genetic risk factor to neuropsychiatric and neurodevelopmental disorders. CDH13 is highly expressed during embryonic brain development, as well as in the adult brain, where it is present in regions including the hippocampus, striatum and thalamus (among others) and is upregulated in response to chronic stress exposure. It is however unclear how CDH13 interacts with environmentally relevant cues, including stressful triggers, in the formation of long-lasting behavioural and molecular changes. It is currently unknown how the environment influences CDH13 and which long term changes in behaviour and gene expression are caused by their interaction. This work therefore investigates the interaction between CDH13 deficiency and neonatal maternal separation (MS) in mice with the aim to elucidate the function of CDH13 and its role in the response to early-life stress (ELS).

For this purpose, mixed litters of wild-type (*Cdh13*$^{+/+}$), heterozygous (*Cdh13*$^{+/-}$) and homozygous knockout (*Cdh13*$^{-/-}$) mice were maternally separated from postnatal day 1 (PN1) to postnatal day 14 (PN14) for 3 hours each day (180MS; PN1-PN14). In a first series of experiments, these mice were subjected to a battery of behavioural tests starting at 8 weeks of age in order to assess motor activity, memory functions as well as measures of anxiety. Subsequently, expression of RNA in various brain regions was measured using quantitativ real-time polymerase chain reaction (qRT-PCR). A second cohort of mice was exposed to the same MS procedure, but was not behaviourally tested, to assess molecular changes in hippocampus using RNA sequencing.

Behavioural analysis revealed that MS had an overall anxiolytic-like effect, with mice after MS spending more time in the open arms of the elevated-plus-maze (EPM) and the

light compartment in the light-dark box (LDB). As a notable exception, *Cdh13*$^{-/-}$ mice did not show an increase of time spent in the light compartment after MS compared to *Cdh13*$^{+/+}$ and *Cdh13*$^{+/-}$ MS mice. During the Barnes-maze learning task, mice of most groups showed a similar ability in learning the location of the escape hole, both in terms of primary latency and primary errors. *Cdh13*$^{-/-}$ control (CTRL) mice however committed more primary errors than *Cdh13*$^{-/-}$ MS mice. In the contextual fear conditioning (cFC) test, *Cdh13*$^{-/-}$ mice showed more freezing responses during the extinction recall, indicating a reduced extinction of fear memory. In the step-down test, an impulsivity task, *Cdh13*$^{-/-}$ mice had a tendency to wait longer before stepping down from the platform, indicative of more hesitant behaviour. In the same animals, qRT-PCR of several brain areas revealed changes in the GABAergic and glutamatergic systems, while also highlighting changes in the gatekeeper enzyme Glykogensynthase-Kinase 3 (*Gsk3a*), both in relation to *Cdh13* deficiency and MS. Results from the RNA sequencing study and subsequent gene-set enrichment analysis revealed changes in adhesion and developmental genes due to *Cdh13* deficiency, while also highlighting a strong link between CDH13 and endoplasmatic reticulum function. In addition, some results suggest that MS increased pro-survival pathways, while a gene x environment analysis showed alterations in apoptotic pathways and migration, as well as immune factors and membrane metabolism. An analysis of the overlap between gene and environment, as well as their interaction, highlighted an effect on cell adhesion factors, underscoring their importance for adaptation to the environment.

Overall, the stress model resulted in increased stress resilience in *Cdh13*$^{+/+}$ and *Cdh13*$^{+/-}$ mice, a change absent in *Cdh13*$^{-/-}$ mice, suggesting a role of CDH13 during programming and adaptation to early-life experiences, that can results in long-lasting consequences on brain functions and associated behaviours. These changes were also visible in the RNA sequencing, where key pathways for cell-cell adhesion, neuronal survival and cell-stress adaptation were altered. In conclusion, these findings further highlight the role of CDH13 during brain development, while also shedding light on its function in the adaptation and response during (early life) environmental challenges.

Zusammenfassung

Neuronale Entwicklungsstörungen (NES), wie Aufmerksamkeitsdefizit-Hyperaktivitätssyndrom (ADHS) oder Autismus Spektrums Störung (ASS), haben eine größtenteils unbekannte Krankheitsentwicklung, deren klinisches Erscheinungsbild bei dem Patienten durch die individuelle Genetik und Umwelt beieinflusst wird. Veränderungen in allen funktionellen Ebenen des Gehirns, von Netzwerkaktivität zwischen unterschiedlichen Gehirnregionen, über synaptischer Verschaltung, axonalem Wachstum und den Überlebenschancen einzelner Neuronen, bis hin zu molekularen und epigenetischen Modifikationen werden als Schlüsselrollen in NES betrachtet, welche schlussendlich zu langfristigen Verhaltensauffälligkeiten führen.

Genome-weite-Assoziations und genomische Kopiezahlvariations Studien haben Cadherin 13 (*CDH13*) als neuartiges Risikogen für neuropsychiatrische und neuronale Entwicklungsstörungen identifizieren können. CDH13 wird sowohl während der embryonalen Entwicklung, als auch im adulten Gehirn, stark exprimiert und kann dort in Regionen wie dem Hippocampus, Striatum und Thalamus gefunden werden. Darüber hinaus wird es als Reaktion auf akuten (physiologischen und psychologischen) Stress exprimiert. Gegenwärtig ist jedoch nicht bekannt, wie Umwelteinflüsse mit CDH13 interagieren und langanhaltende Veränderungen im Verhalten und der Gene Expression im Gehirn herbeiführen. Die vorliegende Arbeit untersucht daher die Interaktion zwischen CDH13 und Stress während eines frühen Lebensabschnitts.

Hierfür wurden Würfe mit wildtyp (*Cdh13*$^{+/+}$), heterozygoten (*Cdh13*$^{+/-}$) und homozygoten knockout (*Cdh13*$^{-/-}$) Mäusen zwischen dem ersten und vierzehnten Tag nach der Geburt für jeweils 3 Stunden von ihren Müttern getrennt (englisch: maternal separation, MS). In einem ersten Experiment wurden diese Mäuse dann im Alter von 8 Wochen in einer Reihe von Verhaltensversuchen auf ihre motorischen Fähigkeiten und Gedächtnisleistung getestet. Im Anschluss daran wurde mittels der quantitativen Polymerase Kettenreaktion (qPCR) verschiedene Gehirnregionen dieser Tiere auf Expressionsunterschiede in Faktoren für Neurotransmittersysteme, Neurogenese und DNA Methylierungsmechanismen hin analysiert. Das Hippocampus-Gewebe einer zweiten gleich aufgebauten Versuchsgruppe wurde mittels RNA Sequenzierung untersucht.

Die Verhaltensanalyse zeigt das MS einen überwiegend angst-lindernden Einfluss auf die Mäuse hatte. Im Vergleich zu Mäusen ohne MS verbrachten MS-Mäuse mehr Zeit auf dem offenen Arm eines erhöhten Plus-Labyrinths, so wie in der hell-erleuchteten Seite einer Hell-Dunkel Box (HDB). Eine auffallende Ausnahme stellten jedoch die *Cdh13*$^{-/-}$ Mäuse dar, welche in der HDB keinen Zeitanstieg wie ihre *Cdh13*$^{+/+}$ und *Cdh13*$^{+/-}$ MS Geschwister auf der hell-erleuchteten Seite aufwiesen. In dem Barnes Labyrinth (einem Test für räumliches Lernen) zeigte sich, dass alle Tiere, gemessen an den Fehlern und der Zeit die sie brauchten bis sie den Ausgang fanden, zunächst ähnliche Lernerfolge hatten. Im zweiten Teil des Experiments, in dem der Ausgang auf eine neue Position gelegt wurde, begangen *Cdh13*$^{-/-}$ Mäuse ohne MS hingegen mehr Fehler als *Cdh13*$^{-/-}$ MS Mäusee. In der Kontext-Angst-Konditionierung (KAK) zeigten männliche *Cdh13*$^{-/-}$ Mäuse mehr Angst-Starre während der Extinktions-Wiederholung; ein Befund der eine reduzierte Angst-Auslöschung impliziert. Im Abstiegs-Test, einem Impulsivitätstest, blieben *Cdh13*$^{-/-}$ Mäuse länger auf einem Podest stehen. Mittels qPCR konnte außerdem gezeigt werden dass sowohl ein *Cdh13*$^{-/-}$ Defizit, als auch MS, Veränderungen von GABAergen und glutamatergen Faktoren, so wie Änderungen in dem wichtigen Signalmolekühl Glykogensynthase-Kinase 3 (*Gsk3a*) verursacht haben. Die Ergebnisse der RNA Sequenzierung zeigten eine Anreicherung von Veränderungen in Adhesions-, Entwicklungs- und Endoplasmatischen Reticulumgenen in *Cdh13*$^{-/-}$ defiziten Tieren im Vergleich zu *Cdh13*$^{+/+}$ Mäusen. Im selben Versuch trug MS zu einer erhöhten Aktivierung von Anti-Apoptotischen Signalwegen im Hippocampus bei, während Zell-Adhesionsmoleküle maßgeblich von der Wechselwirkung beider Faktoren betroffen waren.

Zusammenfassend waren *Cdh13*$^{+/+}$ und *Cdh13*$^{+/-}$ Mäuse im Gegensatz zu *Cdh13*$^{-/-}$ Tieren größtenteils stressresitenter, während die RNA Sequenzierung aufzeigte, dass CDH13 Schlüsselkomponenten von Zell-Zell-Adhesions, Überlebens und Zell-Stress-Signalwegen reguiert. Dies suggeriert, dass CDH13 die Programmierung und Anpassung an Umwelteinflüsse steuert, was wiederum lang anhaltende Auswirkungen auf molekularer Ebene und auf das Verhalten der Mäuse zur Folge hat. Abschließend legen die Befunde eine Rolle von CDH13 in der Umgebungsanpassung während der Entwicklung nahe.

1 Introduction

1.1 ADHD and autism - disorders of genetic origin

Attention-deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD) are neurodevelopmental disorders (NDD) of early brain development with a high prevalence and, depending on their severity, a strong overall impact on the individual's quality of life. Core ASD symptoms include problems in social cognition, emotional learning with anxiety in unpredictable/unexpected events, communication, as well as repetitive and stereotypical behaviours, with frequent comorbid disorders such as anxiety disorders, obsessive-compulsive disorder (OCD) and ADHD (Romero et al 2016). ADHD, in turn, comprises an inattentive and possibly hyperactive phenotype. Individuals with ADHD have a predominantly inattentive presentation (ADHD-PI), a predominantly hyperactive presentation (ADHD-PH) or a combined presentation of hyperactivity and inattention (ADHD-C) (Rowland et al 2008). Core symptoms of ADHD are inattention and (mal)adaptive impulsivity, with frequent comorbid conditions such as learning disability, as well as conduct, anxiety and emotional disorders (Nigg et al 2002, Rowland et al 2002).

It has been pointed out that they might also constitute parts of an even larger, partially overlapping, spectrum, which could be described as "neurodevelopmental disorder". It is estimated that roughly 30% of children with an ASD diagnosis show clinically relevant symptoms of ADHD (Kiser et al 2015, Rao & Landa 2014, Rowland et al 2008). Both ASD and ADHD are NDDs with a clear hereditary background and a strong genetic overlap (Kiser et al 2015, Martin et al 2014, Pettersson et al 2013, Stam et al 2009, van Steijn et al 2012). The current hypothesis for the complex and elusive pathogenesis of both disorders is that they originate from multiple genetic risk factors (van Loo & Martens 2007), which require additional environmental, genetic and/or epigenetic "triggers" to become disorder-relevant (Kendler & Eaves 1986, Kiser et al 2015, Kubota 2016).

In the past years, genome-wide association studies (GWAS) have helped to identify potential genetic markers and risk genes, creating new research targets and potentially also models for explaining the complex nature of ADHD and ASD. GWAS focus on associations between single nucleotide polymorphisms (SNPs) and certain traits,

making use of large cohorts including patients and healthy controls, in order to statistically identify SNP variants that occur more frequent in patients. While such studies are very successful in identifying candidate genes associated with disorders, they require extensive testing in order to elucidate the function and attributes for each of the associated genes. One gene which has been frequently linked to ADHD (Lesch et al 2008, Neale et al 2008, Rivero et al 2013, Salatino-Oliveira et al 2015, Zhou et al 2008), ASD (Sanders et al 2011), anxiety disorders, as well as substance abuse and depression, is *CDH13* (Hawi et al 2018, King et al 2017). Variants of the *CDH13* gene, specifically in children and adolescents, have also been associated directly with some of the behavioural and cognitive phenotypes of ADHD and ASD, such as memory impairments (Salatino-Oliveira et al 2011), changes in verbal working memory (Arias-Vasquez et al 2011), IQ discrepancy in autism (Chapman et al 2011), as well as hyperactivity/impulsivity (Salatino-Oliveira et al 2011). However, little is known about the molecular function of CDH13 in the pathogenesis of these disorders, for example if the observed association roots from a loss- or gain-of-function of the CDH13 protein in patients.

1.2 Calcium-dependent cell adhesion molecule 13 (CDH13)

CDH13 is a member of the calcium-dependent cell adhesion molecule family. During development, CDH13 gradually extends its expression from the hindbrain/midbrain into the frontal cortex (Forero et al 2017). CDH13 appears to be highly conserved and can be found in all vertebrate species (Philippova et al 2009), suggesting an important biologically relevant function. CDH13 is present in many brain areas during development and plays roles in guidance of motor neuron axons (Fredette et al 1996, Hayano et al 2014, Rivero et al 2013), while studies in cultured cells have also highlighted its role in synapse formation (Paradis et al 2007). Changes in synaptic connectivity and axonal growth have a strong impact on neurodevelopmental disorders and are suspected to play a key role in their pathophysiology (Halbleib & Nelson 2006, Rivero et al 2013). Following regular gene and protein nomenclature, cadherin 13 will be written in different styles throughout thesis, depending on the specific contexts. If the gene is mentioned, the symbol will be written in italics, e.g *CDH13* (human or general) or *Cdh13* (animal). If the protein is mentioned, or it is a general context, plain text will be used, e.g. CDH13 (human or general) and Cdh13 (animal). This convention will also be used for other genes in this thesis.

1.2.1 Molecular structure and evolution of CDH13

In an evolutionary context, cadherins represent a very old family of molecules that can be found in cnidaria and sponges (Gul et al 2017), and show a strong adaptive diversification in higher metazoans and a significant explosion of new variants coinciding with the emergence of the chordates, indicating a crucial role not only for multicellular life but also for the development of more complex organisms in general (Hulpiau et al 2013, Hulpiau & van Roy 2011). Five main branches of the superfamily can be identified, using domain, phylogenetic and sequence similarity: conserved elements, classical, flamingo, dachsous, the FAT-family and FAT-like (Hulpiau & van Roy 2011). Additionally, unique and isolated members exist that cannot clearly be placed into one of these branches (Nollet et al 2000). It has been speculated that the overall importance of the gene superfamily could arise from its dual function in both cell-cell adhesion and cell-surface-receptor-signalling (Hulpiau & van Roy 2009). In this context, CDH13 appears to be a rather “recently” developed gene, which is present in all

tetrapods, and thus also in all land vertebrates (Hulpiau & van Roy 2009), where it is encoded by only one gene (Gul et al 2017, Hulpiau et al 2013, Hulpiau & van Roy 2009, Hulpiau & van Roy 2011). According to extracellular cadherin domain 1 (EC1) homology comparison of major cadherins, CDH13 can be placed closest to the 7D cadherin family and is thus stronger related to type I and type-II cadherins than the phylogenetic "older" FAT or FAT-like cadherins (Hulpiau & van Roy 2009).

Mapping of *CDH13* onto the human genome revealed it to be located at the position 16q24.3 (Kremmidiotis et al 1998, Lee 1996), a genomic region of reduced stability, relevant for the development of cancer (Carter et al 1990, Kremmidiotis et al 1998) and with potentially relevant consequences in the evolution and development of brain function (Kiser et al 2015). In mice, *Cdh13* is located at chromosome 8 (Rivero et al 2013). Tanihara and colleagues cloned several cadherins from human samples and identified *CDH13* as a homologue to the previously described chicken T-cadherin (Tanihara et al 1994a, Tanihara et al 1994b).

While having otherwise well-conserved extracellular domains, CDH13 lacks the cytoplasmic domain that is typical not only in classical cadherins (E- or VE-cadherins), but also in non-classical cadherins, like flamingo cadherins with receptor-like seven-helix transmembrane regions, gene-clustered protocadherines and FAT-cadherins (Angst et al 2001a, Angst et al 2001b, Tanihara et al 1994b, Wheelock & Johnson 2003). In addition, CDH13 also lacks the transmembrane domain and is connected to the cell membrane by a glycosylphosphatidylinositol (GPI) –anchor (Ranscht & Dours-Zimmermann 1991). While having a GPI anchor, CDH13 also has a non-classical binding mechanism to establish homophilic interactions (Ciatto et al 2010) and is able to bind to other targets and proteins, such as adiponectin, implying a potential receptor function despite the lack of clear cytoplasmic domain for signal transduction (see review (Andreeva & Kutuzov 2010)). While CDH13 is present in centrosomes, unlike other cadherins, it is absent from the nucleus where cleaved cytoplasmic domains of other cadherins act as transcription factors (Andreeva et al 2009). Histologically, cadherins can be found at cell-cell junctions, while CDH13 is more globularly distributed across the cell but redistributes towards the leading edge of migrating cells (Philippova et al 2003). All these uncommon features distinguish CDH13, making it hard to draw functional parallels to other cadherins (Andreeva & Kutuzov 2010), but suggesting a unique role for it in the cell.

1.2.2 CDH13 during development

Cadherins in general represent a prominent group of molecules that play an important role during neurodevelopment, e.g. by coding segmentation and functional subdivisions (Philippova et al 2009, Redies & Takeichi 1996), providing synaptic specificity by an adhesive framework (Basu et al 2015, Huntley et al 2002, Obst-Pernberg & Redies 1999, Ranscht 2000) and by connecting individual olfactory neurons across vast distances to specific olfactory glomeruli in the olfactory bulb (Yagi 2013).

It is thus not surprising that CDH13 is present throughout neurodevelopment, where it was first described in trunk neuronal crest cells and motor neurons from chicken embryos and original named T-cadherin for its "truncated" structure (Ranscht & Bronner-Fraser 1991). In their original study, Ranscht and Bronner-Faser described CDH13 to be expressed along a rostrocaudal axis, with the first CDH13-positive cells appearing in the caudally last formed somite during the first invasion of neuronal crest cells, followed by a steady progression during the maturation of the embryo into more rostal somites. Surgical ablation of the neuronal tube however had no effect on the emergence of CDH13 along the rostrocaudal axis, leaving them to speculate that CDH13 might have a role in crest cell migration and somite polarity (Ranscht & Bronner-Fraser 1991). Another study showed that CDH13 is transiently present in most growing motor neurons during development, and although its expression deminishes over time, it is continiously expressed in a small subset of motor neurons during later stages (Fredette & Ranscht 1994). Studies also highlight that CDH13 is restricted to regions avoided by the growing axons, suggesting that CDH13 acts as a negative regulator of neurit outgrowth (Bai et al 2006). Other studies however show, that CDH13 positively regulates the density of glutamatergic and GABAergic synapses in cell cultured mouse hippocampus neurons (Paradis et al 2007) while also helping to maintain tissue identity of functionally grouped neurons (see review by (Philippova et al 2009)).

While being prominently visible along rostrocaudal fiber tracts during the entire development, CDH13 can also be detected in mice starting at embryonic stage E13.5, where it stretches ventrodorsally along the mid/hindbrain barrier and is present at the contact points between migrating serotonergic neurons and radial glia cells (RGC) of the same region, suggesting an involvement of CDH13 in RGC-mediated migration of dorsal raphe serotonergic neurons (Forero et al 2017). Following this idea, the same study was also able to show that in *Cdh13*-deficient mouse embryos, the density of serotonergic

neurons in the dorsal raphe (DR), but not the median raphe, as well as the serotonergic innervations into the prefrontal cortex, increase, suggesting a role for CDH13 in the development of the DR–prefrontal cortex (PFC) circuitry (Forero et al 2017). During the same embryonic period of E13.5 to E17.5, *Cdh13* can be detected in specific layers of the neocortex, anterior cingulate cortex, hippocampus, thalamus, locus coeruleus, raphe nuclei, amygdala, substantia nigra, ventral tegmental area, striatum and cerebellum (Forero et al 2017).

1.2.3 CDH13 in the adult brain

Similar to their function during neurodevelopment, cadherins play important roles in the adult brain. A huge body of literature (represented here by a few selected reviews) emphasizes their involvement in synaptogenesis (Ranscht 2000), synapse morphology (Seong et al 2015) and synaptic maintenance (Obst-Pernberg & Redies 1999), thereby playing an important role in synaptic plasticity and memory formation (Huntley et al 2002, Murase & Schuman 1999). Due to their pre- and postsynaptic presence and signalling function, cadherines could potentially even allow for communication without secretion of distinct messengers molecule at the synapse (Murase & Schuman 1999).
CDH13 mRNA is expressed in a variety of adult neurons throughout the brain, like in pyramidal and nonpyramidal neurons, astrocytes, Cajal and Purkinje cells (Takeuchi et al 2000) gamma-butyric acid (GABA)-ergic and glutamatergic cells (Paradis et al 2007, Rivero et al 2015), tryptophan hydroxylase 2 (Tph2) -positive serotonergic cells, catecholaminergic cells (Rivero et al 2013), as well as in oligodendrocytes (Campagnoni et al 2001). Studies show, that Cdh13 in the adult mouse hippocampus localises in the presynaptic compartment of inhibitory synapses, where it acts as a negative regulator of inhibitory GABAergic synaptic transmission (Rivero et al 2015). But Cdh13 is not restricted to the hippocampus in adult mice; Cdh13 can be found in the neocortex, anterior cingulate cortex, cerebral cortex, thalamus, medulla oblongata, raphe nuclei and catecholaminergic areas such as the substantia nigra, ventral tegmental area and locus coeruleus (Paradis et al 2007, Redies et al 2012, Rivero et al 2013, Takeuchi et al 2000), closely linking Cdh13 to regions important for memory, motivation and attention (Rivero et al 2013, Vaidya & Stollstorff 2008). Studies by (Drgonova et al 2016) also suggest that Cdh13 in conditional knockout mice modestly influences their cognitive,

locomotor and drug consumption (cocaine) phenotype, suggesting that Cdh13 influences the reward system in the adult brain. As mentioned earlier, CDH13 has not only been linked to NDDs and psychological disorders, but also to memory impairments, verbal working memory (but not visual-spatial memory), IQ and hyperactivity/impulsivity. Additionally, variations in the CDH13 gene have been associated with increased impulsivity, potentially leading to violent behaviour in a Finnish prison cohort (Tiihonen et al 2015).

These findings can be confirmed in a *Cdh13*-deficient mouse model, which shows altered cognitive flexibility in a hippocampus-dependent spatial learning task (i.e. Barnes-maze) and impaired fear memory in cued fear conditioning (Rivero et al 2015). A study in *Cdh13*-deficient rats also revealed altered Pavlovian conditioning to drug cues, while other behavioural dimensions, such as locomotion, conditioned place preference and reaction to food cues remained unaltered (King et al 2017).

1.2.4 CDH13 as a protective factor during cellular stress

While this thesis focuses on the effects of environmental (i.e. early-life stress, ELS) factors on the behaviour and brain transcriptome of *Cdh13* knockout mice, particularly in the hippocampus, it is is worth noting that there is vast literature on the role of CDH13 during cellular stress for various tissue and cell types. As such, CDH13 has been described as a protective factor which is associated with the survival of endothelial cells (EC) under tumor like stress conditions (Joshi et al 2005, Philippova et al 2008), EC in the retina (Nakamura et al 2013), cardiac tissue (Denzel et al 2010) and cortical interneurons (CI) (Killen et al 2017).

In cancer research, CDH13 has been identified as a potential tumor marker and treatment target for a number of different cancer types, such as breast cancer (Riener et al 2008), lung cancer (Sato et al 1998) and lymphomas (Masuda et al 2007), among others. During cancer and tumor development CDH13 plays a role in the migration and growth of blood vessels in to the tumor tissue (Andreeva & Kutuzov 2010, Rubina & Tkachuk 2004) by interacting with vascular growth factors secreted by cancer cells (Andreeva & Kutuzov 2010, Philippova et al 2006) and stabilizing (vascular) tissue integrity (Andreeva & Kutuzov 2010). This “positive” effect on vascular growth during tumor genesis has been linked to the ability of CDH13 to protect EC from the hypoxic

conditions found in tumors, that would normally induce endoplasmic reticulum (ER) stress and apoptosis (Joshi et al 2009, Joshi et al 2005). Literature indicates that CDH13 promotes this survival by interacting with Hspa5/GRP78/BiP (Kyriakakis et al 2010, Nakamura et al 2013). Hspa5/GRP78/BiP is an important ER protein, involved in protein folding, heat shock response and nucleus signalling of the unfolded protein response (UPR) during ER stress (Kyriakakis et al 2010). As such, CDH13 attenuates PERK, the pro-apoptotic part of the UPR pathway, allowing higher levels of ER stress before apoptosis occurs (Kyriakakis et al 2010). Additionally, CDH13 and Hspa5/GRP78/BiP appear to be associated on the surface of cardiovascular endothelial cell surfaces (Philippova et al 2003) suggesting some form of close interaction between both. Studies in the cardiovascular field have shown that CDH13 binds adipocyte-secreted hormone adiponectin (APN), which has cardioprotective effects during heart stress conditions (Denzel et al 2010) and supports revascularization of disrupted blood vessels (Parker-Duffen et al 2013).

Brain research reveals that *Cdh13* knockout mice possess less interneurons and late born pyramidal neurons, while also showing an increase of apoptosis in the same region (Killen et al 2017). The study by (Killen et al 2017) however needs to be considered with caution, since it features only a small sample size and pending replication. Disorders linked to CDH13, such as ADHD and ASD, however show features of increased expression of genes related to apoptosis (Martin et al 2014), while the previously mentioned lines of research in endothelial cells would also suggest such a link to exist (Joshi et al 2009, Joshi et al 2005).

The exact role of CDH13 in apoptosis and migration however still remains a controversial topic, particularly due to differences in tissue and cells types studied (Andreeva & Kutuzov 2010), with several investigations suggesting CDH13 as a negative regulator of cancer progression (Lee 1996), being able to suppress proliferation and invasiveness of cancer cells (Andreeva & Kutuzov 2010), sensitize melanoma cells to apoptosis (Bosserhoff et al 2014) and change proliferation and migration of HUVEX and human SMC cells (Ivanov et al 2004a, Ivanov et al 2004b). In the brain, *Cdh13*-deficiency in mice changes the density of migrating serotonergic neurons in the developing dorsal raphe nucleus (Forero et al 2017).

1.2.5 Summary of CDH13 and its brain-related to this thesis

In summary, CDH13 serves several important neuronal functions. Developmentally, CDH13 regulates the density of migrating neurons and axonal growth in several key areas, including the dorsal raphe. Similarly, CDH13 can be found in the adult brain in several brain regions, such as the hippocampus, where it is involved in synaptic formation and maintenance. CDH13 might also help maintaining tissue identity of functionally grouped neurons. Location in the brain by histological staining and association to disorders/traits from GWAS support the involvement of CDH13 in cognition, drawing a link to the development of NDDs, while genetic screening also highlighting its modulating influence on the GABAergic and glutamatergic systems.

1.3 Effects of early-life stress on behaviour and development of NDDs

Although environmental influences are important during all phases and aspects of life, they are especially relevant during developmentally active periods, in which body and brain develop (and adapt) in a rapid fashion. Environmental factors, such as, alcohol abuse, smoking and infections have been linked to disorders such as autism, depression and schizophrenia, to name a few (Cattane et al 2018, Hisle-Gorman et al 2018, Hunt et al 2018). Particularly relevant for this thesis are the effects of early-life stressful experiences in the mother-child relationship.

In 1960, Harry Harlow and his colleagues performed their classical experiments that revealed the negative effects of depriving new-born monkeys from a comforting caregiver, resulting in "problematic" social deficits later in life (Harlow 1959, Harlow & Zimmermann 1959). With his elegantly designed pioneering studies, Harlow was able to show that all other necessities such as food, shelter, water and security aside, the skin contact and interaction with other organisms itself is vital to the healthy development of infant monkeys. Subsequent research over the next decades revealed that stress and emotional trauma during early-life can be associated with the development of anxiety disorders (McCauley et al 1997), depression (Heim et al 2004), schizophrenia (Huttunen et al 1994), delayed language acquisition, anxiety disorders, ADHD and autism later in life (Ronald et al 2010, Talge et al 2007). As such, physical and sexual abuse during childhood can be linked to higher anxiety and depression rates during adulthood (Mattera et al 2017, McCauley et al 1997, Springer et al 2003). Similarly, prenatal stress can increase the risk for schizophrenia (Huttunen et al 1994). Population-wide studies, performed in regions with recent or temporally isolated crisis (such as wars, acts of terrorism or natural disasters) have revealed a strong link between prenatal stress and later life health issues (Brand et al 2006, van Os & Selten 1998, Yehuda et al 2005). For example, a study addressing the invasion and defeat of the Netherlands by the German army in 1940 revealed a higher prevalence of schizophrenia of people born in 1941 compared to those born during later or earlier years (van Os & Selten 1998).

1.3.1 Models of early-life stress (ELS) in rodents

In animal research two predominant useful types of early-life stress (ELS) paradigms are currently used in the field. One observes stress during pregnancy (e.g. prenatal stress), the other varies conditions during the first days of life (e.g. postnatal stress). Commonly used prenatal stress paradigms use repeated exposure to restrain stress, bright lights, noises, sleep deprivation, (saline) injections, electric shocks and submerging into cold water as a stressor applied to the mother (Fumagalli et al 2007, Weinstock 2001a, Weinstock 2001b), and thereby indirectly affecting the offspring through maternal stress hormones. Usually, paradigms limit the exposure to a developmentally critical time window, in order to manipulate certain physiological or neurodevelopmental processes. Prenatal stress paradigms for example are usually set up to cover the third trimester of pregnancy, which roughly translates to the embryonic postnatal day 10 (PN10) in rats and mice. During this time, stress hormones like cortisol have the largest effect on the brain and different receptor systems (Plotsky & Meaney 1993, Welberg & Seckl 2001). The influence of stress hormones is biologically attenuated by the placental enzyme 11ß-HSD-2, which inactivates 90-80% of maternal glucocorticoids before they reach the embryo (Benediktsson et al 1997, Jamieson et al 1997, Seckl & Chapman 1997). Several studies suggest that the procedure also interacts with later stages of development by changing the behaviour of the mother during rearing (Champagne & Meaney 2006), resulting in a "double-hit" scenario of ELS. To circumvent this problem, cross-fostering can be performed, although this procedure also produces further stress while increasing the logistical demands on the experiment.

Neonatal stress e.g. involves the separation of the pups from the dams, which is usually done during the first two weeks after birth, while some studies differ in the exact length and timing of the separation during selected periods. Controls range from undisturbed litters during the same period, to animals daily handled during the same times (Qin et al 2011). Conditions during separation have been identified as crucial co-factors, since particularly young pups are unable to properly regulate temperature, moisture and position during the absence of the mother. Regular maternal care after the separation and during the rest of the time also plays an important role. Meaney and colleagues were able to show a marked difference in stress susceptibility and neuronal plasticity between offspring from low and high caring mothers in rats, with specific "care-giver-

styles" being transmitted between generations non-genetically (Francis et al 1999a, Liu et al 1997, Meaney 2001). Limited nesting material and adverse environmental conditions during rearing have also gained attention recently (Ivy et al 2008, Walker et al 2017) as alternative postnatal stress paradigms. Deprivation during pup rearing is simulated by removing nesting material from the cage of rats and mice, while leaving the nest and mothers otherwise undisturbed during the entire time. This procedure results in a discontinued rearing experience with increased susceptibility to stress and contextual fear conditioning (Walker et al 2017). Also importantly, while genetic influences play a role in the susceptibility to postnatal stress, strain-specific differences (Ellenbroek et al 2004), alongside a non-genetic behavioural transmission of care-giving styles (Champagne & Meaney 2001, Francis et al 1999a) have also been shown to influence the outcome of postnatal stress experiments.

1.3.2 Consequences of early-life stress

ELS can cause persistent changes on all biological levels (e.g. behavioural, cellular and molecular) throughout life. The previously mentioned discontinued rearing experience paradigm thus not only increased susceptibility to stress and contextual fear conditioning, but also reduces adult neurogenesis in the hippocampus (Walker et al 2017), while also showing alterations in receptor composition (Champagne & Meaney 2001, Meaney et al 1996). Additionally, MS might also cause behavioural changes not immediantly apparent, but only become noticeable at a later stage of life (Ellenbroek & Cools 2002).

Studies however are not all agreeing on the effects due to developmental stress exposure. As an example, some studies show an increase (Weller et al 1988), a decrease (Lehmann et al 2000, Patin et al 2004) or no change (Koenig et al 2005) in baseline locomotion in various behavioural tasks after MS or other paradigms. Furthermore, altered dopaminergic functioning, inhibition (Bethus et al 2005, Koenig et al 2005), delayed motor development (Patin et al 2004), deficits in learning and spatial learning (Lemaire et al 2000) and reduced cognitive flexibility (Thomas et al 2016) have also been described. Studies in rats also show that pups subjected to maternal deprivation display inhbited hippocampal plasticity due to hypersensitivity to glucocorticoids when they reach adulthood (Mirescu et al 2004).

Postnatal stress in rodents could also be linked to depression-like behaviour (MacQueen et al 2003), changes in cognitive and emotional reactivity (Fumagalli et al 2007), reduced hippocampal adult neurogenesis (Mirescu et al 2004), as well as cognitive and learning deficits (Baudin et al 2012). Maternal separation, together with early weaning as a model for child maltreatment, could not only show behavioural alterations like hyperactivity, anxiety and attention deficits, but also downregulation of genes involved in the PFC for myelination pathways (Carlyle et al 2012). Additionally, many studies highlight the fact that individual susceptibility to postnatal stress interacts with the strain-specific genetic background of the animal (Ellenbroek et al 2004). It is also not clear, whether cognitive changes are in itself a secondary effect (due to alterations in the PFC and hippocampus, among other regions) or caused by the fear and emotional alterations observed in these animals (Fumagalli et al 2007). Reduced exploration for example and higher latency due to stress-induced hesitation might also attenuate the observed changes in other brain areas.

On the other end of the spectrum, repeated mild stress during rearing has been shown to have positive effects on the offspring. Repeated early handling of pups by the researcher as a part of a control group has revealed that mild disturbances during development could act as physiological stimulation that is actually beneficial during development (Francis et al 1999b) and has positive effects on both offspring and maternal behaviour (Levine et al 1956) such as stress resiliency in conditioned avoidance tests (Levine et al 1956). In Squirrel monkeys repeated mild stress also improved their cognitive performance and prefrontal cortex functioning (Parker et al 2005). Higher stress levels, however, have been shown to have negative effects on hippocampal adult neurogenesis in general (Conrad 2008, Sapolsky 1985), suggesting a dose-dependent effect.

1.4 Hypothesis and objectives

Both CDH13 and ELS contribute to neurodevelopment and have influences on similar regions (such as the hippocampus and PFC) where they affect several neuronal functions, ranging from apoptosis to synaptogenesis and neuronal migration. CDH13 is also upregulated in the nervous system after exposure to chronic stress and has been reported as a neuroprotective factor during stress, suggesting that it is playing a role in adaptation towards environmental challenges. The effects of ELS snd CDH13 deficiency on brain function have in turn consequences on behaviour and emotional states later in life. Both also show a clear link to neurodevelopment, NDDs and affective mood disorders. Not much however is known about how the *Cdh13* gene and ELS influence each other.

Therefore, this thesis tries to answer the question how the CDH13, a neurodevelopmentally important protective/risk factor, contributes to brain development in mice, and how *Cdh13* deficiency in mice modulates the effects of ELS at both the behavioural and molecular level. To this end, this thesis investigates the interaction of CDH13 with ELS (in the form of MS) (1) at a behavioural level, testing for changes in anxiety behaviour, cognition, as well as impulsivity and (2) the effect of *Cdh13* deficiency, ELS and its interaction on the hippocampal transcriptome of mice.
To address the first research question *Cdh13* knockout mice were exposed to MS during their first 14 days of life for 3 hours each day. When they were fully matured these mice and a corresponding control group were tested in a battery of behavioural paradigms: the elevated-plus-maze (EPM), light-dark box (LDB), open field (OF), object recognition (OR), Barnes-maze (BM), step-down (SD) and context fear conditioning (cFC). Additionally, RNA from several brain regions of mice in this experiment was collected and analysed using qRT-PCR. The second research question was approached by replicating the ELS conditions from the first experiment. These mice were however not behaviourally tested afterwards but instead used for transcriptome analysis. Two age groups were assessed, i.e. one directly after weaning (PN22), the other one when they were matured and comparable in age to the behaviourally tested group (PN66).

2 Methods

2.1 Animals

All experiments were carried out using a constitutive *Cdh13* knockout (*Cdh13*$^{-/-}$) mouse line on a C57BL/6N background and described in detail previously (Kiser et al 2018, Rivero et al 2015). All behavioural testing and most of the dissection work took place in the Animal Core Facility at the University Hospital of Würzburg (Würzburg, Germany). Mice were housed in sex- and genotype-matched cages (Macrolon type III, see Table 7) of 3-5 animals from different litters. Housing was under controlled environmental conditions with ambient temperature (21±0.5°C) and humidity (50±5%) and a 12/12h light/dark cycle (lights on at 7:00 h). Food and water were given *ad libitum*. Cage bedding was changed once a week, but scheduled in a way that it did not occure on the day before an animal was tested. The animal work of this thesis is in accordance with the regulations and procedures outlined by the European Community guidelines of animal care. The work has been approved by the boards of the University of Würzburg and the Government of Lower Franconia (license 55.2-2531.01-92/13)

2.2 Facility

All behavioural tests were performed in the Center for Experimental and Molecular Medicine (Zentrum für Experimentelle Molekulare Medizin, ZEMM) at the University of Würzburg. The center also set high hygienic standards, strictly regulated environmental conditions and disinfected materials before they were brought in. During the entire time the animals were in the facility, they were daily checked and medical problems were recorded and treated. In a few cases, male mice had to be separated after heavy infighting.

2.3 Maternal separation

Female *Cdh13*$^{+/-}$ virgin mice intended for breeding of all three cohorts were housed in pairs from the age of 2 months and allowed to habituate to the experimental room for a minimum of 1 week before the start of the breeding. Single male mice of the same age were introduced for one week into the female cages. Vaginal plugs and daily weight gain observations were used to recognize pregnancy (see Figure 1).

Initially it was planned to group house two mothers together and allow them to raise pups in pairs. Pups being born at different days, false pregnancies (or unobserved infanticide by the dominate female during the night), as well as stress induced fighting between the mothers during the first few days of MS led to the change of procedure during the pilot study (not presented here). To avoid these problems, mothers of cohort 1 and 2 were therefor isolated into individual experimental cages roughly 5 to 7 days before giving birth. As a result, no further problems occurred in these groups.

The day a new litter was born was considered as postnatal day 0 (PN0) and the animals were counted but left undisturbed. Each litter was randomly assigned to be maternally separated (MS, number of litters in total = 8), or non-stressed but handled control condition (CTRL, number of litters in total = 11). MS was done at random times of the day (between 8:00 and 15:00 o'clock) for 3h by removing the mother from the cage and placing her in an individual cage in an adjacent room for the duration of the separation. Both MS mothers and MS pups were weighted. CTRL litters and CTRL mothers where handled and weighted, but otherwise left undisturbed. MS pups were left in their home cages and placed under a heating lamp to control temperature (25±2°C). A wet cloth was placed over the cage to provide sufficient air moisture (75±5% humidity). Weaning of the pups took place at PN21. Aside from removing the mothers, but not the pups from their home cages, this paradigm constitutes a standard MS180 PN1-PN14 paradigm with a handled control group (Qin et al 2011). After the weaning, all animals matured naturally without further manipulation until the age of 8 weeks, when behavioural testing started. In total our numbers for the MS condition are (n= 13 *Cdh13*$^{+/+}$, n= 23 *Cdh13*$^{+/-}$ and n= 19 *Cdh13*$^{-/-}$) and handled CTRL (n= 15 *Cdh13*$^{+/+}$, n= 24 *Cdh13*$^{+/-}$, n= 20 *Cdh13*$^{-/-}$). All behavioural tests have been previously described by Kiser and colleagues (Kiser et al 2018).

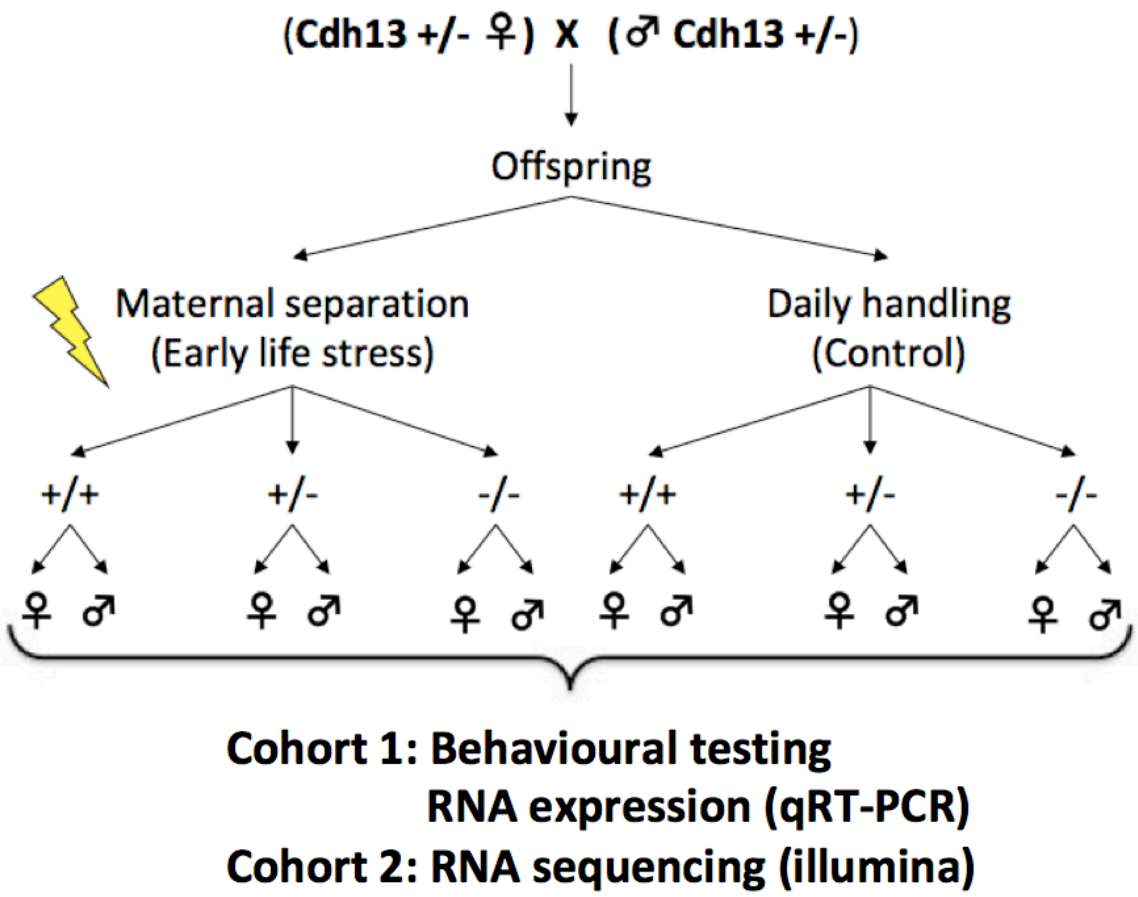

Figure 1 - Breed from heterozygous virgin female and male mice, litters were either maternally separated or daily handled. The resulting offspring encompassed both sexes and all three genotypes. Mice of cohort 1 were then behaviourally tested and RNA from specific brain regions extracted for subsequent RNA analysis using qRT-PCR. In cohort 2, mice were not behaviourally tested prior to being sacrificed in order to increase the chance to detect differences in the RNA sequencing conducted.

2.4 Behavioural tests

All testing occurred during the daylight (12/12h light cycle, 7:00-19:00) phase in the open facilities of the ZEMM. Mice for the behavioural tests were obtained from 8 mothers in the MS condition and 11 mothers in the CTRL condition. In total this lead to 33 male and 28 female offspring in the MS condition and 33 male and 41 female offspring in the CTRL condition. This first cohort was tested in the elevated-plus-maze (EPM), light-dark box (LDB), open field (OF), object recognition (OR), Barnes-maze (BM), step-down (SD) and contextual fear conditioning (cFC). Additionally, brains of this cohort were collected and prepared for RNA extraction.

2.4.1 Elevated-plus-maze

The elevated-plus-maze (EPM) is used as a test for anxiety, approach-avoidance- and exploration behaviour (Bailey & Crawley 2009). The EPM consists of four runways that are connected through a centre section. Opposite arms share the same feature of either being surrounded (closed) by a wall (30 x 5 x 15 cm, 24 lux) or being open at all sides (30 x 5 x 0.25 cm, 70 lux). The maze stands 60 cm elevated above ground and is build out of black Perspex plastic that is semi-permeable to infrared light and illuminated by such from below (TSE Systems, Bad Homburg, Germany, see Figure 2A and Table 7). During testing, mice were placed in the central zone of the EPM, facing to an open arm and away from the experimenter. Their behaviour was then recorded for 5 uninterrupted minutes using an infrared-camera installed above the maze. All tests were recorded on DVD and tracked by VideoMot2 (TSE Systems, Bad Homburg, Germany, see Table 7). Automatically recorded were the time spent in open arm, entries into open and closed arms, as well as total distance travelled. Manually measured were the faecal boli dropped by the animal during testing.

2.4.2 Light-dark box

Another anxiety test used was the Light-Dark-Box (LDB), which highlights the conflict of deciding between avoiding an open illuminated place and exploration of this (unknown) area (Kathleen et al., 2009). The light-dark box (LDB) was build out of a brightly illuminated grey-opaque compartment (40 x 34 x 40 cm, 70 lux, TSE Systems, Bad

Homburg, Germany, see Figure 2B and Table 7) and a small enclosed dark compartment (40 x 16 x 40 cm, 10 lux) with a central gate (5 x 5 cm). Both plastics were transparent to infrared light and illuminated like described in the previous chapter (see 2.4.1).
At the beginning of the experiment, mice were placed in the lower right corner of the dark compartment and recording was started when the light was closed. Mice were then left undisturbed to freely explore the setup for 10 minutes while the experiment was recorded on DVD and analysed using VideoMot2 (TSE Systems, Bad Homburg, Germany, see Table 7). Automatically recorded were the time spent in dark and light compartments, entries to light box and total distance travelled. Manually measured were the faecal boli dropped by the animal during testing.

2.4.3 Open-field test

The Open Field (OF) is a simple test that can be used to measure locomotor activity of an animal, as well as anxiety to new and yet unexplored open spaces (Stanford 2007). The test setup was build out of a black semipermeable to infrared Perspex box (50 x 50 x 40 cm, centre 70 lux, corners 34 lux, see Figure 2 C and Table 7), which again was illuminated with infrared light from below. Mice were placed in the centre of the arena under a non-transparent dark-grey cylinder and habituated for 30 seconds, before the cylinder was lifted and they were allowed to freely explore the OF arena for 30 min. Behavioural measurements included time and distance in the centre (25 x 25 cm placed in the centre) and the time over the entire period, as well as in intervals of 5 minutes, and faecal boli dropped during the experiment.

2.4.4 Object-recognition test

The Object Recognition (OR) test used derived from a variation of previous work from Strekalova and colleagues (Strekalova et al 2013) and is a test measuring the ability of mice to recognize new objects in an environment (Antunes & Biala 2012). In order to reduce the novelty and not measure habituation effects, we used the same OF arena the animals previously visited during the OF test (see Figure 2D and Table 7). The OF was therefor considered to be the habituation phase to the testing arena most other studies use.

For the test, two identical objects (A1 and A2, both stainless steel tally counters) were placed in opposite ends of the arena. Objects were glued to the ground using double-sided tape, with a closest distance of 6 cm from the two surrounding walls. Mice were placed in the centre of the setup and habituated (20 s) under the same non-transparent cylinder described in the previous chapter (2.4.3). When the cylinder was lifted, recording started and the animals could freely explore the arena and the two objects for 10 minutes. During this test, the amount of time the animal interacted with either object was calculated, so that on the next test day (24±1 h later), the object the animal interacted less with could be removed and replaced with a new object (B1, stainless steel wall-mountable tally counter) (Akkerman et al 2012b, Strekalova et al 2013). This new object was very similar to the original object, sharing material, design and texture, but was different in height with the additional feature of a metal leg.

For this test, the distance travelled, time spend with each object and discrimination index was measured. The discrimination index (DI) was calculated as previously described by others, as the percentage of the time exploring the new object ($Time_{object\ new}$) divided by the total exploration time of both objects ($Time_{object\ old+new}$) multiplied by 100 (Akkerman et al 2012a, Akkerman et al 2012b, Antunes & Biala 2012, Ennaceur & Delacour 1988).

2.4.5 Barnes-maze

The Barnes-maze is an escape task, which measures spatial memory (Barnes 1979). For our setup we used a round table (Ø 160 cm) that was elevated 120 cm above the floor and contained 40 rat sized holes (Ø 7 cm) along the edge (see Figure 2E and Table 7). Under one of these holes a small plastic box filled with fresh nesting material was placed, while the other holes were left empty. The table surface was uniformly lit from two sides with neon tubes (70±5 lx). Laminated A4 papers with simple optical cues were placed on three of the four walls, while the forth side was bordered by the exit curtain.

Animals were trained for 4 days with a total number of 10 acquisition trials to find the target hole giving access to the escape box, assessing initial learning and memory abilities. The number of trials per day was two on the first two days, and three on the last two days. After these 10 trials, the box was moved to a position 180° opposite to the original position, and 4 more trials were conducted over the course of two days

(reversal phase), investigating the ability of the animals to relearn and update an existing memory. At the start of each acquisition or reversal trial, animals were placed for 20 seconds under the same non-transparent cylinder used in the previous experiments. After the cylinder was lifted, recording started and the mouse was free to explore the table surface and all the holes for 180 seconds or until the animal escaped through the escape hole. When a mouse did not succeed in escaping, it was gently guided into the box in order to show the animal the escape option (Karabeg et al 2013). Upon escaping, all animals were left undisturbed for 30-45 seconds in order to improve the perception of the box as a safe (escape) place. After a short safety-time (30 seconds) in the escape box, animals were returned to their home cage after the end of each session.

The same VideoMot2 (TSE Systems, Bad Homburg, Germany, and Table 7) system was used to track the animal during each trial. Measurements were previously described in our paper (Kiser et al 2018). As behavioural measures primary latency (time until the mouse found the target the first time), escape latency (time until the mouse entered the target), primary errors (number of errors until the target was first discovered), search strategies and number of dropped faecal boli were recorded. In case the animal did not discover the target, primary latency and escape latency were set to the maximum of 180 seconds. Search strategies were evaluated by the experimenter analogous to O'Leary and colleagues (O'Leary & Brown 2013). A spatial strategy was recorded if the mouse performed 2 or fewer primary errors and found the target within the time limit of 180 seconds. Other strategies were either serial search strategy, when the mouse was inspecting holes in a clock/anti-clock wise order, or had no search pattern with multiple centre crossings but ultimately found the target. Mice which did not find the targets while also committing 2 or less errors were labeled as non-performers (Kiser et al 2018).

2.4.6 Step-down test

The step-down (SD) test is a simple hesitation test (see Figure 2F and Table 7). Animals are again habituated for 20 seconds to the test environment by placing them under a glass cylinder. The entire setup is built inside of the previously described OF box. The only behavioural measurement recorded was the latency to step down from a platform (9.4 cm x 9.4 cm x 2.5 cm). After completion, the animals were returned to their home

cage. The test was repeated three times on three consecutive days. Each time the light conditions were altered to be more safe or aversive. The order of light intensities was day 1: 68 lux, day 2: 6.8 lux and day 3: 308 lux. This was done to ensure no habitation to the light source and allowed to compare if the increased or decreased light intensity affected the measurement.

2.4.7 Context fear conditioning

Context fear conditioning (cFC) is the most basic form of fear conditioning, which tests the ability of the animal to associate an aversive stimulus with a specific environment (see Figure 2G and Table 7). The cFC paradigm used in this work was previously described in (Kiser et al 2018) and was originally adapted from Vignisse and colleagues (Vignisse et al 2014). The cFC chamber from TSE Systems was a moderately lit (100 lux) transparent plastic cube, with a stainless-steel grid floor. From the outside the cube was plastered with a simple geometric pattern. Conditioning was done by habituating the mice first acquisition to the chamber for 2 minutes, after which a single shock of 0.7 mA of alternating current was delivered for 2 seconds. Immediately afterwards the lights in the test chamber were turned off and the animals were moved back to their home cage. 24h after acquisition, fear recall was measured by placing the animals back into the test chamber for 3 minutes, without delivering a shock. After this time, mice were brought back into their home cage. 24h later, extinction recall was performed, testing the animals again for 3 minutes in the test chamber without delivering a shock. In both recall tests, presence or absence of freezing was manually scored every 10 seconds for freezing behaviour. Freezing behaviour was considered, when the posture and movement of the mice indicated a rigid and immobile posture for >1 second. The percentage of freezing was calculated from all 18 individually scored observations (Kiser et al 2018).

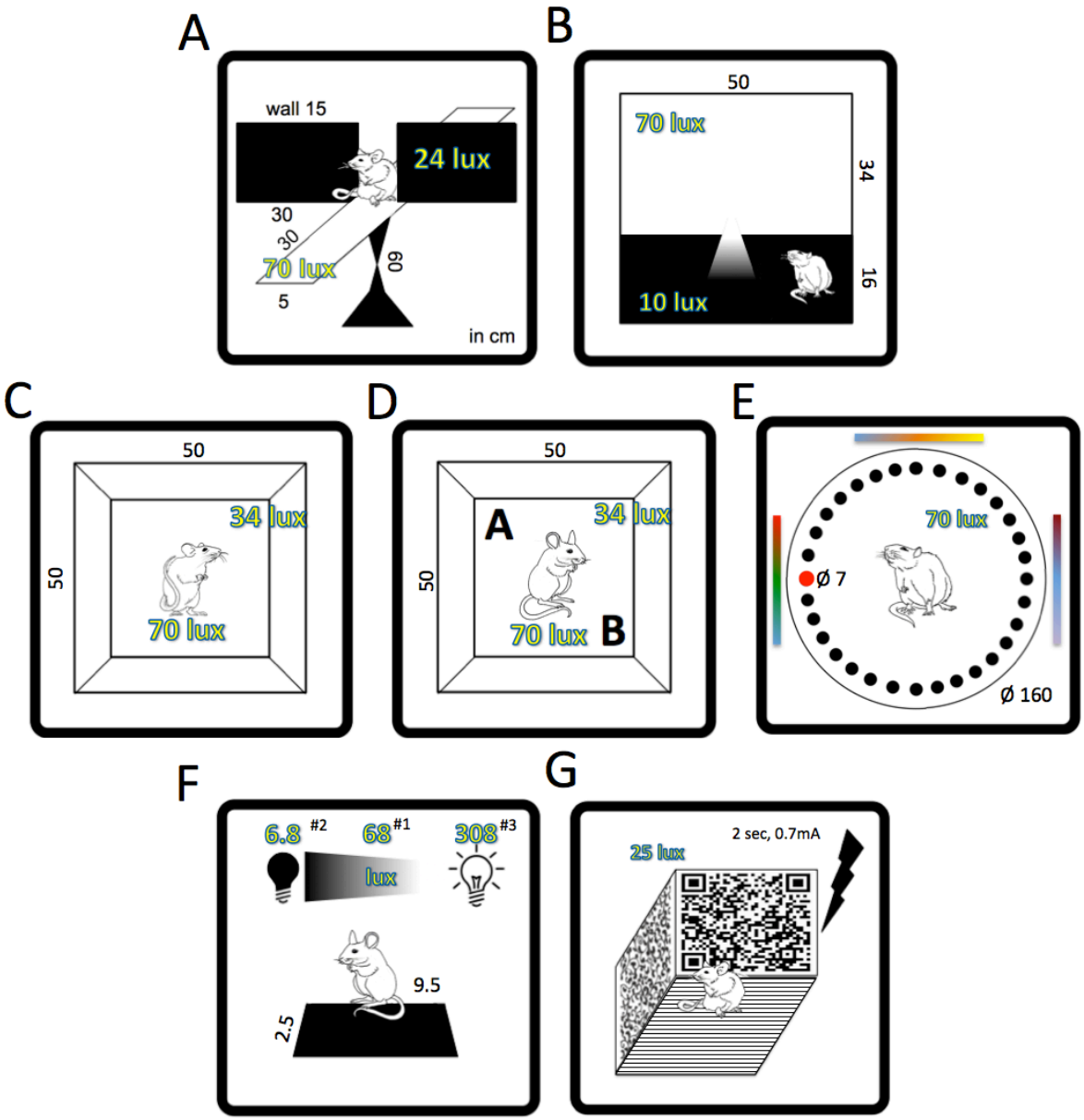

Figure 2 - Graphical representations of all the different behavioural tests used. (**A**) Elevated-plus-maze. (**B**) Light dark box. (**C**) Open field. (**D**) Object recognition. (**E**) Barnes-maze. (**F**) Step-down test. (**G**). Fear conditioning. Image was prepared using power point. Images of mice are donations from Anna Kreis.

2.5 Statistical analysis of the behavioural experiments

For all tests a three-way ANOVA type II was used to analyse genotype, environment and sex effects, alongside their respective interactions. Type II ANOVA is particularly powerful for unbalanced date sets, such as the data presented in this thesis (Langsrud, 2003). Additionally to the degrees of freedom (df), F-value and p-value, eta-square (η^2) was calculated from SSbetween/SSresidual and interpreted according to Cohen (0.02=small, 0.13=medium and 0.26=large). Tukey's HSD (honest significant difference) was used as a posthoc test. For the analysis of the OF and BM data, interval or trial, respectively, were used as a within factor. Significant interactions were then further analysed by comparing individual time points with an ANOVA. When no interaction of time was present, data for each individual were collapsed and analysed without the factor time. All analysis was done using "R" studio (version 0.99 and 0.99.902, see Table 12). The ANOVA function was provided by the package "ez" (Lawrence 2013). Data in graphics are presented as mean ± s.e.m.

2.6 Brain extraction

Mice were sacrificed 2 weeks after the end of the experiments by a lethal dose of anaesthesia (isoflurane) followed by cervical dislocation. Non-perfused brains were then quickly dissected out of the skull and frozen in dry ice cooled 2-methylbutane (-50°C). The entire procedure lasted less than 6 min. During the brain extraction, blood from the heart was collected with a piped and collected in EDTA-containing tubes. Samples were then transported on dry ice and stored at -80°C awaiting further processing.

2.7 Plasma corticosterone (CORT) measurement

Blood plasma was separated from other blood components via centrifugation (20min, 3500g, 4°C). Samples were then stored on dry ice (-70°C) and shipped to Maastricht (School for Mental Health and Neuroscience, Maastricht, Netherlands), where they were analysed using an enzyme-linked immuno-sorbent assay (ELISA, Demetitec Diagnostics GmbH, Kiel, Germany) as previously described (van den Hove et al 2011, Van den Hove et al 2006).

2.8 Analysis of gene expression using quantitative real-time PCR (qRT-PCR)

In order to measure changes of gene expression due to *Cdh13* deficiency and maternal separation in mice, the previously collected brains were dissected, specific brain regions were extracted, their RNA isolated.

2.8.1 Dissection of brain regions

Non-perfused (native) brains were carefully thawed on a sterile cooling table at -8.5°C and cut under a stereo-microscope using scalpels which were cleaned using RNAse free 70% ethanol. Brain regions where then extracted from 6 brain slices (approximately 1 mm thick) using the Allen Brain atlas as a reference (for a detailed guide see Figure). Collection encompassed the prefrontal cortex (PFC), amygdala (AMY), hippocampus (HIPP), dorsal/median raphe (d/mRN), striatum (STR), nucleus accumbens (NAc), thalamus (THAL) and the ventral tegmental area + substantia nigra (VTA/SN). Upon extraction, brain tissue samples were put into RNAse free 2 µL Eppendorf tubes, which were prelabelled and cooled to on dry ice. Samples were then stored at -80°C until RNA extraction for the VTA, d/mRN, PFC, thalamus, striatum, NAc and AMY. The hippocampus was crushed on a dry ice cooled, sterile stainless-steel plate with a metal mortar. The resulting powder was then manually split into two sample tubes to allow separate RNA and protein (not conducted) based analysis on the same sample.

2.8.2 RNA isolation from brain tissue

RNA was isolated from the brain regions using the mi*RNeasy Mini Kit (Qiagen,* all materials are also listed in Table 9). The isolated tissue was lysed and homogenized in 300 µl Qiazol. Segregation of the aqueous phase was done by centrifugation in gel loaded *Maxtract* tubes (5 min/12000g, 17°C). Post-centrifugation, the lysates were mixed with 1.5 x absolute ethanol and transferred to *RNeasy Mini Kit* filter columns (*Qiagen*, 5min/12000 g, RT). Flow-through was discarded and the samples were centrifuged and washed using 350 µl RWT buffer (20 s/12000 g, RT). Following centrifugation, 80 µl DNase Mix (10 µl DNase I and 70 µl of RDD-buffer) was pipetted into each column, in order to eliminate remaining traces of genomic DNA (15 min, RT). Samples were then centrifuged (20 s/12000 g, RT), washed and centrifuged once more using 350 µl RWT buffer (20 s/12000 g, RT). This step was repeated twice with 500 µl RDD buffer (Centrifuge: 20 s/12000 g at RT followed by 2 min/12000g at RT). The RNA was eluted in 50 µl RNase-free water following an on-column incubation (1 min, RT) and centrifugation step (1 min/12000 g, RT). The integrity of the eluted RNA was evaluated using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Science) and Gel electrophoreses (1,5% Agarose gel in TE buffer, 100 bp ladder, 110 V, 30 min). No samples were below the specified optimum ratio for Nanodrop absorption of A260/280 and A260/230 (>1.85, not shown). Gel electrophoresis was used to confirm the integrity of the isolated RNA by observation of clear 28S and 18S rRNA bands (Figure 3).

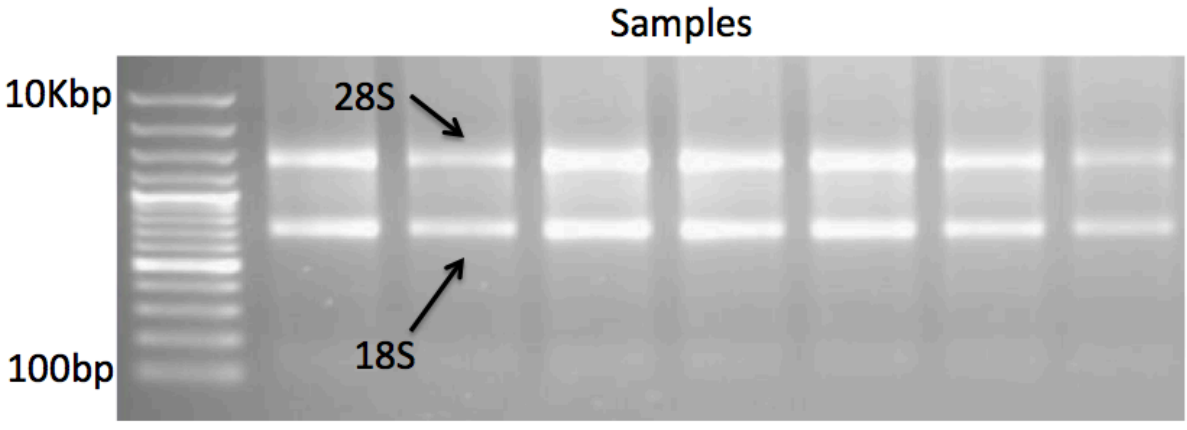

Figure 3 - Quality control of intact RNA isolation using gel electrophoresis, by confirming clear visibility of the 28S and 18S ribosomal RNA bands. Molecular weight ladder is depicted in logarithmic scale starting with 100bp. Image was modified from the master thesis of Emanuela Corradino, 23. December 2016, Faculty of Biology, Julius-Maximilians-Universität Würzburg.

2.8.3 Complementary DNA production

Complementary deoxyribonucleic acid (cDNA) was synthesized from the isolated RNA using the *Quantitect Reverse Transcription Kit (*Qiagen; all materials are also listed in Table 9). All remaining genomic DNA was digested in 4 µg of DNA Wipeout Buffer mixed with RNA extract and water for a total reaction volume of 28 µl with 2 µg RNA content and incubated in a thermocycler (2 min, 42°C). cDNA was synthesized by adding 2 µl of the master mix, 2 µl of the primer-Mix and 8 µl of the buffer-mix of the Reverse Transcription Kit. Additionally, non-reverse transcriptase controls (NRTs), non-template control (NTC) and inter-run-calibrator (IRC) were also synthesized along with the samples as additional quality and calibration samples. The reaction mixture was incubated in a thermocycler for 30 min at 42°C to produce the cDNA. Afterwards, the samples were heated up to 95°C to inactivate the reverse transcriptase. After the cDNA production, the cDNA was diluted in a 1:5 ratio with TE buffer and stored at -20°C. All remaining RNA isolated and not used up in this step was frozen again at -80°C.

2.8.4 Measurement of gene expression using qRT-PCR

The genes under investigation and their respective primers for targets and reference genes are listed in the supplementary material. For the detection of inter-run variation, five IRC were amplified on every plate. 5 µM SYBR Select CFX Master Mix from Life Technologies, 1 µM of target primer, 1 µl of cDNA and 3 µl water were mixed. After loading 10 µl of the mixes in triplicate on a 384-well plate, the plate was covered with an optic foil, centrifuged and kept overnight in the fridge. The qRT-PCR was run in the thermocycler CFX384 from Bio-Rad using a standard cycling protocol. The CFX Manager 3.0 software from Bio-Rad was used to analyse the amplified products. For the analysis, the melt curve, melt peak and the quantification cycle (c_q) were determined.

2.8.5 Statistical analysis of qRT-PCR data

The efficiency of the primers was calculated with the software LinRegPCR (Heart Failure Research Centre, Amsterdam, The Netherlands, version 2014.4, see Table 12). LinRegPCR calculates the efficiency of each amplicon by baseline correcting raw data and calculating the common window-of-linearity, from which the amplification efficiency (AE) per sample can be calculated. The AE was then used to calculate the relative starting concentration of each sample by the qBase+ software (Biogazelle NV, Zwijnaarde, Belgium, version 2.5, see Table 12). qBASE+ suggestions for the most stable

reference genes were used for the normalization of the data. Each region was normalised according to the optimal combination of housekeeping genes: hippocampus (*Gapdh3*, *Act* and *pgK*), PFC (*Rplp0* and *B2M*), m/d Raphe (*Rplp0* and *B2M*) and Amygdala (*pgK* and *Ubc*). This resulted in some regions to missing samples, since the normalization factor could only be calculated with the full set of reference genes. Group differences were analysed using a type II two-way ANOVA. Additionally to the degrees of freedom (df), F-value and p-value, eta-square (η^2) was calculated from SSbetween/SSresidual and interpreted according to Cohen (0.02=small, 0.13=medium and 0.26=large). Tukey's HSD (honest significant difference) was used as a posthoc test. All analysis was done using "R" studio (version 0.99 and 0.99.902). The ANOVA function was provided by the package "ez" (Lawrence 2013). Log2 transformed data in graphics is presented as mean ± s.e.m.

2.9 RNA sequencing

Mice for the RNA sequencing experiment were breed using 19 mothers in the MS condition and 19 mothers in the CTRL condition. In total this led to 73 male and 93 female mice in the MS condition and 79 male and 82 female animals in the CTRL condition. Mice of this group were not tested but daily handled. Half of the mice were sacrificed after weaning, the other half at 66 days of age. Only male *Cdh13*$^{+/+}$ and *Cdh13*$^{-/-}$, but not male *Cdh13*$^{+/-}$ or female mice were used in the RNA sequencing to reduce the complexity of the analysis, effectively also reducing the required samples size required to screen all groups (PN22: (MS: n= 11 *Cdh13*+/+ and n= 10 *Cdh13*-/-) or handled (CTRL: n= 8 *Cdh13* and n= 9 *Cdh13*-/-) and PN66: (MS: n= 10 *Cdh13*+/+ and n= 7 *Cdh13*-/-) or handled (CTRL: n= 7 *Cdh13* and n= 7 *Cdh13*-/-).

2.9.1 Preparation of RNA for Illumina sequencing

Brains from animals of cohort 2 were extracted as described in chapter 2.8.1 and mRNA isolated as described in chapter 2.8.2. Samples of RNA of 100 ng or more were diluted in water up to 10 µl and frozen at -80°C and sent to the RNA sequencing core unit, where all the remaining work was carried out (medical faculty, CU systems medicine, IMIB, Margarete Göbel and Konrad Förstner, all materials are also listed in Table 11) according to the TruSeq Stranded mRNA sample preparation guide from illumina (Illumina 2013). A detailed description of each step can be found at the illumina homepage (see link in the references). Using the Low sample protocol starting from page 16, RNA was first purified using magnetic bead poly-T oligo molecules to which the polyA tails of the sample mRNA could bind and be separated from its dilution. After two washing steps, mRNA was fragmented. Deviating from the illumina protocol, the step 1 of the subsection “incubate RFP” was carried out with 94°C for 12 min, after which samples were immediately put on water ice. First and second cDNA strand were synthesised using random primers. During synthesis of the first strand, Actinomycin D was added to the solution to prevent any remaining DNA to be synthesised during the following steps. In the next step, chimera formation of concatenated template formation was prevented by adding a single “A” nucleotide to the 3’ end of each fragment, with a complementary “T” nucleotide on the 3’ end of the adapter. Then dual indexing adapters (i7 and i5) were ligated to the cDNA. After ligation of the index, selective DNA enrichment of fragments with adapters on either end of the fragment was done by PCR

using adapter specific primers. In order to prevent skewing of the representation of each fragment, only 12 cycles of PCR were used. After the enrichment PCR, quality and quantity control were done using Qubit and Bioanalyzer DNA 1000, determining concentration and fragment size in order to standardise the fragment count in each pool to an equal 5 nmol for each sample. Processing 69 samples thus was spread to 5 individual runs on the Illumina 500 Next Generation Sequencer, with 75 nt single end reads, resulting in 28 million reads for each sample.

2.9.2 Statistical analysis of RNA sequencing data using the voom-lima pipeline

RNA sequencing data from the Illumina 500 Next Sequencer machine was then processed and mapped in the RNA sequencing core unit in Würzburg (CU systems medicine, IMIB, Margarete Göbel and Konrad Förstner). Data for each sample consisted of an ENSEMBLE ID and reads counted. In total, 21973 genes were detected. Data were then converted into an edgeR object and analysed using the voom-Limma pipeline described by Bioconductor (Law et al 2016). For the further analysis, the two age groups were analysed individually to simplify the model and increase power. First, genes with no counts in any sample were removed from the analysis. The remaining reads were then transformed into log2 counts per million (CPM). To identify main effects and interactions, an adjusted version of the voom-limma pipeline from Bioconductor was used (Law et al 2016). The first function, Voom, calculates the mean variance trends, which are later fed into a linear model using the lmFit function. In the next step, empirical Bayes statistics are calculated for the differential expressions using the function eBayes (Law et al 2014). The results are then filtered for genes that have a p-value smaller than 0.01 and a CPM fold change higher than 0.2 or lower than -0.2. The resulting lists were then used for further enrichment using an automated online search for common Gene Ontology (GO)/KEGG terms in them, as well as an extensive literature search of the top 20 genes with the highest p-values (Jensen et al 2009, The UniProt 2017, UniProt Consortium 2018). Analysis of interaction was carried out, using t-tests (corrected using fdr) on the read data to highlight the direction of the changes.

3 Results

3.1 Behavioural consequences of *Cdh13* deficiency and maternal separation

In the first experiment, animals were tested in a battery of behavioural tests to ascertain the effects of *Cdh13* deficiency and maternal separation on anxiety, learning and cognition. The average litter size of this first cohort was 7.86±1.8 pups, with an average weight of 9.57±1.59g at the day of weaning (P21-23). 50.28% of the pups were male, and the distribution of genotypes was 27% *Cdh13*$^{+/+}$, 57% *Cdh13*$^{+/-}$ and 18% *Cdh13*$^{-/-}$. Animals were either assigned to be maternally separated between PN1 and PN14 (MS: n= 13 *Cdh13*$^{+/+}$, n= 23 *Cdh13*$^{+/-}$ and n= 19 *Cdh13*$^{-/-}$) or daily handled during the same time (CTRL: n= 15 *Cdh13*$^{+/+}$, n= 24 *Cdh13*$^{+/-}$, n= 20 *Cdh13*$^{-/-}$). Chi-Square analysis of the gene distribution did not reveal a deviation of the expected Mendelian distribution; neither did the sex distribution differ from the expected. No group differences for weight could be observed. Data from the elevated-plus-maze, light-dark-box, open-field, Barnes-maze, step-down test and context fear conditioning, but not the object recognition task, presented in this section was also previously published by the author of this thesis (Kiser et al., 2018).

3.1.1 Elevated-plus-maze test

Analysis for *total distance* revealed a significant main effect of environment ($F_{1,104}$=5.071, η^2=0.046, p=0.026, Figure 4A), indicating that CTRL mice covered less distances when compared to MS mice. A tendency also existed for genotype x sex ($F_{1,104}$=2.371, η^2=0.043, p=0.098, not displayed), revealing that female *Cdh13*$^{-/-}$ mice covered more distance than male *Cdh13*$^{-/-}$ mice (p=0.077). Locomotion however was not affected by sex in *Cdh13*$^{+/+}$ and *Cdh13*$^{+/-}$ mice.

Analysis for *open arm time* showed a significant interaction for environment x sex ($F_{1,104}$=10.137, η^2=0.089, p=0.002, Figure 4B), showing that CTRL males spent significantly less time in the open arms compared to male MS (p=0.001) and female CTRL mice (p=0.033).

Analysis for *number of faecal boli* in the EPM showed both, an environment x sex ($F_{1,102}$=4.591, η^2=0.043, p=0.035), as well as a genotype x sex interaction ($F_{1,102}$=3.227, η^2=0.059, p=0.044, Figure 4C). Individual post-hoc test for either interaction revealed

that CTRL females deposited significantly more *faecal boli* than CTRL males (p=0.011) and *Cdh13*$^{-/-}$ females had a trend to defecate more when compared to *Cdh13*$^{+/+}$ females (p=0.076) and *Cdh13*$^{-/-}$ males (p=0.062).

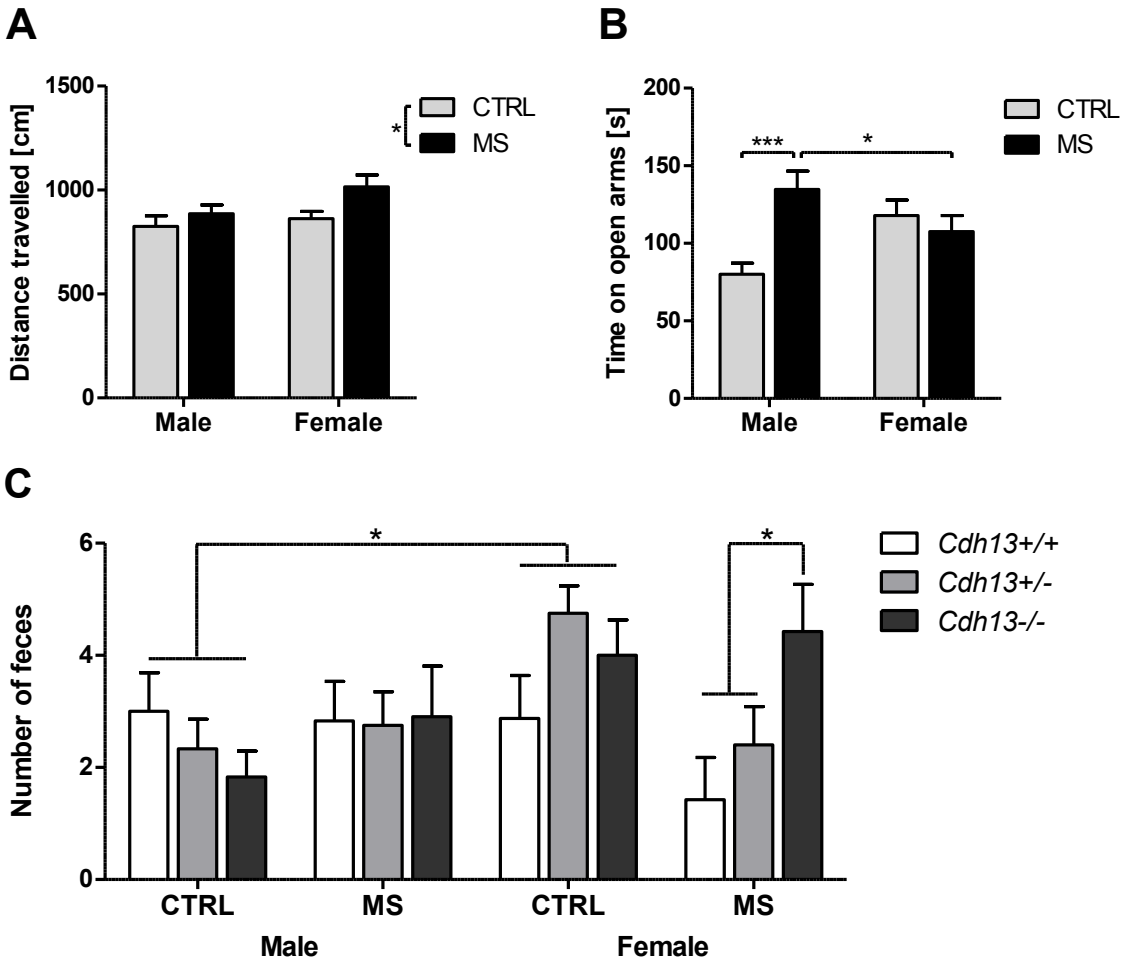

Figure 4 - Effects of gene, environment and sex in the EPM. (A) Total distance in the EPM showed an environmental difference between CTRL and MS animals, but no genotype or sex difference. **(B)** Time spend on the open arm of the EPM was higher for MS male animals, compare to CTRL male and MS female mice. **(C)** Female CTRL mice dropped more faecal boli during the experiment compared to their male CTRL counterparts. Among female mice after MS, *Cdh13*-deficient mice show a marked increase compared to the other two genotypes within the same group. Data are presented as mean ± s.e.m., *P<0.05, ***P<0.001. CTRL, control; MS, maternal separation. Figures are modified from Kiser et al., 2018.

3.1.2 Light-dark transition test

Analysis for *Total distance* detected a main effect of sex ($F_{1,103}$=8.578, η^2=0.076, p=0.004, Figure 5C), revealing that males covered significantly shorter distances than females. Additionally, a main effect of environment can be detected ($F_{1,103}$=4.166, η^2=0.038, p=0.043), revealing that CTRL mice travelled significantly shorter distances compared to MS mice.

Analysis of *time spent in the light compartment* detected a genotype x environment interaction ($F_{1,103}$=4.375, η^2=0.078, p=0.015, Figure 5D), revealed that CTRL mice spend significantly less time in the light compartment compared to MS mice (p=0.015). This effect was mainly driven by MS *Cdh13*$^{+/+}$ and MS *Cdh13*$^{+/-}$ mice, while MS *Cdh13*$^{-/-}$ mice spend significantly less time in the light side compared to either of these groups (p≤0.025). MS *Cdh13*$^{+/-}$ mice also showed a significant increase in time in the light compartment compared to their control group (p=0.006). There were no interaction effects in the *number of faecal boli*, but a main sex effect ($F_{1,104}$=4.526, η^2=0.042, p=0.036), indicating that females produced more *faecal boli* than males.

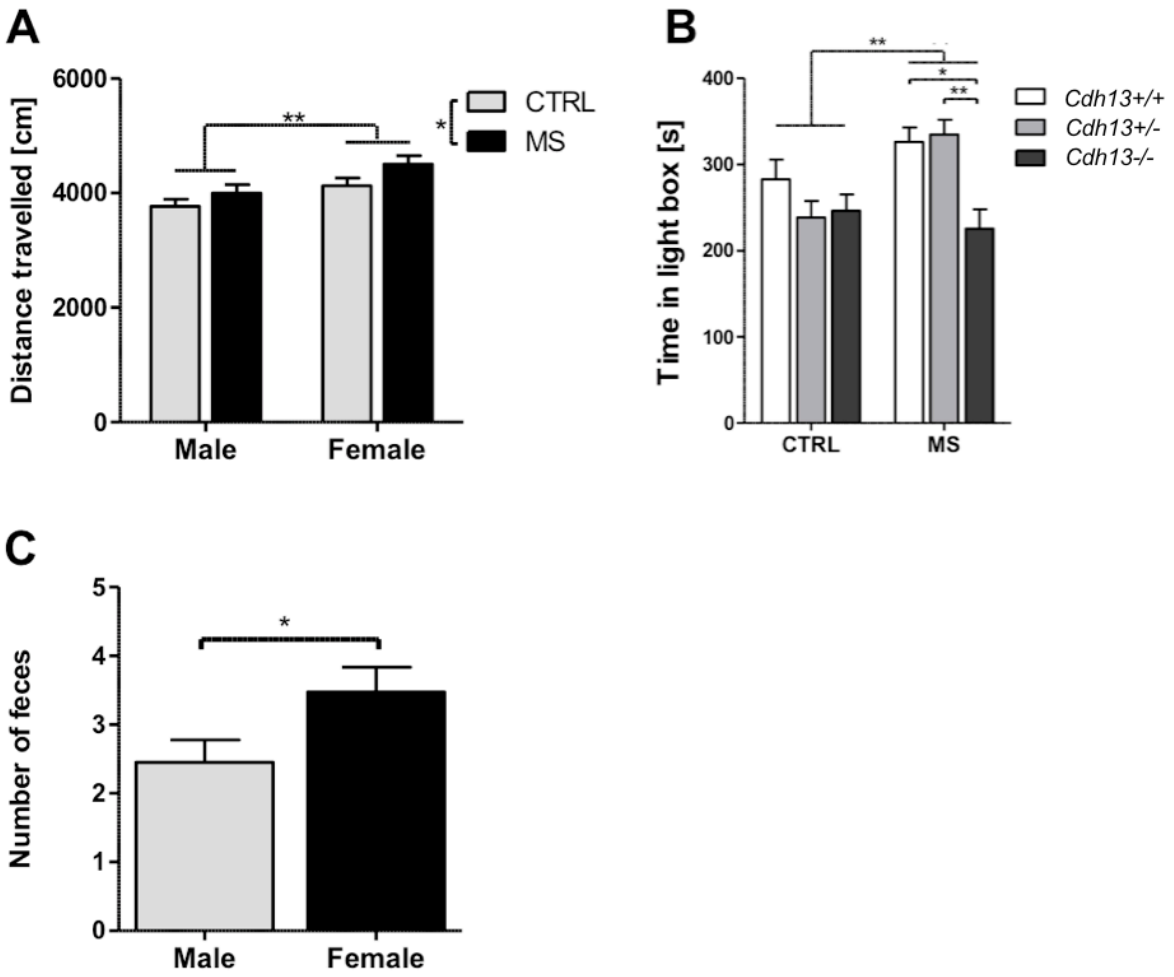

Figure 5 - Effects of gene, environment and sex in the LDB. (C) Distance travelled in the LDB indicates both, a higher activity by female as well as my MS mice. **(B)** Time spend in the light box as a measure of reduced anxiety and increased exploration, indicates an anxiolytic effect of MS, as well as a genotype x environment interaction in *Cdh13*$^{-/-}$. Data are presented as mean ± s.e.m., *P<0.05, **P<0.01, ***P<0.001. CTRL, control; MS, maternal separation. Figures are modified from Kiser et al., 2018.

3.1.3 Open-field test

Repeated measures ANOVA (rmANOVA) revealed multiple significant effects for the factor time in the dependent variables of *time spent in the centre, total distance travelled* and *number of rearings* ($F_{5,510/340}$≥6.422, η^2=0.086, p<0.001). Horizontal (distance) and vertical (rearing) activity decreased while at the same time *centre time* increased over the 30-min session of each test, showing that the animals habituate to the experimental situation. No significant interactions between time and other factors were detected for the variables *distance travelled* and number of *rearings*. Irrespective of time there was a significant sex x genotype interaction for both variables ($F_{2,102/68}$≥3.748, η^2≥0.068, p≤0.027), revealing that male *Cdh13*$^{-/-}$ mice covered shorter distances than male *Cdh13*$^{+/-}$ (p=0.035) and female *Cdh13*$^{-/-}$ mice (p=0.010, Figure 6E), while male *Cdh13*$^{+/-}$ mice reared more frequently when compared to male *Cdh13*$^{-/-}$ (p=0.009) and female *Cdh13*$^{+/-}$ mice (p=0.039, Figure 6F). Additionally, a significant main effect of environment for the number of *rearings* was detected ($F_{1/68}$=7.843, η^2=0.103, p=0.006, not shown), showing that CTRL mice were less vertically active when compared to MS mice (p=0.006).

Analysis of *time spent in the centre*, revealed a 4-way interaction between genotype, environment sex and time ($F_{10,510}$=2.017, η^2=0.016, p=0.030, fig. 1G), that can also be broken down to a 3-way interaction of genotype, environment sex and time ($F_{2,102}$=3.813, η^2=0.041, p=0.025, Figure 6G). A detailed post-hoc analysis of the second half of the 30 minute experiment, contrasting individual comparisons against each other, showed that MS not only increased centre time when compared to CTRL mice (p=0.009), but also highlighted that specifically male *Cdh13*$^{-/-}$ MS mice spend more time in the centre, compared to male *Cdh13*$^{-/-}$ CTRL mice (p=0.018), as well as a sex difference with female mice spending less time in the centre when compared to male mice (p=0.050). ANOVA of faecal boli did not show any significant changes between groups.

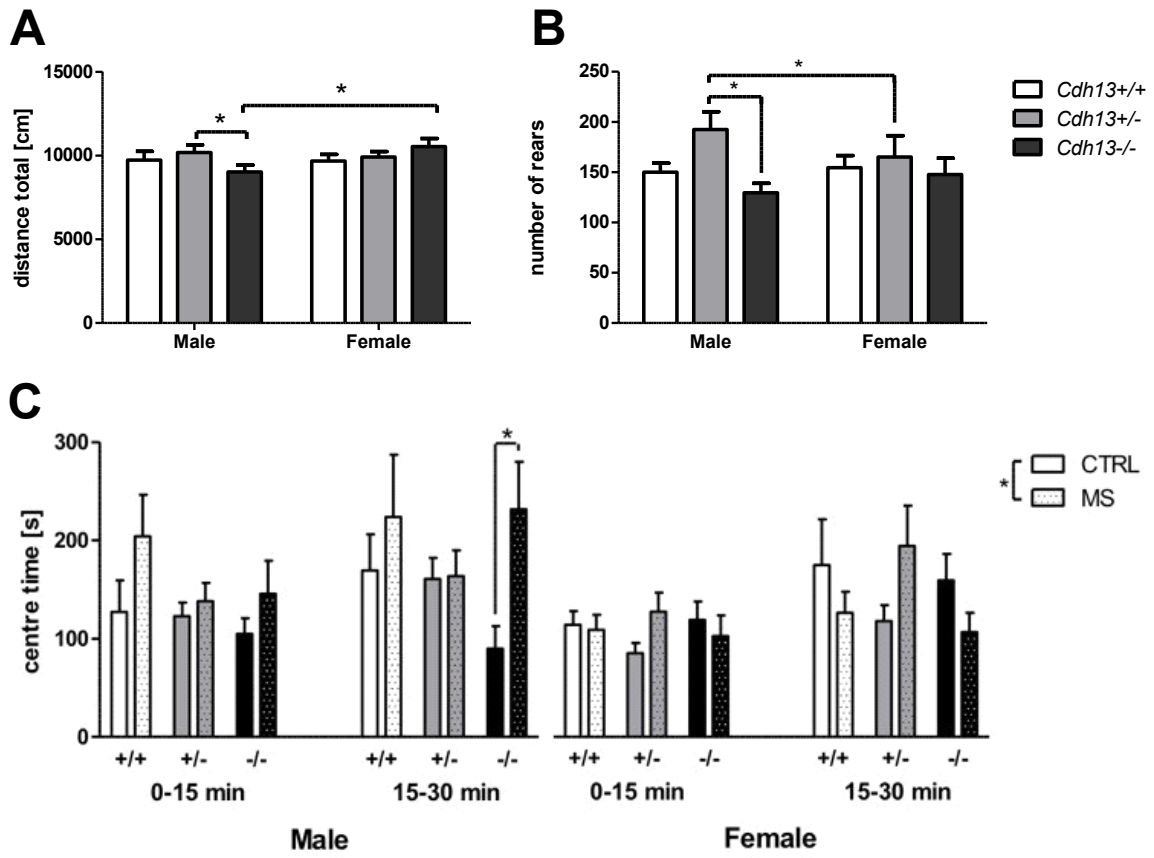

Figure 6 - Effects of gene, environment and sex effects in the OF (E, F). (E) Total distance covered in the OF. **(F)** Number of rears in the OF. **(G)** Time in the centre of the OF during the first and second half of the test. Data are presented as mean + s.e.m., #P<0.1, *P<0.05, **P<0.01, ***P<0.001. CTRL, control; MS, maternal separation; +/+, *Cdh13*$^{+/+}$; +/-, *Cdh13*$^{+/-}$; -/-, *Cdh13*$^{-/-}$. Figures are modified from Kiser et al., 2018.

3.1.4 Object-recognition test

rmANOVA for *total distance* revealed a genotype x sex x day interaction ($F_{2,102}$=7.562, η^2=0.011, p=0.000) which was further studied by analysing each day individually. During day 1 there was only a tendency for a genotype x sex interaction ($F_{2,102}$=2.378, η^2=0.044, p=0.098, Figure 7A), with male *Cdh13*$^{-/-}$ mice travelling less than female *Cdh13*$^{-/-}$ mice (p=0.066). During day 2, this effect became stronger ($F_{2,102}$=3.986, η^2=0.072, p=0.022), but post-hoc tests failed to reach significance (p=0.158), probably due to a lack of statistical power.

Analysis of averaged *object exploration time* showed no day effect but an interaction between sex and environment ($F_{1,102}$=3.984, η^2=0.021, p=0.049, Figure 7B), although post-hoc tests did not reveal significant group differences.

Furthermore, we found no differences in the *discrimination index* among experimental groups, which indicates that all mice displayed similar recognition memory (Figure 7C).

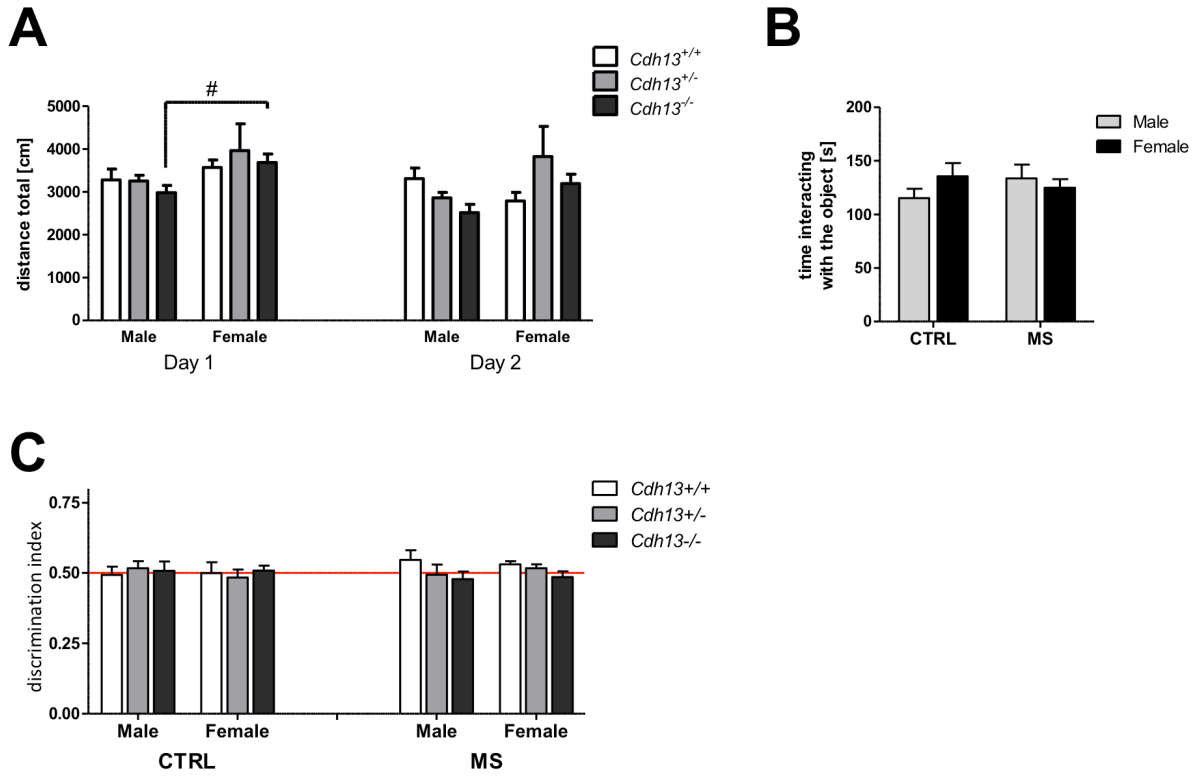

Figure 7 - Gene and sex interaction in the object recognition task. (A) Distance travelled in the object recognition task. **(B)**. Time spend interacting with objects, averaged for both test days. **(C)** The discrimination index showed no significant change from chance level (red line), suggesting that neither the new nor the older object were preferred. Data are presented as mean + s.e.m., *P<0.05. G, genotype; E, environment; S, sex; D, day; CTRL, control; MS, maternal separation.

3.1.5 Barnes-maze test

The factor test day had a strong effect on the dependent variables *total distance*, *primary errors, escape latency* and *target latency* ($F_{5,475}$≥37.39, η^2≥0.282, p≤0.001), with all parameters gradually decreasing throughout acquisition (day 1 to day 4) and reversal training (day 5 to day 6). This indicates that all groups successfully learned the task.

A significant day x genotype x sex interaction for the *total distance* travelled was found ($F_{10,475}$=1.92, η^2=0.028, p=0.040). Particularly male *Cdh13*$^{-/-}$ mice traveling shorter distances during the 1st day of acquisition ($F_{2,101}$=3.908, η^2=0.072, p=0.023) compared to male *Cdh13*$^{+/-}$ mice (p=0.025) and female *Cdh13*$^{-/-}$ (p=0.021).

Male mice also took significantly more time compared to female mice finding the target hole and escape into it (main effect on *escape latency* and *primary target latency*, $F_{1,95}$≥9.104, η^2≥0.039, p≤0.003).

Additionally, an overall genotype x environment interaction could be found for the number of primary errors ($F_{2,95}$=3.462, η^2=0.067, p=0.035, Figure 8A), with a clear trend for MS *Cdh13*$^{-/-}$ mice committing less *primary errors* before finding the target compared to CTRL *Cdh13*$^{-/-}$ mice (p=0.054). Similarly, a weak significant genotype x environment interaction could be found for non-spatial search strategies ($F_{2,95}$=2.756, η^2=0.055, p=0.069; Figure 8B) with CTRL *Cdh13*$^{-/-}$ mice making more use of random or serial search strategies compared to MS *Cdh13*$^{-/-}$ mice.

Analysis of *primary target latency* showed a strong day x environment interaction ($F_{5,475}$=4.526, η^2=0.029, p<0.001, Figure 8C), revealing a significant increase in *primary latency* for MS mice during the first day of reversal training compared to CTRL mice. Similarly, a significant day x environment interaction could also be detected for *reversal errors* ($F_{1,95}$=5.127, η^2=0.051, p=0.026), with CTRL mice less frequently visiting the previous target than MS mice.

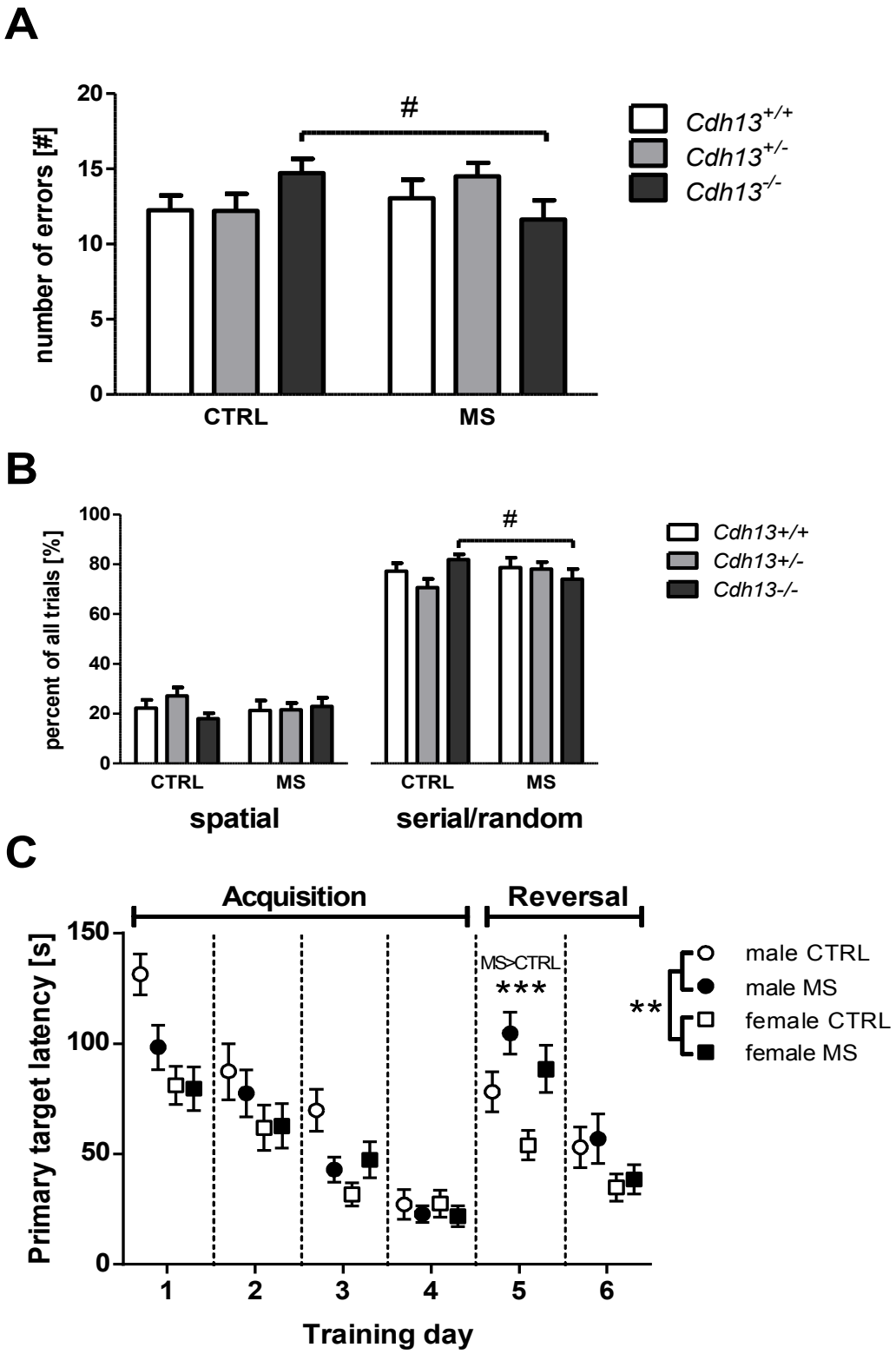

Figure 8 - Barnes Maze. (A) Primary errors, averaged across all test day. **(B)** Search strategies selected by each individual were recorded, summarized across all 14 trials for each group and presented as percent of all trials. **(C)** Primary latency to find the target, displayed for environment and sex. Data are presented as mean + s.e.m, #P<0.1, *P<0.05, **P<0.01, ***P<0.001. G, genotype; E, environment; CTRL, control; MS, maternal separation; M, male; F, female. Figures are modified from Kiser et al., 2018.

3.1.6 Step-down test

Repeated measures analysis for the step-down latency did not reveal any main effect of genotype, environment, session or an interaction between these factors. Averaging all three sessions a tendency for a genotype effect became apparent ($F_{1,72}$=2.441, η^2=0.063, p=0.094) (Figure 9), showing that *Cdh13*$^{-/-}$ mice remained slightly longer on the platform when compared to *Cdh13*$^{+/-}$ mice (p=0.1). Descriptively, this effect also exists between *Cdh13*$^{-/-}$ and *Cdh13*$^{+/+}$ mice, without reaching significance.

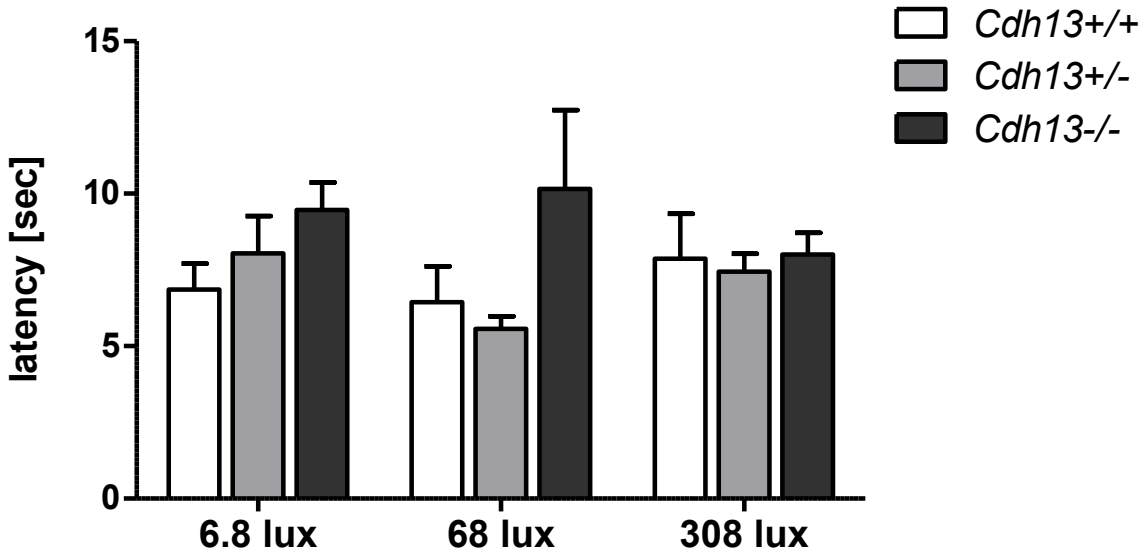

Figure 9 - Genotype effect for latency during the step-down test. When combining all three sessions with different illumination intensities, a genotyped effect revealed that *Cdh13*$^{-/-}$ mice by tendency take longer to step down from the platform. No effect of repeated measures or environmental influence could be detected. Data are presented as mean + s.e.m., $^{\#}P<0.1$. G, genotype; M, male; CTRL, control; MS, maternal separation; F, female CTRL, control; MS, maternal separation; M, male; F, female.

3.1.7 Context-fear conditioning test

24 hours after fear conditioning (context recall) no significant effects or interactions on freezing were detected between any of the groups. Only after 48 hours (extinction recall) a significant interaction between genotype and sex could be found ($F_{2,72}$=4.558, η^2=0.112, p=0.013) (Figure 10). Post hoc analysis showing that male *Cdh13*$^{+/-}$ and *Cdh13*$^{+/+}$ mice by tendency freeze less than their respective female *Cdh13*$^{+/-}$ (p=0.05) and *Cdh13*$^{+/+}$ (p=0.09) counterparts. Additionally, *Cdh13*$^{-/-}$ males tended to freeze more than male *Cdh13*$^{+/+}$ mice (p=0.07).

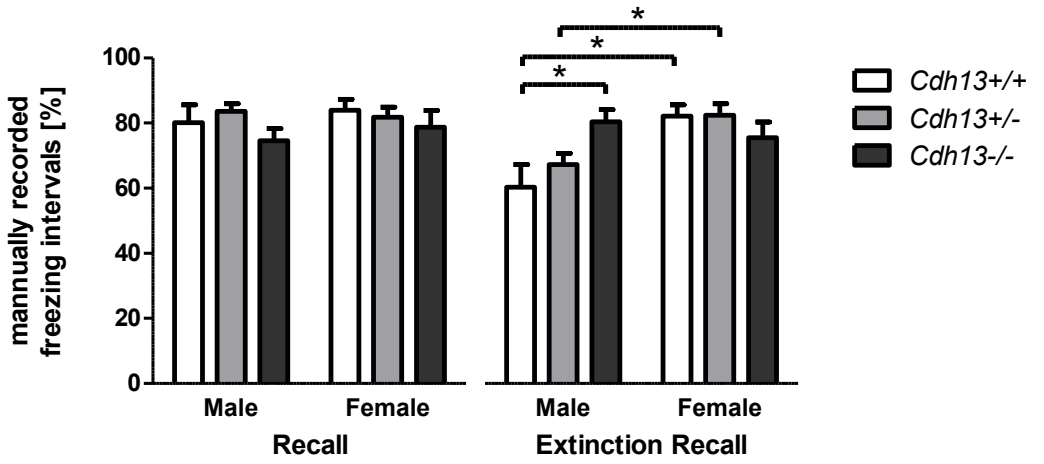

Figure 10 – Genotype x sex interaction during the extinction recall phase of the fear conditioning test. While no differences in freezing can be detected between groups for the context recall (day 1), showing that all groups acquired the conditioning similarly. During extinction recall, only male *Cdh13*$^{+/+}$ and *Cdh13*$^{+/-}$ mice show a noticeable reduction, while male *Cdh13*$^{-/-}$ mice and female mice in general show impaired extinction recall by persistent high freezing rates. Data is presented as mean + s.e.m., *P<0.05. G, genotype; S, sex; CTRL, control; MS, maternal separation; M, male; F, female. Figure is modified from Kiser et al., 2018.

3.2 CORT measurments

Analysis of the CORT concentration using 3-way ANOVA revealed a strong significant effect on sex ($F_{1,103}$=147.243, η^2=0.588, p<0.0001), with CORT levels being two times higher in females compared to males. A significant main effect on genotype ($F_{2,103}$=3.422, η^2=0.063, p=0.035) revealed in the subsequent post-hoc analysis that both *Cdh13*$^{+/-}$ (p=0.044) and *Cdh13*$^{-/-}$ (p=0.001) mice had significantly lower CORT levels when compared to *Cdh13*$^{+/+}$ mice (Figure 5).

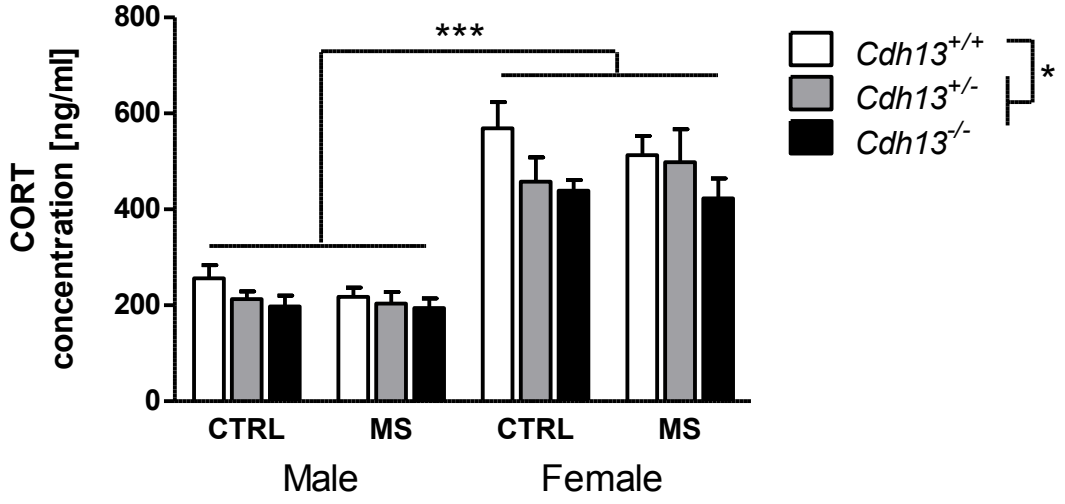

Figure 11 - Blood corticosterone concentrations of mice, which have not been tested for the last 2 weeks. Data is presented as mean + s.e.m. *P<0.05, ***P<0.001. Additional post-hoc comparisons are available in Supplementary table 1. G, genotype; E, environment; S, sex; CTRL, control; MS, maternal separation. Figure previously published Kiser et al., 2018.

3.3 Results of the quantitative real-time PCR

After the completion of the behavioural test, RNA from several brain regions of the male mice were collected and analysed using quantitative real-time PCR. This was done to ascertain if changes due to *Cdh13* deficiency and maternal separation could be found. Specific pathways of interest were addressed in each region individually. This part also was set up to prepare a follow up RNA sequencing and identify suitable targets for it. Additionally, outliers in the amygdala, hippocampus und PFC were removed, resulting in a final sample sizes are for the AMY (N=54), the hippocampus (N=52), the PFC (N=53) and the raphe nucleus (N=55). A 2-way ANOVA for the individual genes revealed many strong effects for genotype and environment, as well as a single interaction. Subsequently results are summarized by factors and interaction.

3.3.1 Prefrontal cortex

Genotype effects could be detected on the expression of *Vgat* and *Htr2a* ($F_{2,53}$≥3.861, η^2≥0.027, p≤0.027), with posthoc tests showing that these two genes are upregulated in *Cdh13*$^{-/-}$ mice compared to *Cdh13*$^{+/-}$ mice (p≤0.027) as well as *Cdh13*$^{+/+}$ mice in the case of *Vgat* (p=0.061). Additionally, several tendencies towards a genotype effect in *Htr1a*, *Gsk3a* and *Gabra1* ($F_{2,53}$≥2,606, η^2≥0.076, p≤0.083) were detected, all rising from a slight increase of expression of these genes in *Cdh13*$^{-/-}$ mice (Figure 12).

The environmental manipulation of maternal separation significantly affected *Htr1a*, *Htr2a*, *ActB, Gsk3a, Gsk3b* and *Dnmt3a* ($F_{1,53}$≥4.171, η^2≥0.061, p≤0.046), with all showing an upregulation in MS mice compared to controls (p≤0.046). There was also a tendency for an environmental effect in *Vgat* ($F_{1,53}$=3.112, η^2=0.045, p=0.083), showing an upregulation after MS (Figure 13).

No interaction was detected.

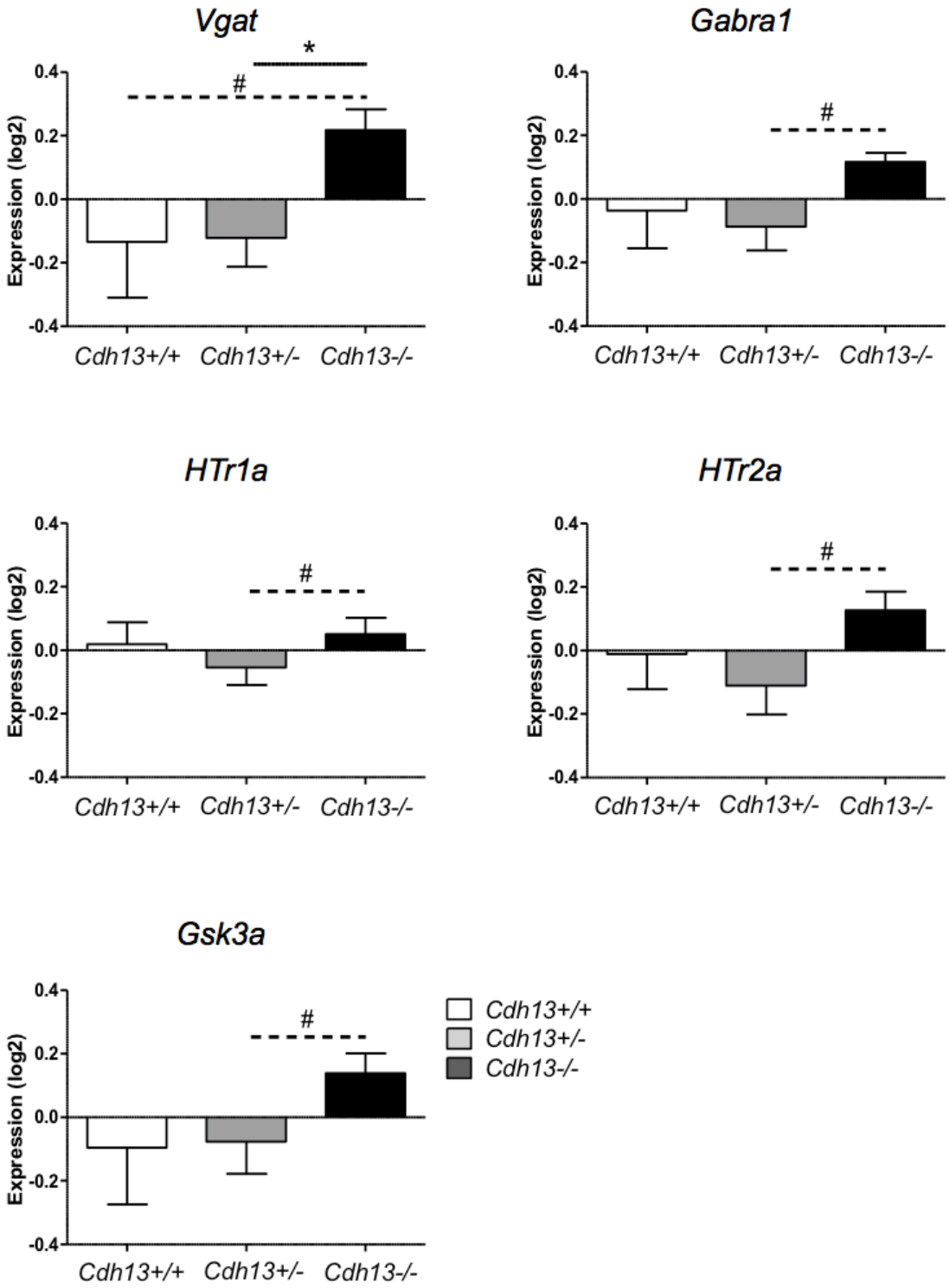

Figure 12 - Relative expressions of *Vgat, Gabra1, Htr1a, Htr2* and *Gsk3a* in the PFC summarised for genotype. The data showed a general increase of expression in *Cdh13*$^{-/-}$ compared to *Cdh13*$^{+/-}$ and in some cases even *Cdh13*$^{+/+}$. Data are presented as mean + s.e.m, $^{\#}P<0.1$, $^{*}P<0.05$.

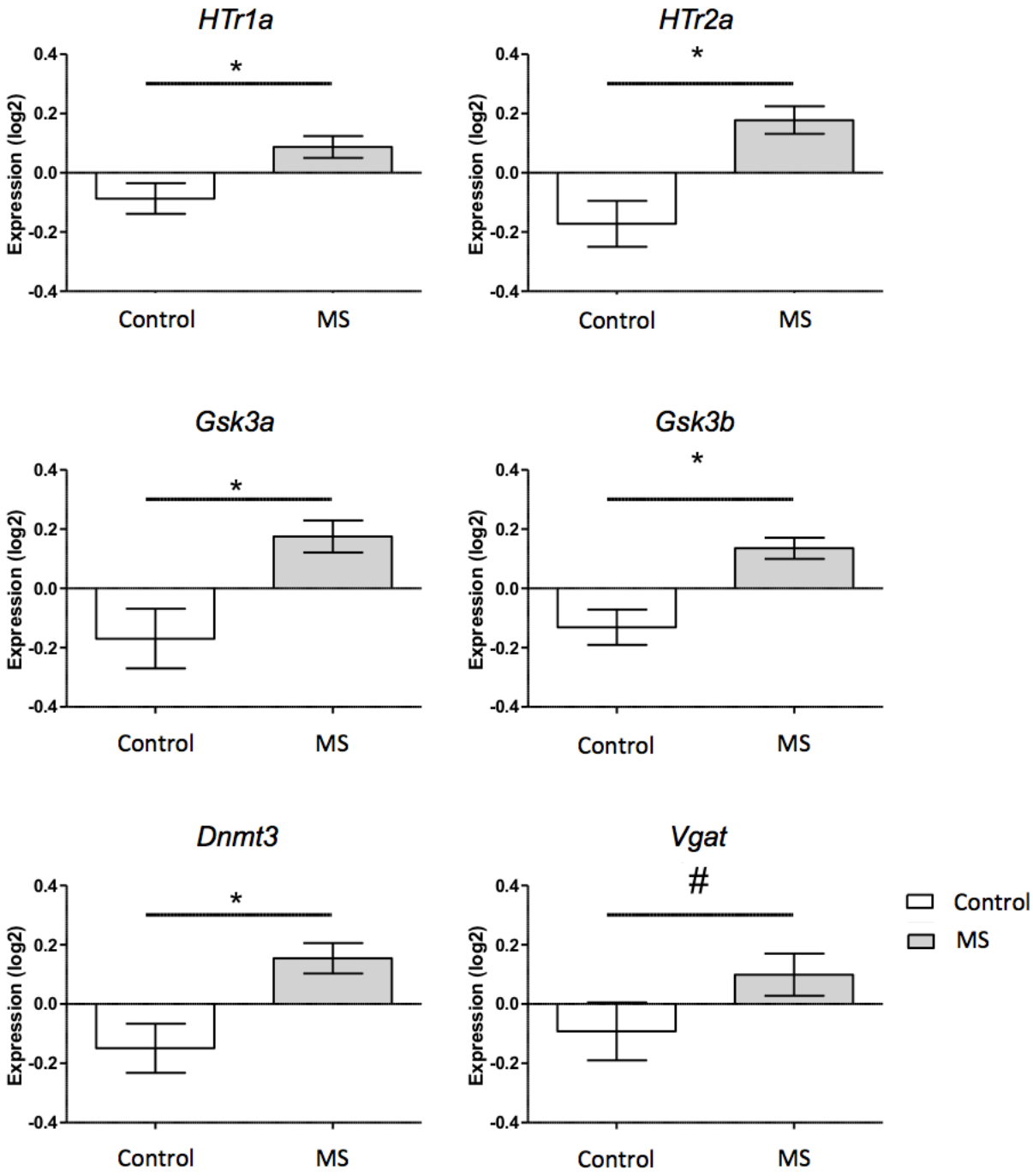

Figure 13 - Relative expressions of *Htr1a, Htr2, Gsk3a, Gsk3b*, *Dnmt3a* and *Vgat* in the PFC summarised for environment. Maternal separation lead to an increase in expression of all factors, when compared to control animals. Data are presented as mean + s.e.m, #P<0.1, *P<0.05.

3.3.2 Hippocampus

2-way ANOVA revealed a strong genotype effect on *Gsk3a* ($F_{2,52}$=4.622, η^2=0.15, p=0.0142), but not for *Gsk3b* ($F_{2,52}$=0.702, η^2=0.025 p=0.5). Post-hoc testing revealed a significant increase for *Cdh13*$^{-/-}$ compared to *Cdh13*$^{+/-}$ animals (p=0.014) (Figure 14). No other significant effects or interactions could be found. All other genes measured did not show a significant change in expression due to *Cdh13* deficiency or maternal separation.

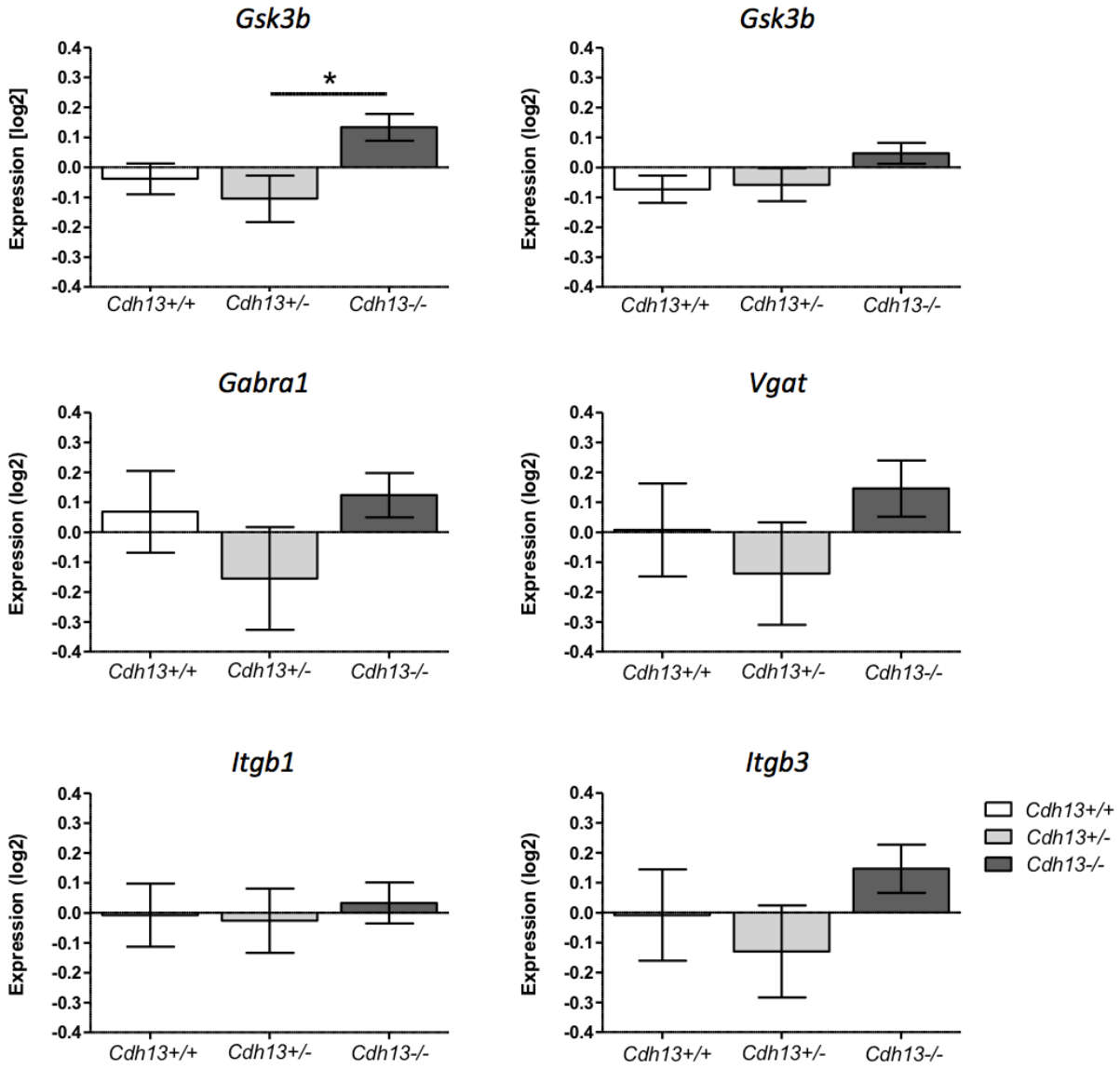

Figure 14 - Relative expressions of *Gsk3a* and *Gsk3b* in the hippocampus summarised for genotype. A significant increase of Gsk3a could be detected in *Cdh13*$^{-/-}$ when compared to *Cdh13*$^{+/-}$. No significant changes could be detected for *Gsk3b*, *Gabra1*, *Vgat*, *Itgb1* or *Itgb3*. Data are presented as mean + s.e.m, *P<0.05. **Integrin beta-1**

3.3.3 Dorsal/median raphe

2-way ANOVA revealed an interaction for *Mao*, *Tph2*, *Trdmt1* (formerly known as *Dnmt2*) and *Dnmt3a* ($F_{2,55}$≥4.058, η^2≥0.128, p≤0.023). No comparisons survived post-hoc comparison due to the huge variance and the overall small power in the 3 by 2 factor comparison (3 gene and 2 environment factors) (Figure 15).

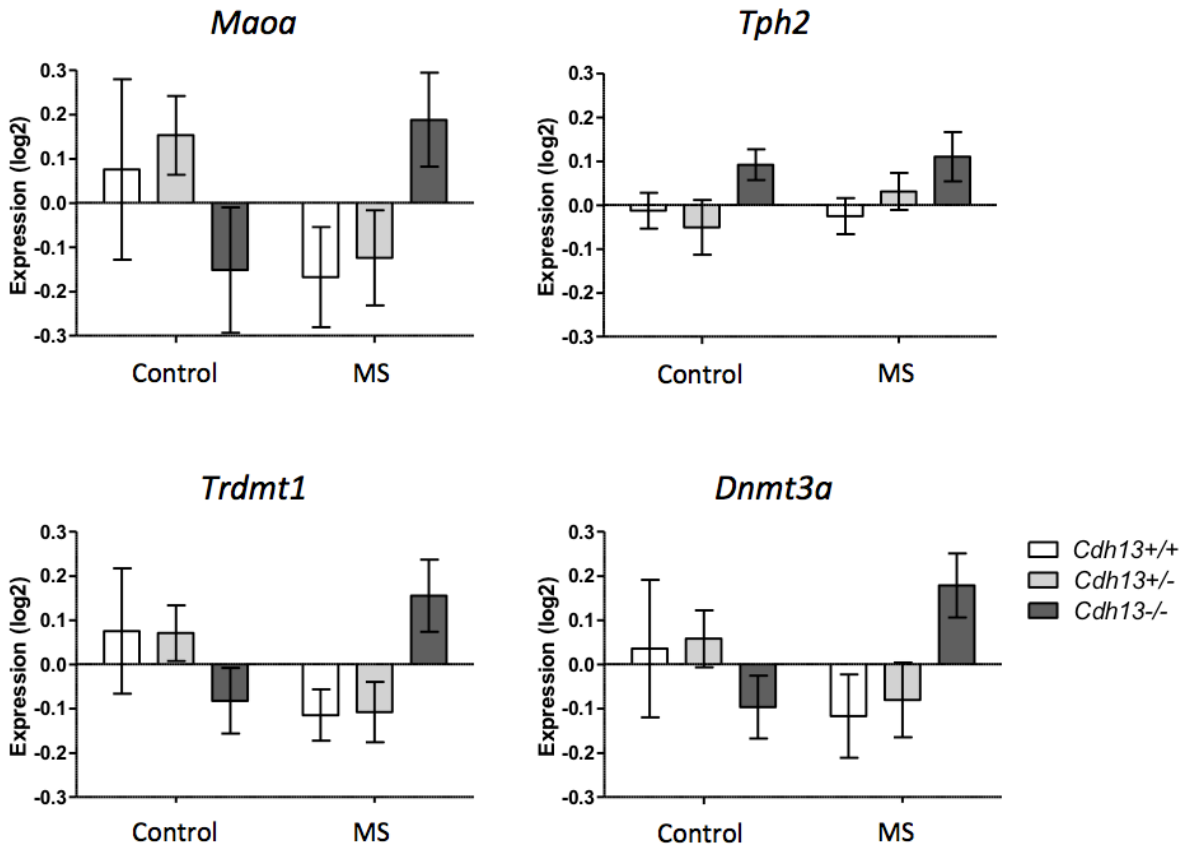

Figure 15 - Relative expressions of *Maoa, Tph2, Trdmt1* and *Dnmt3* in the dorsal/median raphe. A 2-way ANOVA revealed a significant interaction, but no effect survived post-hoc comparison. Data are presented as mean + s.e.m.

3.3.4 Amygdala

2-way ANOVA revealed a genotype effect on *Dnmt3a, Gad1, Gad2, Mc4r* and *Vglut3* ($F_{2,54}$≥3.404, η^2≥0.102, p≤0.041). Post-hoc comparisons showed that *Cdh13*$^{-/-}$ animals have increased expression of *Gad1, Gad2* and *Vglut3* when compared to *Cdh13*$^{+/+}$ and *Cdh13*$^{+/-}$ mice (p≤0.048). This effect was also present in *Gad1* and Dnmt3a but only reached significant levels compared to *Cdh13*$^{+/-}$ mice (p≤0.054). Contrary to this, *Mc4r* sticks out as the only gene with a downregulation in *Cdh13*$^{+/-}$ mice compared to the two other groups (p≤0.108) (Figure 16).

Additionally, environmental effects for *Trdmt1* and *Dnmt3a* could be detected ($F_{2,54}$≥4.633, η^2≥0.072, p≤0.036), alongside a tendency for *Nr3c* ($F_{2,54}$=0.702, η^2=0.025 p=0.088). Both *Trdmt1* and *Dnmt3a* are slightly upregulated after MS, while no effect survived post hoc comparison with respect to *Nr3c* (Figure 17).

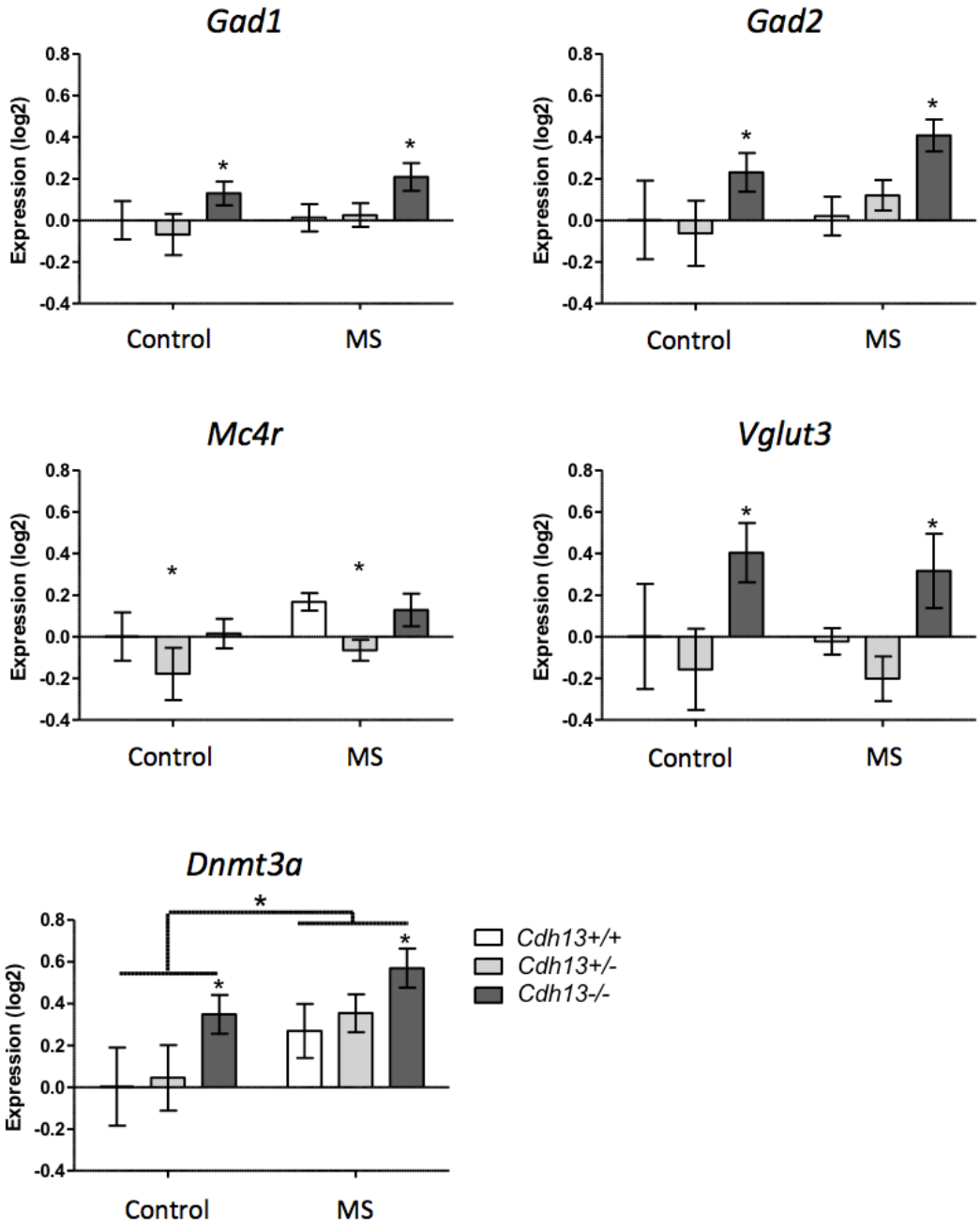

Figure 16 - Relative expressions of *Gad1*, *Gad2*, Dnmt3a Vglut3 and Mc4r in the amygdala. *Cdh13*$^{-/-}$ genotype increased GAD1 and GAD2 expression, irrespective of environment. Maternal separation and *Cdh13*$^{-/-}$ genotype both lead to an increase in expression of *Dnmt3a*. In *Cdh13*$^{+/-}$ mice *Mc4r* was significantly decreased compared to the other two groups. Data are presented as mean + s.e.m, *P<0.05.

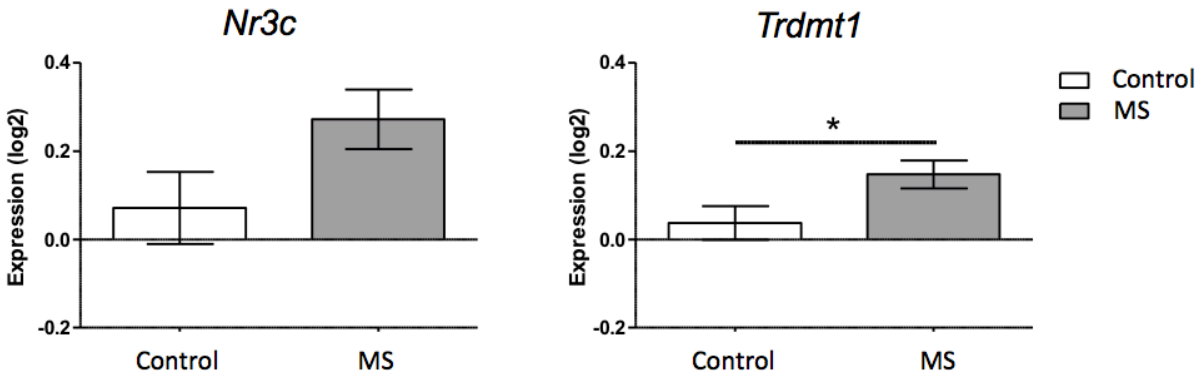

Figure 17: Relative expressions of *Gsk3a* and *Gsk3b* in the amygdala summarised for genotype. A significant increase of *Trdmt1* after MS could be detected. Data are presented as mean + s.e.m, *P<0.05.

3.4 Effects of *Cdh13* deficiency and MS on the expression of selected genes using Illumina RNA sequencing

To more accurately measure the change of expression due to *Cdh13* deficiency in mice a second cohort of animals was raised. In summary, this cohort had a similar litter size with 8.61±1.48 pups, an average pup weight of 10.73±1.28 g during weaning (PN22) and a sex distribution of 46.48% male animals. Genotype distribution was 26% $Cdh13^{+/+}$, 51% $Cdh13^{+/-}$and 23% $Cdh13^{-/-}$. Only male homozygote mice were used for the RNA sequencing at age PN22 (CTRL: $Cdh13^{+/+}$ N=8, $Cdh13^{-/-}$ N=9; MS: $Cdh13^{+/+}$ N=11, $Cdh13^{-/-}$ N=10) or after reaching adulthood at PN66 (CTRL: $Cdh13^{+/+}$ N=7, $Cdh13^{-/-}$ N=7; MS: $Cdh13^{+/+}$ N=10, $Cdh13^{-/-}$ N=7). Chi-Square analysis of the gene distribution did not reveal a deviation of the expected Mendelian distribution; neither did the population differ from the expected. In neither group differences for weight could be observed. There was also no weight difference between MS and CTRL conditions. The RNA-Sequencing data presented here can be accessed through the NCBI Gene Expression Omnibus by its GEO accession number GSE119082 (Edgar et al 2002) and was also published and partially discussed in (Kiser et al 2018).

3.4.1 Age as the strongest contributing factor

In the transcriptomic analysis, mRNA for a total of 21973 genes was detected. An initial analysis of the factor “Age” revealed 1387 genes (6,31%) to significantly express in an age-dependent manner, even after p-adjustment was performed (see Figure 18). GO and KEGG terms encompassed well over 600 entries, allowing for further filtering among all GO terms with a false discovery rate (fdr) below $1.0x10^{-04}$. This resulted in 91 GO and 3 KEGG terms remaining in the analysis, almost 20% (N=19, fdr<$1.0x10^{-04}$) of which were linked to developmental processes and classes, another 10% (n=9, fdr<$2.42\ x10^{-05}$) to cell cycle and proliferation, and 10% (N=9) to membrane adhesion, 10% (N=9) to the extracellular space of the plasma membrane. The remaining half was miscellaneous entries of various functions. None of the KEGG terms belong to any of these groups. The overall strong changes of developmental, proliferation and membrane factors and classes in the comparison suggested to split the two age groups to not lose the much weaker effects of the factors gene and environment.

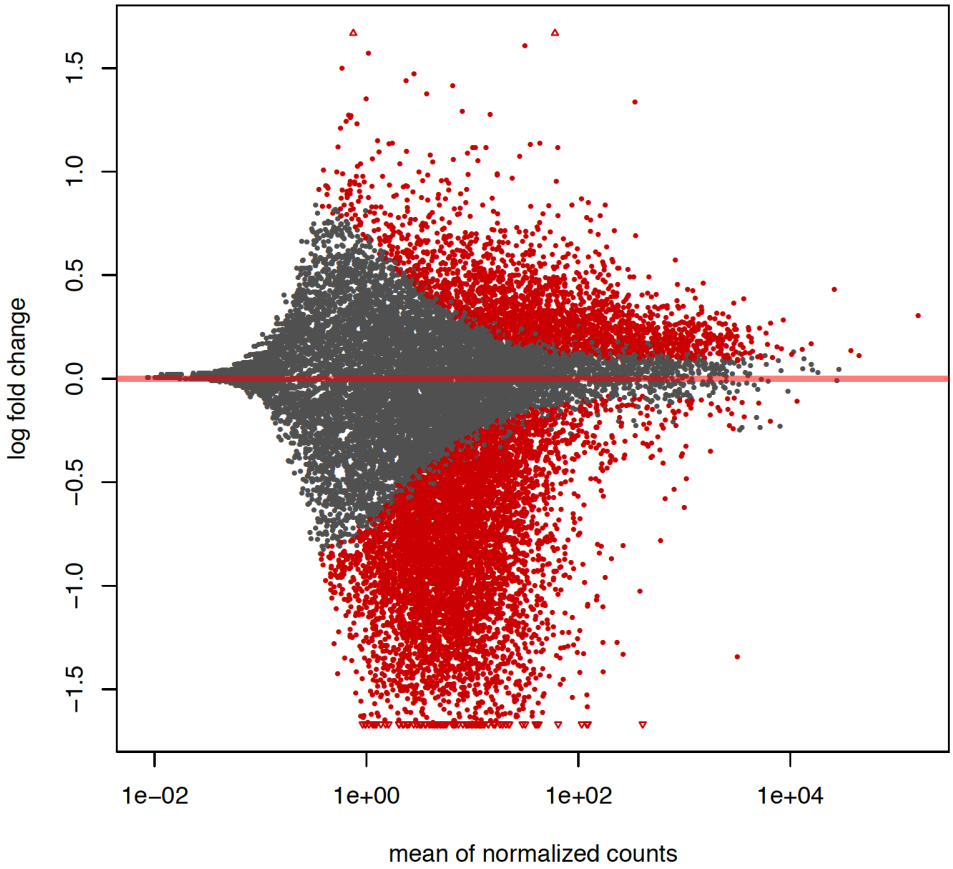

Figure 18 - Scatter plot showing base means of normalized counts (x-axis) and log fold changes (y-axis) (MA-plot). Marked red are all p-adjusted genes in the distribution. The red line represents a log fold change of zero. Graphic was calculated from results of the DESeq2 pipeline, not from the Limma-voom-edgeR.

3.4.2 Initial filtering and subsequent enrichment using GO/KEGG terms of changes due to *Cdh13* deficiency in mice

Several hundred differentially expressed genes (DEGs) could be detected to be altered by *Cdh13* deficiency (factor G, 310 genes), MS (factor E, 225 genes) and their interaction (interaction G x E, 402 genes). As mentioned in chapter 2.9.2, these results were filtered for genes that have a p-value smaller than 0.01 and a CPM fold change higher than 0.2 or lower than -0.2.

GO and KEGG term based enrichment for G revealed a significantly enriched pathway for cell adhesion (n=11, fdr=0.002, Table 1) and two classes of membrane components enriched due to the E (n>71, fdr<0.004, Table 2). No significant pathway enrichment could be identified for the interaction. The enrichment of the 46 overlapping genes for G, E, and G x E however revealed three enriched pathways, one for cell adhesion molecules (CAMs) (n=4, fdr=0.033, Table 3), which included 4 different cell adhesion molecules (synaptic cell adhesion molecule, *SynCAM*; Claudin 20, *Cldn20*; Integrin alpha 4, *Itga4*; neural cell adhesion molecule 2, *Ncam2*), as well as one for vascular smooth muscle contraction (n=4, fdr=0.027, Table 4) and one for Salivary secretion (n=3, fdr=0.041, Table 5).

Analysis of the PN66 group revealed one to two hundred DEGs due to CDH13 deficiency (factor G, 119 genes), MS (factor E, 186 genes) or their interaction (interaction G x E, 142 genes) (Table 2). GO and KEGG term based enrichment for G revealed a class for focal adhesion (GO #0005925, n=10, fdr=0.003, Table 2), but also several significantly enriched classes for the cell surface and the external side of the plasma membrane (GO: #09897, #05576, #44421, #70062, n>9, fdr<0.003, Table 2), as well as several classes related to the endoplasmic reticulum (ER), ER protein processing and vesicle transport could be identified to be enriched (GO: #05788, #34663, #31988, #05790, KEGG: #4141, n>3, fdr<0.027, Table 2). Analysis for the factor E showed two classes/pathways related to the ER that were enriched in the sample (GO: #034663, #05788 n>6, fdr<0.016, Table 2). No significant pathway enrichment could be identified for the interaction nor for the overlap between G, E and G x E. The summary of all gene lists is not printed with this thesis but can be accessed in a separate excel file that is provided with the digital version of the thesis or by using the uploaded files to the publication by (Kiser et al 2018).

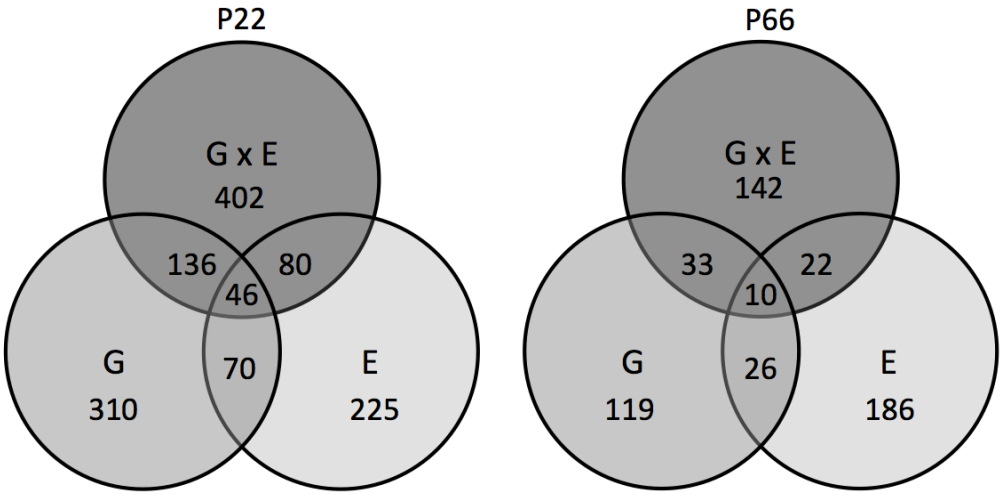

Figure 19 - Venn diagram of differentially expressed genes in the hippocampus of behaviourally naïve male mice in the two age groups, PN22 and PN66. The diagram shows the effects of genotype (*Cdh13*+/+ versus *Cdh13*-/-), environment (maternally separated versus control mice) and their interaction. G (genotype), E (environment), G x E (gene by environment interaction). Adapted from Kiser et al., 2018.

Table 1 - GO and KEGG gene enrichment analysis of the PN22 dataset filtered for the highest change (p<0,01 and (log2 FC > 0.2 or log2 FC < -0.2)). Abbreviations: G (Gene factor), E (Environment factor), Intersection, number of genes that have been significantly changed by gene, environment and the gene by environment interaction (see Venn diagram); # pathway ID, KEGG or GO pathway ID for the discovered enriched pathway; Overlap, number of genes from pathway/class contained in the filtered list of differently expressed genes; fdr, false discovery rate; Names for matches in the list, list of the names of genes contained in the sample that are part of the pathway/class. Table has been modified from supplementary material of Kiser et al., 2018.

Comparison	#pathway ID	pathway description	Overlap	fdr	Names for matches in list
Gene	KEGG.4514	Cell adhesion molecules (CAMs)	11/309	0.0021	*Cadm1, Cd6, Cdh15, Cldn20, Cldn9, H2-DMb1, H2-Ob, Itga4, Lmnt1, Lrrc4c, Ncam2*
Environment	GO.0031224	intrinsic component of membrane	74/224	0.0029	*Abca8a, Abca9, Adra1a, Adra2a, Awat1, Bdkrb2, Btla, Cadm1, Cd36, Cd52, Cd83, Chl1, Cklf, Cldn14, Clec4a2, Cntnap5b, Csmd1, Dcc, Dlk1, Dll3, 4930522H14Rik, Epor, Evi2a, Fcrl5, Fras1, Fxyd7, Gpr128, Gria3, Grik1, Grin2b, Grpr, Gucy2f, Gxylt2, Hcn1, Igsf6, Il6ra, Itga4, Kcna3, Kcnn3, Lmnt1, Lpar5, Ly75, Ncam2, Npr3, Opalin, Pcdh15, Plekhh1, Pllp, Plxnc1, Prtg, Ptprt, Rgr, Rnf133, Rnf148, Rom1, Scn11a, Slamf6, Slc24a4, Slc38a11, Spink10, St6galnac1, Tectb, Tenm1, Tmc8, Tmem132e, Tmem203, Tmem63a, Tmem98, Tnfrsf9, Tnfsf10, Tpcn2, Trpa1, Trpc4, Trpc5*
Environment	GO.0016021	integral component of membrane	71/224	0.0041	
Intersection	KEGG.4270	Vascular smooth muscle contraction	4/45	0.0267	*Adra1a, Gucy1a2, Pla2g3, Prkg1*
Intersection	KEGG.4514	Cell adhesion molecules (CAMs)	4/45	0.0332	*SynCAM, Cldn20, Itga4, Ncam2*
Intersection	KEGG.4270	Vascular smooth muscle contraction	4/45	0.0267	*Adra1a, Gucy1a2, Pla2g3, Prkg1*
Intersection	KEGG.4970	Salivary secretion	3/45	0.0407	*Adra1a, Gucy1a2, Prkg1*

Table 2 - GO and KEGG enrichment of genes from the PN66 comparison filtered for the highest change (p<0,01 and (log2 FC > 0.2 or log2 FC < -0.2)). G (Gene factor), E (Environment factor) G x E (Interaction); #pathway ID, KEGG or GO pathway ID for the discovered enriched pathway; Overlap, number of genes from pathway/class contained in the filtered list of differently expressed genes; fdr, false discovery rate; Names for matches in the list, list of the names of genes contained in the sample that are part of the pathway/class. Table has been modified from supplementary material of Kiser et al., 2018.

Comparison	#pathway ID	pathway description	Overlap	fdr	Names for matches in list
PN66 Gene	GO.0009986	cell surface	17/118	0.0001	*Bmp10, Calr, Cdh13, Cftr, Defb19, Defb29, Hspa5, Il2ra, Il6, Itga2b, Klrk1, Ms4a2, Pdia3, Procr, Spn, Tnfrsf9, Tnfsf4*
PN66 Gene	GO.0005576	extracellular region	41/118	0.0005	*Acot11, Ang5, Bmp10, Calr, Cdh13, Cftr, Cldn5, Cnn2, Defb19, Defb29, Fam59b, Grem2, Hist2h2be, Hist2h4, Hpx, Hsp90b1, Hspa5, Hspb1, Hsph1, Htra1, Il6, Itga2b, Itih3, Klk11, Ly6g5b, Marcksl1, Nqo2, Nyx, Ovgp1, Pdia3, Pdia6, Procr, Pycard, Pygl, Rp1l1, Sectm1a, Spn, Steap4, Tnfrsf9, Tnfsf4, Tspan8*
PN66 Gene	GO.0005788	endoplasmic reticulum lumen	7/118	0.0005	*Calr, Cercam, Fkbp10, Hsp90b1, Hspa5, Pdia3, Pdia6*
PN66 Gene	GO.0044421	extracellular region part	36/18	0.0011	*Acot11, Ang5, Bmp10, Calr, Cdh13, Cftr, Cldn5, Cnn2, Fam59b, Grem2, Hist2h2be, Hist2h4, Hpx, Hsp90b1, Hspa5, Hspb1, Hsph1, Htra1, Il6, Itga2b, Itih3, Klk11, Marcksl1, Nqo2, Nyx, Pdia3, Pdia6, Procr, Pygl, Rp1l1, Sectm1a, Spn, Steap4, Tnfrsf9, Tnfsf4, Tspan8*
PN66 Gene	GO.0009897	external side of plasma membrane	9/118	0.0018	*Calr, Cdh13, Il2ra, Il6, Itga2b, Klrk1, Ms4a2, Spn, Tnfrsf9*
PN66 Gene	GO.0005925	focal adhesion	10/118	0.0031	*Akap12, Calr, Cdh13, Cnn2, Hsp90b1, Hspa5, Hspb1, Itga2b, Pdia3, Procr*
PN66 Gene	GO.0034663	endoplasmic reticulum chaperone complex	3/118	0.0034	*Hsp90b1, Hspa5, Pdia6*
PN66 Gene	GO.0070062	extracellular exosome	28/118	0.0034	*Acot11, Calr, Cdh13, Cftr, Cldn5, Cnn2, Fam59b, Hist2h4, Hpx, Hsp90b1, Hspa5, Hspb1, Hsph1, Htra1, Itga2b, Itih3, Klk11, Marcksl1, Nqo2, Pdia3, Pdia6, Procr, Pygl, Rp1l1, Sectm1a, Spn, Steap4, Tspan8*
PN66 Gene	GO.0031988	membrane-bounded vesicle	30/118	0.0177	*Acot11, Ap1g2, Cdh13, Cftr, Cldn5, Cnn2, Fam59b, Hist2h4, Hpx, Hsp90b1, Hspa5, Hspb1, Hsph1, Htra1, Itga2b, Itih3, Klk11, Marcksl1, Nqo2, Ovgp1, Pdia3, Pdia6, Procr, Pygl, Rinl, Rp1l1, Sectm1a, Spn, Steap4, Tspan8*
PN66 Gene	GO.0005790	smooth endoplasmic reticulum	3/118	0.0274	*Calr, Hspa5, Pdia3*
PN66 Gene	KEGG.4141	Protein processing in endoplasmic reticulum	7/118	0.0065	*Calr, Derl3, Hsp90b1, Hspa5, Hsph1, Pdia3, Pdia6*
PN66 Environment	GO.0034663	endoplasmic reticulum chaperone complex	6/185	0.0000	*Dnajb11, Hsp90b1, Hspa5, Pdia4, Pdia6, Sdf2l1*
PN66 Environment	GO.0005788	endoplasmic reticulum lumen	8/185	0.0012	*Calr, Dnajb11, Hsp90b1, Hspa5, Pdia3, Pdia4, Pdia6, Sdf2l1*
PN66 Environment	KEGG:4141	Protein processing in ER	8/185	0.0159	*Calr, Dnajb11, Hsp90b1, Hspa5, Pdia3, Pdia4, Pdia6, Xbp1*

3.4.3 Manual screening of top 20 most significantly changed genes

As a further detailed analysis for each age group, the top 20 lists of genes changed by either *Cdh13* deficiency (factor G), MS (factor E) or their interaction (G x E) was manually screened for interesting associations found in the literature.

3.4.3.1 Top 20 genes affected by *Cdh13* deficiency in mice

To further understand the effect that *Cdh13* deficiency has on mice, the 20 genes with the smallest p-value in both age groups were investigated by an extensive literature research through PubMed and UniProt. In the PN22 group 3 genes that are linked with long-term depression of glutamatergic synapses in the hippocampus were found to be downregulated in *Cdh13*$^{-/-}$ mice (*SynCAM*, *Akap5* and *Prkg*). Another gene that is important for synaptic adhesion was also downregulated (*Tenm1*) (Table 4). *SynCAM* is a nectin-like protein that mediates homophilic adhesion between neurons and mast cells (Furuno et al 2005), that promotes glutamatergic synapse formation in the hippocampus (Biederer et al 2002) and is speculated to play a role in neuronal migration and differentiation (Fujita et al 2005). *Akap5* is a kinase anchor protein, which plays a role in long term depression (LTD) of neurons in the CA1 excitatory synapses in the hippocampus (Sanderson et al 2012). While only found putatively, *Prkg1* has been identified as an interaction partner of *Akap4* in humans (Hein et al 2015). *Tenm1* is a transmembrane protein, with a regulatory active C-terminus (*Tcap-1*), that can be cleaved off the rest of the protein (Chand et al 2013). *Tcap-1* has a dose dependent effect on stress in the amygdala (Wang et al 2005) and positively regulates neurite and axonal growth in hippocampal cell (Al Chawaf et al 2007), probably by supporting filapodium formation (Chand et al 2012). Additionally, 5 inflammation and immune factors (downregulated: *H2-Ob*; upregulated: *Unc13c*, *Sash3*, *Plcd1* and *Gdf15*) could be identified to be altered by *Cdh13* deficiency in the PN22 group. These factors play roles in mast-cell maturation (*Unc13d*) (Higashio et al 2008), apoptosis (*Plcd1*) (Mu et al 2015), pro cell survival (*Bdh2*)(Yang et al 2013), early immediate injury signalling (*Gdf15*) (Zimmers et al 2005) and regulation of T-cell invasion into the brain (*H2-Ob*)(Walter et al 2000).

The top 20 list of smallest p-values due to *Cdh13* deficiency in PN66 mice revealed a different picture, compared to their siblings that were analysed at the younger age of PN22. Most notably, 7 of these genes are either directly or indirectly linked to the endoplasmic reticulum (ER) (Table 4). 6 of these genes were downregulated (*Calr, Derl3, Hsp90b1, Hsph1, Pdia3* and *Cyp2j9*) while one was upregulated (*Fkbp10*). Calreticulin (*Calr*) is a calcium binding chaperone that also plays a role in Ca^{2+} homeostasis of the ER (Llewellyn et al 2000, Michalak et al 1999). *Hsp90b1* and *Hsph1 (*alias *Hsph110*) are two heat shock proteins. In particular, *Hsph1* is expressed in the hippocampus (Hylander et al 2000) and has been associated with neurodevelopmental disorders, such as Parkinson's disease (Gorenberg & Chandra 2017). The cytochrome *Cyp2j9* is widely expressed in the brain (Qu et al 2001), where it is involved in proliferation and neurogenesis (Miller et al 2013). *Derl3* is involved in the ER stress response (Belmont et al 2010) and folding of membrane proteins (Lilley & Ploegh 2004). Both *Pdia3* and *Fkbp10* are also involved in protein folding and ER quality control (Bork et al 2017). Other members of the *Pdia3* family are associated with *Calr* and *Calnexin* function (Solda et al 2006), while binding partners of the *Fkbp* family are known to have neuroprotective roles in the brain (Tanaka et al 2002). Explorative enrichment analysis of these 7 genes revealed *Calr, Pdia3, Fkbp10, Derl3, Hsp90b1* and *Hsph1* to be part of the ER lumen (GO:0005788, fdr=0.003) and involved in protein processing in the ER (KEGG:4141, fdr<0.000, Table 3).

Table 3 - GO and KEGG annotated pathways, acquired by calculating the network enrichment for the top 20 regulated genes of the PN66 dataset in the comparison between *Cdh13*$^{-/-}$ and *Cdh13*$^{+/+}$. Enrichment shows multiple functional and regional interconnections between all 7 factors, highlighting a dysregulation of the associated networks. fdr=False Discovery Rate.

pathway ID	pathway description	Genes in our sample	fdr
GO:0006457	protein folding	*Calr, Pdia4, Hsp90b1, Hsph1, Fkbp10*	0.002
GO:0061077	chaperone-mediated protein folding	*Calr, Hsph1, Fkbp10*	0.048
GO:0005788	endoplasmic reticulum lumen	*Calr, Pdia3, Hsp90b1, Fkbp10*	0.003

In both age groups Cdh13 also appears in the list. Compared to wild type mice *Cdh13* is significantly downregulated in *Cdh13*-deficient mice. This finding could be explained by the fact that a bit of *Cdh13* mRNA up to the modified stop-codon is still expressed. This piece is non-functional and should usually be degraded by nonsense-mediated mRNA decay (Wada et al 2018). *Insitu* stainings in our laboratory however consistently reveal small quantities of non-functional *Cdh13* mRNA to remain within the cells of *Cdh13* knockout mice. This finding can be considered not only as a replication, but also a verification that our analysis pipeline worked.

Table 4 - Top 20 genes differentially regulated in *Cdh13*$^{-/-}$ compared to *Cdh13*$^{+/+}$ in PN22 and PN66 mice. The base list of 21973 individual genes was first processed and then filtered for a p-value smaller than 0.01 and a logarithmic fold change larger than 0.2 or smaller than -0.2. Downregulated genes are coded in red, upregulated genes in green.

	SYMBOL	ENSEMBLE-ID	p value	p.adj	log2FC	Full Name
PN22	*Fabp12*	ENSMUSG00000027530	0,000	0,420	-1,678	Fatty acid binding protein 12
	Cyp2ab1	ENSMUSG00000022818	0,000	0,420	-1,621	Cytochrome P450, family 2, subfamily ab, polypeptide 1
	Olfr1395	ENSMUSG00000050763	0,000	0,420	-1,317	olfactory receptor 1395
	Tnni2	ENSMUSG00000031097	0,001	0,420	-1,264	Troponin I
	Cdh13	ENSMUSG00000031841	0,000	0,000	-0,678	Cadherin 13
	Tenm1	ENSMUSG00000016150	0,001	0,420	-0,447	Teneurin transmembrane protein 1
	Mcf2	ENSMUSG00000031139	0,000	0,420	-0,353	proto-oncogene DBL
	Prkg1	ENSMUSG00000052920	0,000	0,420	-0,351	Protein kinase, cGMP-dependent, type 1
	SynCAM	ENSMUSG00000032076	0,000	0,420	-0,301	synaptic cell adhesion molecule
	Kiaa1109	ENSMUSG00000037270	0,001	0,420	-0,259	Fragile Site-Associated Protein; Tweek
	Pacrgl	ENSMUSG00000029089	0,000	0,420	-0,226	PARK2 co-regulated-like
	Akap5	ENSMUSG00000021057	0,000	0,420	-0,226	A-Kinase Anchor protein 5
	Mllt3	ENSMUSG00000028496	0,000	0,420	-0,210	Myeloid/Lymphoid Or Mixed-Lineage Leukemia Translocated To Chromosome 3
	Plcd1	ENSMUSG00000010660	0,000	0,420	0,296	Phospholipase C, delta 1
	Fxyd1	ENSMUSG00000036570	0,001	0,420	0,359	Phospholemman
	Sash3	ENSMUSG00000031101	0,000	0,420	0,443	SAM And SH3 Domain Containing 3
	Bdh2	ENSMUSG00000028167	0,001	0,420	0,524	3-Hydroxybutyrate Dehydrogenase 2
	Unc13d	ENSMUSG00000057948	0,000	0,420	0,567	Unc-13 homolog D
	Fbxw14	ENSMUSG00000105589	0,001	0,420	1,242	F-Box And WD Repeat Domain Containing 14
	Gdf15	ENSMUSG00000038508	0,001	0,420	1,415	Growth differentiation factor 15
PN66	*Pnpla5*	ENSMUSG00000018868	0,000	0,193	-2,002	Patatin Like Phospholipase Domain Containing 5
	Ms4a2	ENSMUSG00000024680	0,000	0,193	-1,835	Membrane Spanning 4-Domains A2
	Gimap9	ENSMUSG00000051124	0,001	0,527	-1,206	GTPase, IMAP family member 9
	Defb29	ENSMUSG00000044249	0,000	0,193	-1,191	Beta-Defensin 29
	Tas1r3	ENSMUSG00000029072	0,000	0,193	-1,148	Taste receptor type 1 member 3
	Derl3	ENSMUSG00000009092	0,000	0,526	-0,837	Derlin-3
	Cdh13	ENSMUSG00000031841	0,000	0,108	-0,609	Cadherin 13
	Nyx	ENSMUSG00000051228	0,000	0,492	-0,545	Nyctalopin
	Hsp90b1	ENSMUSG00000020048	0,000	0,526	-0,337	Endoplasmin
	Calr	ENSMUSG00000003814	0,000	0,526	-0,325	Calreticulin
	Acot11	ENSMUSG00000034853	0,000	0,461	-0,310	Acyl-coenzyme A thioesterase 11
	Cyp2j9	ENSMUSG00000015224	0,000	0,526	-0,268	Cytochrome P450, family 2, subfamily j, polypeptide 9
	Hsph1	ENSMUSG00000029657	0,000	0,193	-0,245	Heat shock protein 105 kDa
	Pdia3	ENSMUSG00000027248	0,000	0,526	-0,235	Protein disulfide-isomerase A3
	Garem2	ENSMUSG00000044576	0,001	0,555	0,240	GRB2-associated and regulator of MAPK protein 2
	Fkbp10	ENSMUSG00000001555	0,000	0,526	0,315	Peptidyl-prolyl cis-trans isomerase FKBP10
	Muc3a	ENSMUSG00000094840	0,001	0,568	0,387	Mucin 3A, cell surface-associated
	Rbm3	ENSMUSG00000031167	0,000	0,461	0,433	RNA-binding protein 3
	Ciart	ENSMUSG00000038550	0,000	0,193	0,513	Circadian-associated transcriptional repressor
	Fmo9	ENSMUSG00000026560	0,000	0,526	1,507	Flavin-containing monooxygenase

3.4.3.2 Top 20 genes affected by *maternal separation* in mice

The list of top 20 smallest p-value genes in PN22 contained a number of genes associated with apoptosis, cell survival, differentiation and migration and five apoptosis-related genes (Table 5). Interestingly, the two downregulated genes Sushi-repeat-containing protein (*Srpx*), as well as L-amino oxidase 1 (*Lao1*), encode pro-apoptotic factors that induce apoptosis (Suhr & Kim 1996, Tambe et al 2009), while the three upregulated genes, 3-hydroxybutyrate dehydrogenase type 2 (*Bdh2*), Protein Mis18-beta (*Oip5*) and Cell division cycle-associated protein 2 (*Cdca2*), are all pro-survival factors that promote cell survival (Naemura et al 2018, Yang et al 2013) and neurogenesis (Sansom et al 2009). Three genes involved in hippocampal cell migration and differentiation (*Adra1a*, *Tenm1* and *Fndc1*) were also significantly altered. *Adra1a* is expressed in the hippocampus (Alonso-Llamazares et al 1995) and activated during active-avoidance learning in rats, modulating synaptic efficiency (Lv et al 2016). Inhibition of *Adra1a* can cause motivational and motor activity depression while keeping spatial memory intact (Levcik et al 2013). *Tenm1* positively regulates neurite and axonal growth in hippocampal cells (Al Chawaf et al 2007). *Fndc1* interacts with integrin splice variants (Liao et al 2002) and plays an important role in migration, differentiation, cell adhesion and growth (Pankov & Yamada 2002).

Among the 20 genes that were most affected by MS in PN66 were many associated with ER stress, immune response, membrane binding or neurogenesis (Table 5). The most prominent pathway in this list includes ER- related genes, among which *Dnajb11, Pdia3, Pdia6, Hspa5* and *Sdf2l1* are upregulated, while *Cirbp, Cyp4f15* and *Fbxw10* are downregulated. *Dnajb11* promotes *Hspa5* recruitment (Shen et al 2005) and is an indicator for ER stress (Fukuda et al 2001). The other four factors identified have also been implicated in ER stress (*Pia3* and *Pia6*) (Ramos et al 2015), apoptosis and inflammation (*Cirbp*) (Nishiyama et al 1997) and might play a role in ER function (*Cyp4f15*) (Neve & Ingelman-Sundberg 2010). Particularly upregulation of *Pdia3* and *Pdia6* are clear signs of ER stress in cancer (Ramos et al 2015).

GO/KEGG terms presented in chapter 3.4.2 underscored the finding of ER changes due to MS with the same genes (*Calr, Pdia3, Fkbp10, Derl3, Hsp90b1* and *Hsph1*) being part of the same GO term for the ER lumen (GO:0005788, fdr=0.003) and the KEGG class involved in protein processing in the ER (KEGG:4141, fdr<0.000, Table 2). *DnaJ* homolog

subfamily B member 11 (*DnaJb11*) together with heat shock protein 5 (*Hspa5*) forms a complex that exists (spatially) apart of the calnexin/calreticulin system in the ER and binds to unfolded proteins (Meunier et al 2002), facilitating their correct folding (Cunnea et al 2003, Shen et al 2005).

Table 5 - Top 20 genes differentially regulated after maternal separation in PN22 and PN66 mice. The base list of 21973 individual genes was first processed and then filtered for a p-value smaller than 0.01 and a logarithmic fold change larger than 0.2 or smaller than -0.2. Downregulated genes are coded in red, upregulated genes in green.

	SYMBOL	ENSEMBLE-ID	p value	p.adj	log2FC	Full Name
PN22	*Vmn2r40*	ENSMUSG00000090864	0,000	0,625	-2,141	Vomeronasal 2, receptor 40
	Bpi	ENSMUSG00000052922	0,000	0.233	-1,755	Bactericidal permeability-increasing protein
	Adam3	ENSMUSG00000031553	0,001	0,625	-1,493	A disintegrin and metallopeptidase domain 3
	Fabp12	ENSMUSG00000027530	0,000	0,625	-1,462	Fatty acid-binding protein 12
	4930522 H14Rik	ENSMUSG00000060491	0,001	0,625	-1,432	Uncharacterized protein C1orf185 homolog
	Olfr1395	ENSMUSG00000050763	0,000	0,625	-1,289	Olfactory receptor 1395
	Lao1	ENSMUSG00000024903	0,001	0,625	-1,064	Amine oxidase
	Srpx	ENSMUSG00000090084	0,000	0,625	-0,880	Sushi-repeat-containing protein SRPX
	Gm1027 1	ENSMUSG00000069518	0,000	0,625	-0,845	Predicted gene 10271
	Gm2156 0	ENSMUSG00000094460	0,001	0,625	-0,788	Predicted gene, 21560
	Gucy2f	ENSMUSG00000042282	0,001	0,625	-0,726	Retinal guanylyl cyclase 2
	Adra1a	ENSMUSG00000045875	0,000	0,625	-0,514	Alpha-1A adrenergic receptor
	Cda	ENSMUSG00000028755	0,000	0,625	-0,452	Cytidine deaminase
	B230307 C23Rik	ENSMUSG00000080717	0,001	0,625	-0,415	MCG4787, isoform CRA_b
	Tenm1	ENSMUSG00000016150	0,001	0,625	-0,413	Teneurin-1
	Fndc1	ENSMUSG00000071984	0,001	0,625	-0,293	Fibronectin type III domain-containing 1
	Bdh2	ENSMUSG00000028167	0,000	0,625	0,533	3-hydroxybutyrate dehydrogenase type 2
	Oip5	ENSMUSG00000072980	0,001	0,625	0,567	Protein Mis18-beta
	Cdca2	ENSMUSG00000048922	0,000	0,625	0,610	Cell division cycle-associated protein 2
	Tectb	ENSMUSG00000024979	0,000	0,390	1,263	Beta-tectorin
PN66	*Bpifb6*	ENSMUSG00000068009	0,000	0,458	-1,743	Bacterial/permeability increasing protein, fold-containing family B member 6
	Pnpla5	ENSMUSG00000018868	0,000	0,458	-1,654	Patatin-like phospholipase domain-containing protein 5
	Tnfsf4	ENSMUSG00000026700	0,000	0,458	-1,546	Tumor necrosis factor ligand superfamily member 4
	Defb26	ENSMUSG00000074680	0,001	0,511	-1,173	Beta-defensin 26
	Defb29	ENSMUSG00000044249	0,000	0,313	-1,173	Beta-defensin 29
	Tas1r3	ENSMUSG00000029072	0,000	0,458	-0,894	Taste receptor type 1 member 3
	Meltf	ENSMUSG00000022780	0,000	0,458	-0,664	Melanotransferrin
	Gli1	ENSMUSG00000025407	0,001	0,511	-0,589	Zinc finger protein GLI1
	Cdhr3	ENSMUSG00000035860	0,000	0,313	-0,568	Cadherin related family member 3
	Fbxw10	ENSMUSG00000090173	0,000	0,458	-0,450	F-box/WD repeat-containing protein 10
	Itga2b	ENSMUSG00000034664	0,000	0,458	-0,426	Integrin alpha-2b
	Fcgr2b	ENSMUSG00000026656	0,000	0,458	-0,358	Fc receptor, IgG, low affinity Iib
	Cyp4f15	ENSMUSG00000073424	0,000	0,458	-0,335	Cytochrome P450 CYP4F15
	Cirbp	ENSMUSG00000045193	0,000	0,458	-0,321	Cold-inducible RNA-binding protein
	Dnajb11	ENSMUSG00000004460	0,001	0,509	0,214	DnaJ homolog subfamily B member 11
	Pdia3	ENSMUSG00000027248	0,000	0,458	0,219	Protein disulfide-isomerase A3
	Pdia6	ENSMUSG00000020571	0,000	0,458	0,340	Protein disulfide-isomerase A6
	Hspa5	ENSMUSG00000026864	0,000	0,313	0,517	Endoplasmic reticulum chaperone BiP
	Sdf2l1	ENSMUSG00000022769	0,000	0,458	0,585	Stromal cell-derived factor 2-like protein 1
	Rnf224	ENSMUSG00000089953	0,000	0,458	1,668	RING finger protein 224

3.4.3.3 Top 20 genes affected by the interaction between *Cdh13* deficiency and maternal separation in mice

The top 20 list of genes which were most significantly changed by the interaction between genotype and environment in the PN22 group included genes associated with neuronal migration, synapse formation, brain development, endoplasmic reticulum (ER) stress and inflammation, but also a group of five genes with unclear or unknown functions. Analysis of interaction was carried out, using t-tests (corrected using fdr) on the read data to highlight the direction of the changes (Table 6). Galanin receptor type 1 (Galr1) shows a robust increase in expression in *Cdh13*-deficient mice after MS ($p=0.021$) and a downregulation of *Galr1* in wildtype animals after MS ($p=0.003$). The opposite direction is visible in Claudin (*Cldn20*), homeobox protein DLX-4 (*Dlx4*), thrombospondin-4 (*Thbs4*), cell division cycle-associated protein 2 (*Cdca2*) and retinol-binding protein 2 (*Rbp2*), for which a marked downregulation in *Cdh13*-deficient animals after MS ($p<0.082$) and an upregulation for wildtype mice after MS ($p<0.082$), relative to their Ctrl groups can be observed. Additionally, all but *Rbp2* and *Cdca2* also show a difference between wildtype and *Cdh13*-deficient mice, but only in between the control groups ($p<0.082$).

The top 20 list of genes with expressional changes by an interaction of *Cdh13* genotype and environment (MS) included genes associated with cell-cell adhesion and regulation of the cytoskeleton (Table 6). *Rhoh* is an important regulator of cell survival and growth and belongs to an entire family of factors that is strongly involved in migration and cell-cell adhesion (Nelson 2008, Wittchen et al 2005). *Spn* promotes de-adhesion and cell-cell interactions in macrophages (van den Berg et al 2001) and is suggested to be involved in the removal of inhibitory proteins from the immunological synapse (Allenspach et al 2001). Integrin alpha 2b (*Itga2b*) has been shown to be involved in Ca^{2+} dependent adhesion (Calvete 1995) as an adhesion cell-surface receptor (Takada et al 2007).

The remaining genes can be summarised by immune function, including high affinity immunoglobulin epsilon receptor subunit beta (*Ms4a2*), Beta-defensin 29 (*Defb29*), two variants of B cell translocation gene 3 (*Btg3*) and Leukosialin (*Spn*). There were 6 genes with unclear or various functions.

Table 6 - Top 20 genes differentially regulated due to gene by environment interactions in PN22 and PN66 mice. The base list 21973 individual genes was first processed and then filtered for a p-value smaller than 0.01 and a logarithmic fold change larger than 0.2 or smaller than -0.2.

	SYMBOL	ENSEMBLE-ID	p value	p.adj	Alternative Names
PN22	*Rbp2*	ENSMUSG00000032454	0,000	0,358	Retinol-binding protein 2
	Cldn20	ENSMUSG00000091530	0,000	0,358	Claudin
	Dlx4	ENSMUSG00000020871	0,000	0,358	Homeobox protein DLX-4
	Thbs4	ENSMUSG00000021702	0,000	0,358	Thrombospondin-4
	Bdh2	ENSMUSG00000028167	0.000	0,300	3-hydroxybutyrate dehydrogenase type 2
	Cdca2	ENSMUSG00000048922	0,000	0,379	Cell division cycle-associated protein 2
	Unc13d	ENSMUSG00000057948	0,000	0,386	Protein unc-13 homolog D, Munc13-4
	Naaa	ENSMUSG00000029413	0,000	0,358	N-acylethanolamine-hydrolyzing acid amidase
	Gtf3c6	ENSMUSG00000019837	0,000	0,386	General transcription factor 3C polypeptide 6
	Usp13	ENSMUSG00000056900	0,000	0,379	Ubiquitin carboxyl-terminal hydrolase 13
	Zfp804b	ENSMUSG00000092094	0,000	0,386	Zinc finger protein 804B
	Pla2g3	ENSMUSG00000034579	0,000	0,358	Phospholipase A2, group III
	Galr1	ENSMUSG00000024553	0,000	0,358	Galanin receptor type 1
	Tnni2	ENSMUSG00000031097	0,000	0,358	Troponin I, fast skeletal muscle
	Gm15557	ENSMUSG00000078972	0,000	0,358	Predicted gene 15557
	Fabp12	ENSMUSG00000027530	0,000	0,379	Fatty acid-binding protein 12
	Tex19.1	ENSMUSG00000039329	0,000	0,181	Testis-expressed protein 19A
	4930522H14Rik	ENSMUSG00000060491	0,000	0,339	Uncharacterized protein C1orf185 homolog
	Cebpe	ENSMUSG00000052435	0,000	0,358	CCAAT/enhancer-binding protein epsilon
	Vmn2r40	ENSMUSG00000090864	0,000	0,358	Vomeronasal 2, receptor 40
PN66	*Noxa1*	ENSMUSG00000036805	0,001	0,813	NADPH oxidase activator 1
	Stpg3	ENSMUSG00000036770	0,001	0,813	Sperm-tail PG-rich repeat-containing protein 3
	Olfr1384	ENSMUSG00000044170	0,001	0,813	Olfactory receptor 1384
	Traip	ENSMUSG00000032586	0,000	0,756	E3 ubiquitin-protein ligase TRAIP
	Per3	ENSMUSG00000028957	0,001	0,813	Period circadian protein homolog 3
	Btg3	ENSMUSG00000022863	0,001	0,813	Protein BTG3
	Gm7334	ENSMUSG00000044645	0,000	0,813	Protein BTG3
	Ccdc91	ENSMUSG00000030301	0,001	0,813	Coiled-coil domain-containing protein 91
	Lpcat3	ENSMUSG00000004270	0,001	0,813	Lysophospholipid acyltransferase 5
	Yipf2	ENSMUSG00000032182	0,001	0,813	Protein YIPF2
	Acot11	ENSMUSG00000034853	0,001	0,813	Acyl-coenzyme A thioesterase 11
	Dnah1	ENSMUSG00000019027	0,001	0,813	Dynein heavy chain 1, axonemal
	Itga2b	ENSMUSG00000034664	0,000	0,813	Integrin alpha-2b
	Rhoh	ENSMUSG00000029204	0,001	0,813	Rho-related GTP-binding protein RhoH
	Spn	ENSMUSG00000051457	0,001	0,813	Leukosialin;
	Defb29	ENSMUSG00000044249	0,001	0,813	Beta-defensin 29
	Gm11541	ENSMUSG00000056008	0,001	0,813	Predicted gene 11541
	Tas1r3	ENSMUSG00000029072	0,000	0,756	Taste receptor type 1 member 3
	Ms4a2	ENSMUSG00000024680	0,001	0,813	High affinity immunoglobulin epsilon receptor subunit beta
	Pnpla5	ENSMUSG00000018868	0,000	0,756	Patatin-like phospholipase domain-containing protein 5

4 Discussion

The aim of this thesis was to investigate how *Cdh13* deficiency in mice modulates the effects of ELS, both at a behavioural and RNA expression level. To answer this question, mice of litters containing all three *Cdh13* genotypes (*Cdh13*$^{+/+}$, *Cdh13*$^{+/-}$ and *Cdh13*$^{-/-}$) were separated from their mothers for 3 h during each day for the first 14 days of their lives (PN1-PN14). Another group of mice were daily handled during the same time to act as a control group. One cohort was behaviourally tested and RNA expression was analysed using qRT-PCR. The results of this cohort are presented in chapters 4.1 to 4.4. A second cohort of behaviourally naïve mice was then raised to analyse changes in the hippocampal transcriptome. The results of this experiment are presented in chapter 4.5.

4.1 *Cdh13* deficiency modifies the response to MS and changes the balance of the serotonergic and GABAergic neurotransmitter systems

The first cohort of mice were tested in a battery of behavioural tests to evaluate anxiety behaviour, memory and locomotion. Changes in behaviour could be found for all three investigated factors (genotype, sex and environment) and highlighted a complex interaction of gene, environment and sex. Additionally, RNA from 4 different brain regions of these mice was isolated and analysed using qRT-PCR.

4.1.1 No adaptation to MS in *Cdh13*-deficient mice

While maternal separation is often considered to induce maladaptive changes, our results show a reduced anxiety phenotype in many of our MS groups during testing, particularly in *Cdh13* wildtype (and heterozygous) mice. These two MS groups show increased open arm time in the EPM, increased time in light side of the LDB and increased centre time in the OF (only in male MS mice). In contrast, *Cdh13*$^{-/-}$ MS mice show no increased time on the light side of the LDB, when compared to the two other MS groups, a delayed entering of the central area of the OF (with a recovery towards the end of the experiment) in the *Cdh13*$^{-/-}$ MS mice when compared to *Cdh13*$^{-/-}$ control mice, a tendency for increased step-down latency in the SD test in *Cdh13*$^{-/-}$ mice compared to the other two groups (as seen by a more cautious/hesitant phenotype) and reduced fear extinction in the cFC for *Cdh13*$^{-/-}$ male mice when compared to the other two male groups.

Many studies which use MS, report an increase in anxiety behaviour (Shin et al 2016), depression-like phenotypes (Strekalova et al 2005), reduction in learning (Baudin et al 2012), alongside cognitive deficits in their animals after exposure to MS (Baudin et al 2012, Veenema et al 2007, Wong et al 2015). A large body of literature however also highlights the ambiguity of the paradigm with respect to various influences such as: small variations in the environment (Coutellier et al 2009), the genetic background of the mice (Kember et al 2012, Savignac et al 2011), their sex (Kember et al 2012, Korosi et al 2012), the quality and duration and the intensity of maternal care (Ashbrook et al 2015, Pedersen et al 2011,

Walker et al 2008) as well as the duration of the stressful experience (Savignac et al 2011, Strekalova et al 2005, Strekalova & Steinbusch 2010). While publications on the issue are hard to come by, many colleagues regularly share little "anecdotes" about the differences they observed in behavioural tests due to the layout of laboratory rooms, cage placement, distance to ventilator systems, cleaning schedules, seasons and even "Monday morning effects" during shift changes. Another challenge in conducting reliable ELS experiments could arise from compromises committed to the design integrity and power in order to please ethical boards (see for example (George et al 2010, Own & Patel 2013).

On a genetic/strain level, the mouse strain used in this study (C57BL/6) has been reported to have lower anxiety scores in the LDB and OF when compared to strains such as the BALB/c mice (O'Leary & Brown 2013). Other studies have also revealed that C57BL/6 mice, when subjected to MS, can show a phenotype of reduced anxiety behaviour in the EPM and LDB (Savignac et al 2011), strongly mirroring the results presented in this thesis. C57Bl/6 mice also appear to be particularly resilient towards MS, with mothers showing no changes in long term but increased short term maternal behaviour after 3 hours of MS and a decrease in anxiety behaviour in the offspring when they reach adulthood (Own & Patel 2013). Cross-fostering more stress reactive BALB/6 mice to C57Bl/6 mothers reduced anxiety in these animals, while C57Bl/6 pups cross-fostered to "low-quality" maternal care BALB/6 mothers did not show a change in their anxiety phenotype (Anisman et al 1998), suggesting that the MS outcome in this thesis might be affected by a mix of maternal (rescue) behaviour and genetic predisposition in C57Bl/6 mice to be resilient towards MS.

On a more functional level, it has been hypothesised that stress, malnutrition and environmental adversities integrate into the development, since they contain valuable information for demands later encountered in life (Agrawal et al 1999, Heiming et al 2011, Low et al 2012). Studies also highlight the fact that mild postnatal stress increases later life stress resilience in mice (Glover 2011), while short periods of handling (Weiner et al 1987) or short separations from the mother for up to 15 minutes in length, provoke an increase in maternal care

behaviour in an attempt to compensate for the disturbance (Millstein et al 2006, Moles et al 2004). Other studies again show that more severe forms of early-life stress, such as the *Maternal Separation and Early Weaning* paradigm presented by (George et al 2010) are better suited to produce an early strong adverse environment.

Overall, our results would be in agreement with the reduction of anxiety-related scores seen after MS in C57Bl/6 mouse lines (Own & Patel 2013). This is particularly true for our *Cdh13* wildtype (and heterozygote) mice which appear to have adapted to the treatment by displaying increased open arm time in the EPM, increase light side time in the LDB and increased centre time in the OF (only in male MS mice). This apparent adaptation is however absent in *Cdh13*$^{-/-}$ mice, which show no difference compared to control mice and thus lack the integration of their early-life experience into their behavioural phenotype. These findings suggest that CDH13 could be essential for adaptation to early-life experiences, helping in the programming of behavioural phenotypes. Absence of Cdh13 in turn disrupts early-life programming and even strain specific effects of reduced anxiety seen in many studies. In humans, CDH13 is not really "absent" like in our knockout model but potentially differentially expressed and concentrated within the brain. It would thus be interesting to see how *Cdh13* overexpression would have affected the behavioural phenotype of these animals after MS. Additionally, a more adverse model then our MS procedure might also be used in future studies to investigate if *Cdh13*$^{-/-}$ mice poses a general (aka. "for better and for worse") or specific programming deficiency.

4.1.2 Changes to the serotonergic system in the PFC and d/mRN

The serotonergic system plays an important role in the modulation of many cognitive and emotional processes and can be linked to emotional disorders. As such we investigated the changes of serotonergic receptors and metabolic factors in the PFC and dorsal/median raphe nuclei. Our results revealed an upregulation of *Htr1a* and *Htr2a* in the PFC of *Cdh13*-deficient male mice, while also exhibiting an interaction of the *Cdh13* genotype and maternal separation which affected the expression of two important genes for serotonin metabolism in the dorsal/median raphe. The latter interaction however does not produce a statistically detectable post hoc difference between expression levels, therefore only allowing the tentative postulation that the serotonergic metabolism in the dorsal/median raphe might be altered.

Anxiety behaviour in rats and mice can be linked to the proper balance of the inhibitory *Htr1a* (present both on excitatory and inhibitory neurons) and the excitatory *Htr2a* (mostly present on excitatory neurons) receptor activation in the PFC (Albert 2012). A disturbance in this balance can either occur due to a low concentration of available serotonin (Albert 2012, Savignac et al 2011), or improperly balanced receptor "sensitivity" on excitatory (pyramidal) versus inhibitory (inter-) neurons. As such, a low (but not absent) serotonin concentration has been linked to increased anxiety after (mild) stress exposure, due to a stronger sensitivity of the *Htr1a* receptor to inhibit the inhibitory interneurons. This in turn results in a disinhibition of pyramidal neurons and subsequent establishment of an anxiety phenotype (Albert 2012, Holmes 2008). It has also been shown that perturbed *Htr2* functionality following MS contributes to an anxiety phenotype (Benekareddy et al 2011). This would be in agreement with our observations of an increased expression of the *Htr2a* gene and increase of anxiety in *Cdh13*$^{-/-}$ mice, suggesting that the observed phenotype is supported by changes in the serotonin receptor composition of the PFC. While post hoc tests did not show a statistical difference, the initial ANOVA revealed a significant interaction for *Cdh13* deficiency and maternal separation in the expression of the two main serotonin metabolic enzymes *Tph2* and *Maoa* in the

dorsal/median raphe, suggesting that the serotonergic system, as a whole, undergoes changes due to *Cdh13* deficiency. This interpretation is further supported by a previous study from our laboratory showing increased density of serotonergic neurons in the developing dorsal raphe as well as increased innervation in the PFC in *Cdh13*-deficient mouse embryos (Forero et al 2017). Other studies have shown that early-life stress can increase the expression of *Htr1a* and *Htr2a* receptors, which results in an increased sensitivity of the PFC to serotonergic innervations (Benekareddy et al 2010, Goodfellow et al 2009) and has been interpreted as a potentially (protective) adaptation towards stress (Goodfellow et al 2009). There are also indications in rats that receptor sensitivity in the PFC switches between *Htr2a* and *Htr1a* when comparing PN6-PN12 with PN16-PN19 animals (Beique et al 2004), right when in our study MS was conducted, potentially affecting the two receptor systems differently at that time.

While $Cdh13^{+/+}$ and $Cdh13^{+/-}$ MS mice in our study show a phenotype of decreased anxiety, $Cdh13^{-/-}$ mice did not. As mentioned before, the balance between *Htr1a* and *Htr2a* alongside lower serotonin concentrations is essential for developing the anxiety like phenotype (Albert 2012, Savignac et al 2011). Considering that the increased innervation from the dorsal raphe is present already during embryonic development (Forero et al 2017) and that the expression of serotonergic receptors is already higher in *Cdh13*-deficient mice *per se*, one might speculate that the non-adaptive phenotype of our *Cdh13*-deficient mice is reflective of an imbalanced serotonergic system that is "unable" to adapt to the environmental challenge of MS from birth. Altered levels of serotonin and/or a different connectivity in the raphe could very well account for the observed changes in response to MS, as others have mentioned such alteration in the serotonergic system as a cause for higher sensitivity to MS procedures (Savignac et al 2011).

In summary, *Cdh13*-deficient mice show an upregulation of two serotonergic receptors in the PFC, irrespective of the early-life environment, leading us to speculate that the genotype lacks the ability to adapt, when compared to the

general strain specific phenotype of decreased anxiety after MS. There are some limitations to this interpretation however, as it is unclear from our work, if the change in gene expression indeed resulted in a change of receptor density, or even a change in inhibitory receptors at all. The *Ht1a* receptor for example also serves as an autoreceptor on the presynaptic end of the synapse, which is also in the homogenate we created. Additionally, it is unclear if the observed change corresponds to changes in pyramidal neurons as well as interneurons, or if they were asymmetrically stronger in one of the two neuronal PFC subpopulations.

4.1.3 *Cdh13* deficiency changes the GABAergic system of the PFC and AMY

A previous study already reported reductions in synaptic density of glutamatergic and GABAergic synapses in cultured hippocampal neurons after inhibition of *CDH13* (Paradis et al 2007). A more recent study from our laboratory however shows increased inhibitory synaptic transmission of cornus ammonis 1 (CA1) pyramidal cells in ventral hippocampal slices from *Cdh13*-deficient mice (PN20-22) (Rivero et al 2015), establishing *CDH13* as a negative regulator for inhibitory synapses and a "safe-guard" for proper excitatory-inhibitory balance in the region. Results from this thesis show that these changes could extend to other brain regions, such as the PFC and amygdala. Both the PFC and the amygdala of behaviourally tested mice show signs of increased expression of genes involved in GABAergic neurotransmission, a system also relevant for the serotonergic system. In the amygdala, *Gad1* and *Gad2* are upregulated, while in the PFC *Gabra1* and *Vgat* are upregulated, both times in $Cdh13^{-/-}$ mice compared to $Cdh13^{+/+}$ and $Cdh13^{+/-}$ mice. While a change is also observed in the glutamatergic transporter gene *Vglut3* in the amygdala, the overall result indicates an alteration in the GABAergic/glutamatergic systems in $Cdh13^{-/-}$ mice. No changes in the GABAergic system could be detected in the hippocampus samples.

These alterations, along with the changes to the serotonergic receptors, suggest a stronger GABAergic inhibition of the PFC in $Cdh13^{-/-}$ mice. Analogue to the argumentation of low serotonin resulting in stronger inhibition of GABAergic neurons in the PFC, the increase in GABAergic receptors and transporters could have been an adaptive change to counterbalance the overall increase of serotonin in $Cdh13^{-/-}$ CTRL mice. In fact, GABAergic neurons in the PFC mature shortly around birth (Flores & Morales Medina 2016), where they also play a critical role in the development of pyramidal neurons in the PFC. The PFC is also a part of the "default mode network", a network active during resting tasks, which has been shown to be directly influenced by GABA and glutamatergic concentrations (Hu et al 2013) and has been shown to be differently activated in children with ADHD (Fair et al 2007).

As such, $Cdh13^{-/-}$ mice, irrespective of environment, indeed show the tendency for an increased latency in the SD, while $Cdh13^{-/-}$ CTRL mice also enter the

central area of the OF with a delay, compared to the other genotypes. Both results would be supportive of a cautious/hesitant phenotype in *Cdh13*-deficient mice. While the direction is different than expected from an ADHD model, it nevertheless affects the same spectrum of phenotypes, suggesting that maybe just another type of CDH13 dysregulation could be active in ADHD patients, as compared to our *Cdh13*-deficient mice.

4.2 Learning and memory deficits due to MS and *Cdh13* deficiency

Overall, no strong genotype effect on memory could be detected in this study, with the exception of *Cdh13*$^{+/+}$ as well as the *Cdh13*$^{+/-}$ male mice significantly decreasing their freezing time during extinction recall in th cFC when compared to *Cdh13*$^{-/-}$ male mice. *Cdh13*$^{-/-}$ male mice therefore revealed a diminished ability to change existing behavioural patterns in the cFC. During the BM test, a similar pattern could be observed, with male *Cdh13*$^{-/-}$ CTRL mice showing a trend to commit more primary errors and utilise less efficient search strategies compared to male *Cdh13*$^{-/-}$ MS mice. During the same test, MS animals in general committed a greater number of primary errors on the first day of the reversal phase.

Alterations in learning and memory are key features in neurodevelopmental disorders such as ADHD and ASD. The three tasks used in this study are the Object recognition task (OR), the Barnes-maze (BM) and context Fear conditioning (cFC). The OR task, as a test for long and short term memory, involves the entorhinal cortex, the main interface between the hippocampus and neocortex (Lueptow 2017, Tsao et al 2018), the BM, as a spatial task, involves dorsolateral prefrontal cortex and hippocampus, amongst other areas (van Asselen et al 2005), while the cFC, mainly utilises amygdala and the hippocampus (Curzon et al 2009). *Cdh13* deficiency also contributes to increased inhibition of CA1 pyramidal neurons (Rivero et al 2015), a region involved in spatial and contextual long-term memory (Jung et al 2017). Additionally, *Cdh13* expression peaks during the same postnatal developmental weeks (Rivero et al 2015), during which hippocampal development and synaptogenesis in the mice forebrain are strongest (Steward & Falk 1991). *Cdh13* is also expressed in the PFC and hippocampus of mice during adulthood (Rivero et al 2013). Matching the function of this region, previous studies from our laboratory revealed a mild impaired cognitive flexibility in *Cdh13*-deficient mice, visible in significantly more primary errors to find the escape hole during the reversal phase of the BM and a tendency for a reduced freezing response in cued fear conditioning (Rivero et al 2015). Studies in *Cdh13*$^{-/-}$ rats show a decreased choice learning in the reaction time task compared to wildtype rats, while not differing in other

learning paradigms, such as the conditioned place preference or the approach to Pavlovian food cues (King et al 2017).

While we did not find the same effect, we could find a tendency for *Cdh13*$^{-/-}$ MS mice to commit less primary errors in the BM when compared to *Cdh13*$^{-/-}$ CTRL mice, which can potentially be explained by *Cdh13*$^{-/-}$ CTRL mice also using slightly more "inefficient" search strategies in the BM. We however detected a marked reduction in fear extinction during the cFC in *Cdh13*$^{-/-}$ males when compared to *Cdh13*$^{+/+}$ or *Cdh13*$^{+/-}$ male mice, which is contrary to previously published results, in which *Cdh13*-deficient mice show reduced freezing response towards cue conditioning. Due to the fact that there was no "learning" in any of the groups during the OR task, no further insight could be gained from this test.

While these results are supportive of a phenotype exhibiting mild cognitive impairments in *Cdh13*-deficient mice, they once again highlight the importance of recognising the mediating factors of environment and sex, as presented in other chapters (see chapters 4.1 and 4.3). In general, the tests perfromed only support the mild learning disadvantage in the cFC test for *Cdh13* deficient (male) mice. The previously reported results from the BM could only been shown for the comparison between MS and control *Cdh13* deficient mice, suggesting some level of learning disability that interacts with the environment at some level.

4.3 Increased locomotion in female *Cdh13*$^{-/-}$

Consistent changes in locomotor activity could be identified across all behavioural tests. These changes were again influenced by all three factors: genotype, environment and sex. A robust genotype by sex interaction highlighted greater distances covered by female *Cdh13*$^{-/-}$ mice in the EPM, OF, OR and BM, when compared to their male *Cdh13*$^{-/-}$ counterparts. Females in our study overall where more active (significant only in the LDB, but descriptively visible also in the EPM, OR and BM), with *Cdh13*$^{-/-}$ male mice showing reduced locomotion even compared to the other two male groups (significant in the OF and BM). While expressional analysis using qRT-PCR or RNA expression might have helped to elucidate some of the observed differences between male and female mice, female RNA samples were not analysed. This was mainly due to the fact that the increase in comparisons would have further reduced the power of each test.

The interaction between genotype and sex raises interesting questions, since ADHD and ASD are neurodevelopmental disorders, which are more prevalently recognised in males than in females (Rucklidge & Harrison 2010). While some etiopathology-relevant genes have been linked to sex chromosomes (Jamain et al 2003), diagnostic criteria are more in favour for recognising male compared to female symptoms (Loomes et al 2017, Rucklidge & Harrison 2010). In detail, ADHD in boys is expressed by a stronger hyperactive phenotype when compared to girls, resulting in a higher degree of diagnostic recognition through its “more extravert” nature, while girls show higher degree of inattentiveness, anxiety disorders and depression due to ADHD (Barbaresi et al 2006, Kok et al 2016, Quinn & Madhoo 2014, Thorell & Rydell 2008). As suggested by Quinn and colleagues, girls may have “better” coping strategies, alongside different hormonal influences contributing to the overall phenotype and differentiating them from the ADHD phenotype in males (Quinn & Madhoo 2014). These studies strongly suggest that “hyperactive” or “hypoactive” are not traits formed by the gene alone, but by a complex interaction with the environment and sex. With regards to our mice, one might speculate that (stressed) dams might display a sex bias in rearing pups. Other studies have established that low vs. high rearing quality constitutes a key factor in stress resilience (Coutellier et al 2009),

suggesting that if a (sex) bias exists in rearing quality, it would translate into a behaviourally observable phenotype. It is notoriously hard to observe and record sex of pups in an undisturbed way (or genotype for that matter), and thus it was not conducted in this project. Studies by (Yin et al 2016) however show that female pups in a maternal separation experiment vocalise more during the age of PN7-PN8 after the separation, which might result in a difference of maternal care they might receive.

It is also worth mentioning, that previous studies in the same mouse line have shown an increase in locomotor activity in *Cdh13*$^{-/-}$ mice (Rivero et al 2015), which is at odds with the results for our male mice reported here. As such the mice in our study were daily handled by the experimenter from birth while in the previously published study, mice were naïve to handling up to the point they entered experimentation. While it is always possible to attribute the locomotor activity to differences in procedures between studies, another less obvious variation exists between both studies: the seasons they were conducted in. Our previous study consisted of mice from summer cohorts (Rivero et al 2015), while mice from this study were born and tested during winter/early spring. Season-dependent changes in gene expression can be shown in natural environments (Eccard & Herde 2013, Lodewijckx 1984), but also in laboratory rodents that are "naturally" deprived of outside information for over 300 generations (Ferguson & Bailey 2013), alongside behavioural changes of higher levels of activity during the summer and lower activity during winter (Lodewijckx 1984). Moreover season-dependent downregulation of *Cdh13* during spring compared to autumn was shown in song birds (*Melospiza melodia*) (Mukai et al 2009), raising the possibility of a similar seasonal change in *Cdh13* expression mitigating the group differences between genotypes in our study. Studies in humans reveal that there are seasonal effects in ADHD populations due to its close relation to seasonal affective disorder (SAD), with increased levels of depression, lack of motivation, increased anxiety and reduced activity (Levine et al 1956, Wynchank et al 2016). By this, our findings raise a possible link between CDH13 and seasonal affective disorders (SAD), which is highly prevalent in ADHD patients (Levitan et al 1999, Wynchank et al 2016).

In the light of this finding, it is possible that the previously reported results could have been masked in this study by a stronger seasonal effect, which we did not account for. Consequently, we suggest that reporting the season of testing might be useful in behavioural neurosciences in order to increase reproducibility of studies involving animals and track behavioural changes that are season dependent.

4.4 Increase in expression of *Dnmt3a* and *Trdmt1*

While this thesis did not directly measure epigenetic changes such as DNA methylation, expression of methylation related genes still provides a clue to possibly existing modifications due to Cdh13 deficiency and MS in mice. As such, we identified that MS and *Cdh13* deficiency both increase the expression of *Dnmt3a* (see chapter 3.3.4). In the PFC, only MS caused a significant increase in the expression of *Dnmt3a* (see chapter 3.3.1). Most interestingly, in the dorsal/median raphe nuclei, a GxE interaction revealed an increase of *Dnmt3a* and *Trdmt1* (formerly known as *Dnmt2*) in $Cdh13^{-/-}$ mice that had been subjected to MS, when compared to $Cdh13^{-/-}$ CTRL mice and $Cdh13^{+/+}$ or $Cdh13^{+/-}$ MS mice (see chapter 3.3.3).

There are 3 known DNA methyltransferases 1, 3a and 3b, while the previously described DNMT2 actually uses RNA as substrate and was renamed into tRNA-methyltransferase 1 (*Trdmt1*). DNMT1 is mainly considered to be involved in maintenance methylation since it prefers hemi-methylated DNA (Razin & Riggs 1980), while DNMT3a and DNMT3b are thought to be *de novo* methyltransferases with no specific preference for the methylation status of their DNA substrate (Okano et al 1998). A vast body of literature highlights the changes in methylation patterns occurring due to early-life stress (Anier et al 2014, Blaze & Roth 2017, Doherty et al 2016), chronic stress (Le Francois et al 2015), the intensity of maternal care (Seckl & Meaney 2006, Szyf et al 2007) and early-life experiences (Mattern et al 2018).

As such, studies in animals and humans have shown that all three DNMTs can methylate specific promoters (Seckl & Meaney 2006, Szyf et al 2007, Zhang et al 2010) as well as "globally" change methylation patterns in response to special environmental exposures (Anier et al 2014, Klose & Bird 2006, Vrettou et al 2017). A broad spectrum of literature, albeit potentially impacted by publication bias, highlights the pronounced epigenetic "susceptibility" of the glucocorticoid receptor (GR), which appears downregulated and hypermethylated due to stressful experiences and early-life stress (Park et al 2017). As such, the promoter of the GR and *Gad1* show a reduced methylation and increased expression in pups with "high" maternal care behaviour, as compared to pups with lower maternal care behaviour (Zhang et al 2010). This has also been

shown in schizophrenic patients where an upregulation of DNMT1 leads to an increased methylation and consequential downregulation of the *GAD1* gene (Okano et al 1998, Veldic et al 2005).

While our results cannot pinpoint the exact genes which might be affected by the upregulation of DNMTs, and since increased expression is not always accompanied by an increase in methylation (Anier et al 2014), it usually indicates a broader adaptive trend towards environmental changes. Studies in cancer research also suggest DNMT1 and DNMT3a upregulation in aggressive tumours with hypermethylation of *CDH13* (Ma et al 2018). As such, these observations are suggestive for broader epigenetic changes due to MS as well as *Cdh13* deficiency in mice. They also highlight the PFC, amygdala and dorsal/median raphe nuclei as most likely candidates for follow up studies to investigate epigenetic changes due to *Cdh13* deficiency.

4.5 Impact of C*dh13* deficiency and MS on RNA expression in the hippocampus

Some additional insights could also be gained from the RNA sequencing analysis. While these animals had not been behaviourally tested, they represent the "blank slate" that could be considered similar to the situation the behaviourally tested animals started with. We decided to analyse the hippocampus, since previously published studies suggest larger changes in this brain region due to Cdh13 deficiency (Rivero et al 2015, Rivero et al 2013). Overall lack of power, strong inter-individual variability in RNA expression of live animals, higher noise and lower reliability of the qRT-PCR method, alongside the behavioural manipulation in our first cohort might have had an overall negative impact on the ability to detect changes. We therefore limited the RNA sequencing to *Cdh13*$^{+/+}$ and *Cdh13*$^{-/-}$ male mice that had either been maternally separated or daily handled, as in the previous study. Leaving out the *Cdh13*$^{+/-}$ genotype as well as all female groups increase the overall power of or tests. This second cohort of behaviourally naïve mice was raised and either sacrificed directly after weaning (PN21-PN22) or when they were adult (PN66) to extract RNA from their hippocampus to investigate changes in their transcriptome in response to genotype and early-life stress. This added further complexity and introduced additional variability. We therefore also decided to compare both age groups independently from each other. Enrichment of the results, as well as a literature research of the most significant findings reveal, among other observations, that the *Cdh13* deficiency strongly affects pathways of cellular adhesion, but also influences pathways related to ER function.

4.5.1 Expression changes in cell-cell adhesion factors due to *Cdh13* deficiency

Consistent with the function of CDH13 as a membrane protein and a member of the calcium adhesion protein family, an enrichment analysis of the core dataset showed several GO classes and KEGG pathways associated with cell surface function and cell adhesion to be modified in both age groups (PN22 and PN66). In PN22 mice, the more general pathway of "cell adhesion molecules" (CAM, KEGG.4514) shows promising enrichment with 11 genes showing that members of the cadherin, integrin and immunoglobulin super-families are affected by *Cdh13* deficiency at this young age. This pathway contains 4 major families of molecules: integrins, cadherins, immunoglobulins and selectins, which are strongly associated with neuronal development, synaptogenesis, learning/memory in the hippocampus and the the formation of tight junctions (Ahn et al 2018, Gerrow & El-Husseini 2006, Heiskala et al 2001, Robbins et al 2010). In our adult mice (PN66), the pathway of focal adhesion (GO.0005925) is represented with 10 genes. Additionally, 4 other pathways relevant in cell-surface and membrane function (GO.0009986, GO.00005576, GO.0009897, GO.0070062) and 1 relevant for membrane bound vesicles (GO.0031988) were enriched in our sample. Both age groups also show significant changes in the expression of two integrins, i.e. Itga4 (PN22) and Itga2b (PN66), which are essential factors for cell motility and cell adhesion. Both are also showing importance as adhesion receptors (Giancotti & Ruoslahti 1999, Harburger & Calderwood 2009, Kummer & Ginsberg 2006). This is not surprising, considering that many of these pathways share common factors among each other and thus overlap partially. Additionally, they also often play roles during cell migration and adhesion by providing and modifying the extracellular matrix which is used as a scaffold in this process (Janik et al 2010). Our enrichment thus shows a strong association not only with adhesion pathways, but also with pathways relevant for the extracellular-side of the cell membrane.

When the lists of all genes in the PN22 group that were significantly changed by CDH13 deficiency and MS, as well as their interaction, were compared with each other, they showed a remarkable overlap of 46 genes in total with again an overall enrichment for the cell adhesion pathway CAM (KEGG.4514). It is

particularly interesting that such a robust change exists in this pathway across both factors, alongside an interaction of them. In combination, they show a potential link between *Cdh13* and early-life stress through changes cell-cell adhesion after MS (PN22). Considering that the tissue sampled came from the hippocampus, one might speculate that these changes could have also impacted synaptic transmission in the hippocampus, since proper adhesion between the post and presynaptic side is essential for synaptic formation, maintenance and survival (Biederer et al 2002, Gerrow & El-Husseini 2006). In the PN66 group, an interesting interaction could be found for three differentially expressed genes, *Rhoh, Spn* and *Itga2b*. *Rhoh* is an important regulator of cell survival and growth and belongs to a family of factors that is strongly involved in migration and cell-cell adhesion (Nelson 2008, Wittchen et al 2005). Other Rho factors are well known to interact with integrins and cadherins during cell-cell adhesion and migration. *RhoA* for example is an important regulatory factor for cytoskeletal restructuring during migration and cell-cell adhesion and interacts with both integrins as well as cadherins during cell-cell adhesion and migration (Evers et al 2000, Fukata & Kaibuchi 2001, Marjoram et al 2014, Nelson 2008). *RhoA* and *Shn* both play roles in regulation of the cytoskeleton by interacting with the cytoskeletal adaptor proteins ezrin-radixin-moesin (ERM) (Allenspach et al 2001). *Spn* promotes de-adhesion and cell-cell interactions in macrophages (van den Berg et al 2001) and is suggested to be involved in the removal of inhibitory proteins from the immunological synapse (Allenspach et al 2001). Integrin alpha 2b (*Itga2b*) is an important Ca^{2+} dependent adhesion factor by functioning as an adhesion cell-surface receptor (Takada et al 2007) (Calvete 1995). These results also strengthen previously published GWAS data, showing both Cdh13 and the integrin system are linked to ADHD (Franke et al 2009), as well as unpublished results from one of our collaboration partners also showing that *Itga2b* is an interaction partner of *Cdh13*.

From structural analysis we know that CDH13 does not possess an intracellular domain and lacks the ability to relay intracellular signals itself. Our findings of altered expression of 3 factors related to cell-surface adhesion signalling would support the idea that CDH13 might use other factors for signalling. Indeed other groups have already identified potential signalling interaction partners of

CDH13, for example Hspa5/GRP78/BiP in endothelial cells (Kyriakakis et al 2010, Nakamura et al 2013), which is also featured in our enrichment lists changed by Cdh13 deficiency and maternal separation. Although speculative, the atypical structure of CDH13 would suggest a specialised role for this molecule, while the broad and wide expression of Cdh13 during development would suggest a more common function for this gene. It could thus be possible that many cell types use CDH13 as a promiscuous adhesion factor, which can easily be modified to interact with a variety of different intracellular signalling pathways by replacing the binding partner compliment of receptors and signalling-proteins.

4.5.2 *Cdh13* and maternal separation affect the expression of genes involved in endoplasmatic reticulum function

Another interesting observation in our RNA sequencing results indicates that *Cdh13* and maternal separation both interact with several endoplasmic reticulum (ER) and ER stress response pathways (see chapters 3.4.3.1, 3.4.3.2 and 3.4.3.3). Several reviews highlight the growing evidence for the involvement of ER function in neurodegenerative diseases (Garcia-Gonzalez et al 2018, Oslowski & Urano 2011), ER regulated calcium homeostasis in synaptic plasticity (Paula-Lima et al 2014) and ER mediated regulation of neuronal apoptosis (Haughey & Mattson 2002, Jia et al 2018, Oslowski & Urano 2011), suggesting ER mediated processes as a key factor for neuronal plasticity and survival.

Both *Cdh13* deficiency as well as maternal separation significantly enrich three ER-related pathways (GO.0005788, GO.0034663 and KEGG.4141). Additionally, *Cdh13* deficiency enriched an additional ER related pathway (GO.0005790) along with a pathway for membrane bound vesicles (GO.0031988). As discussed in the introduction, *CDH13* is broadly known as a survival factor under cell stress conditions in endothelial cells (Joshi et al 2005, Philippova et al 2008), as well as in cortical interneurons (Killen et al 2017). In vascular cells, CDH13 promotes cell survival by interacting with Hspa5/GRP78/BiP, an ER heat shock and nucleus signalling protein involved in protein folding heat shock response (Kyriakakis et al 2010, Nakamura et al 2013). CDH13 and Hspa5/GRP78/BiP appear to be also associated on the surface of endothelial cell surface (Philippova et al 2003) suggesting some form of close interaction between both. In vascular cells, CDH13 interacts with the unfolded protein response (UPR) pathways during ER stress (Kyriakakis et al 2010) attenuating the PERK mediated pro-apoptotic part of the UPR pathway, allowing cells under ER stress to survive longer (Kyriakakis et al 2010). While Hspa5/GRP78/BiP is not in our PN66 top 20 list of most significantly changed genes due to *Cdh13* deficiency, it is very prominently featured in the enrichment analysis, where it is present in 10 of the 11 pathways we reported and overall downregulated in *Cdh13*-deficient mice compared to wildtype mice ($p=0.004$, $log2FC=-0.365$).

Furthermore, CDH13 has been shown to act as a tumour-supporting factor, by preventing endoplasmic reticulum induced apoptosis due to hypoxic conditions (Joshi et al 2009, Joshi et al 2005). In cortical interneurons, knockdown of Cdh13 in mice results in a reduction of interneurons and late born pyramidal neurons, with a parallel increase in apoptotic cells in the same region (Killen et al 2017). Combining the results of a strong presence of ER pathways, the widely reported interaction of Cdh13 with Hspa5/GRP78/BiP and the reports of CDH13 as a pro-survival factor both in endothelial cells and cortical interneurons, strengthens CDH13 function as a pro-survival factor. Our results also suggest that the underlying function could be similar, if not identical, to the previously reported mechanism in endothelial cells, by attenuation of the pro-apoptotic part of the unfolded protein response pathway. The other genes we report loosely tie into this reading, with at least two other genes of the same UPR pathway being also differently expressed (Derl3 and *Dnajb11*), alongside several ER stress "markers" (*Sdf2l1, Pia3* and *Pia6*).

Most interestingly, MS also affected the expression of ER-related genes in our sample. Results broadly overlap, with the same set of pathways being detected in our enrichment. This is particularly interesting with regard to recent studies that support the involvement of the unfolded protein response (UPR) pathway in neurodegenerative disorders (Garcia-Gonzalez et al 2018) and post-traumatic apoptosis in the hippocampus of chronically stressed rats (Jia et al 2018). Alongside other studies showing that anxiety disorders might be caused by ER related oxidative stress (Fedoce et al 2018) and ER regulated homeostasis of Ca^{2+} at the (hippocampal) synapsis (Paula-Lima et al 2014), it is clear that ER mediated apoptosis is a key modulator of neuronal plasticity in the brain. While our findings are in agreement with this, it is interesting to see that both, Cdh13 deficiency and MS, affect hippocampal ER pathways, but both factors are not interacting in our model. While it is feasible that the sample size, combined with the aforementioned inter-individual variance, resulted in a diminished power to detect such an interaction in our study, it might also be possible that they do generally not interact with each other, but rather "add up". When cellular stress accumulates, it will eventually lead to apoptosis, which defines an end-state of

the process, or will diminish again when the stressor is overcome. As such, our RNA sequencing reveals a snapshot of the ongoing process in the hippocampus. Many cells might have already died in both *Cdh13*-deficient MS mice and thereby not contribute further to the overall RNA expressed. The fact that persistent changes in ER-related pathways are present in both conditions thus indicates ongoing adaptation and plasticity in the hippocampus of our mice. The fact that *Cdh13*-deficiency elicits a similar ER stress-like phenotype suggests that they are under a persistent and constant challenge, comparable to classically evoked ER stress during trauma or neurodegenerative disorders. Our behavioural results indicate as much, since they show diminished adaptation to the environmental challenge (LDB, OF and cFC). A more robust and chronic exposure to a stressful environment might thus have produced a more severe phenotype. Similar to the "double hit" hypothesis, *Cdh13*-deficient mice might already have the first hit, but not yet the adequate second hit to follow up with.

5 Conclusion

The work presented in this thesis indicates that, overall, MS resulted in an anxiolytic phenotype. This response to MS however was negated by *Cdh13* deficiency, which in turn led to delayed habituation, lower exploration, lack of decrease in anxiety behaviour compared to the other MS groups, reduced fear extinction and delayed step-down latency. RNA expression from the PFC, Amygdala and raphe region of these animals also indicate that *Cdh13* deficiency alters the receptor expression for at least two major neurotransmitter systems, while also affecting serotonin production in the raphe in a GxE way. These results highlight that *Cdh13* deficiency plays a critical role during developmental programming and adaptation to early-life stress. The results also support the previously reported mild cognitive impairments found in *Cdh13*-deficient mice but identified them in a different set of behavioural measurements.

Additionally, RNA sequencing in a second, behaviourally untested set of animals revealed combined alterations in adhesion pathways. These are relevant for synaptic formation that might be related to the differential composition of inhibitory synapses that other studies identified in the hippocampus of *Cdh13*-deficient mice. While not being able to exclusively show the connection, the same change in gene expression related to synaptic factors could also be responsible for the behavioural un-adaptive phenotype observed. RNA sequencing also revealed a strong connection between *Cdh13* deficiency and ER stress, extending previously described neuroprotective properties of *Cdh13* to the hippocampus. Any imbalance of CDH13 thus might change the structural and molecular landscape of the brain in a complex interaction with other factors.

Loss of *Cdh13* in our model ultimately revealed a non-adaptive phenotype, which showed little alterations after experiencing MS. While GWA studies linked CDH13 to ADHD and ASD, *Cdh13* deficiency in our mice did not induce a hyperactive or impulsive-like phenotype. On the contrary, the loss resulted in a reduction of impulsive behaviour. There are however some similarities to the behavioural dimensions in which the changes were discovered. The overall link to anxiety behaviour strikes a resemblance to the seasonal anxiety disorder frequently found in ADHD patients; mild cognitive impairments could be

reminiscent of much more complex cognitive alterations that become more "pronounced" in humans due to the higher cognitive demands posed on them in everyday life.

Remarkably, gene expression data from both qRT-PCR and RNA sequencing indicate that changes to the inhibitory system and general synaptic plasticity are to be expected in *Cdh13*-deficient mice. Combined with alterations in the serotonin system and the ER stress response, they would support a much larger, and thus more pronounced change in brain-network topography. Particularly the change to the GABAergic and serotonergic systems in the PFC could have profound effects on the function of the ADHD relevant "default network", suggesting that CDH13 might contribute to some of the observed phenotypes of ADHD and ASD via a disruption of GABAergic (and glutamatergic) systems in the brain. While not showing the complete set of behavioural (and cognitive) alterations found in these disorders, CDH13 might well act in concert with other factors compounding their influences and ultimately leading to the mixed bag of phenotypes seen in both "spectrum disorders": ADHD and ASD.

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7 Supplementary

7.1 Materials for the behavioural experiment

Table 7 - List of materials used during the behavioural testing.

Item	Manufacturer
VideoMod2	TSE Systems, Bad Homburg, Germany
Fear Conditioning System	TSE Systems, Bad Homburg, Germany
Panasonic WV-BP330	Panasonic
Infrared Light Box	TSE Systems, Bad Homburg, Germany
Webcam	Logitech
Elevated-plus-maze	TSE Systems, Bad Homburg, Germany
Light-dark box	TSE Systems, Bad Homburg, Germany
Open field	TSE Systems, Bad Homburg, Germany
Object recognition task (OR)	TSE Systems, Bad Homburg, Germany
OR - objects	ASIN: B00O1SPJ12
Barnes-maze	TSE Systems, Bad Homburg, Germany
Step-down test	Laboratory made
Fear Conditioning System	TSE Systems, Bad Homburg, Germany
Terralin Liquid	Schülke
Makrolon type II	Tecniplast, Hohenpeißenberg, Germany
Makrolon type III	Tecniplast, Hohenpeißenberg, Germany
Polycarbonate water bottles	Tecniplast, Hohenpeißenberg, Germany
Standard rodent chow diet	Ssniff Spezialdiäten GmbH, Soest, Germany

7.2 Brain dissection - Guide

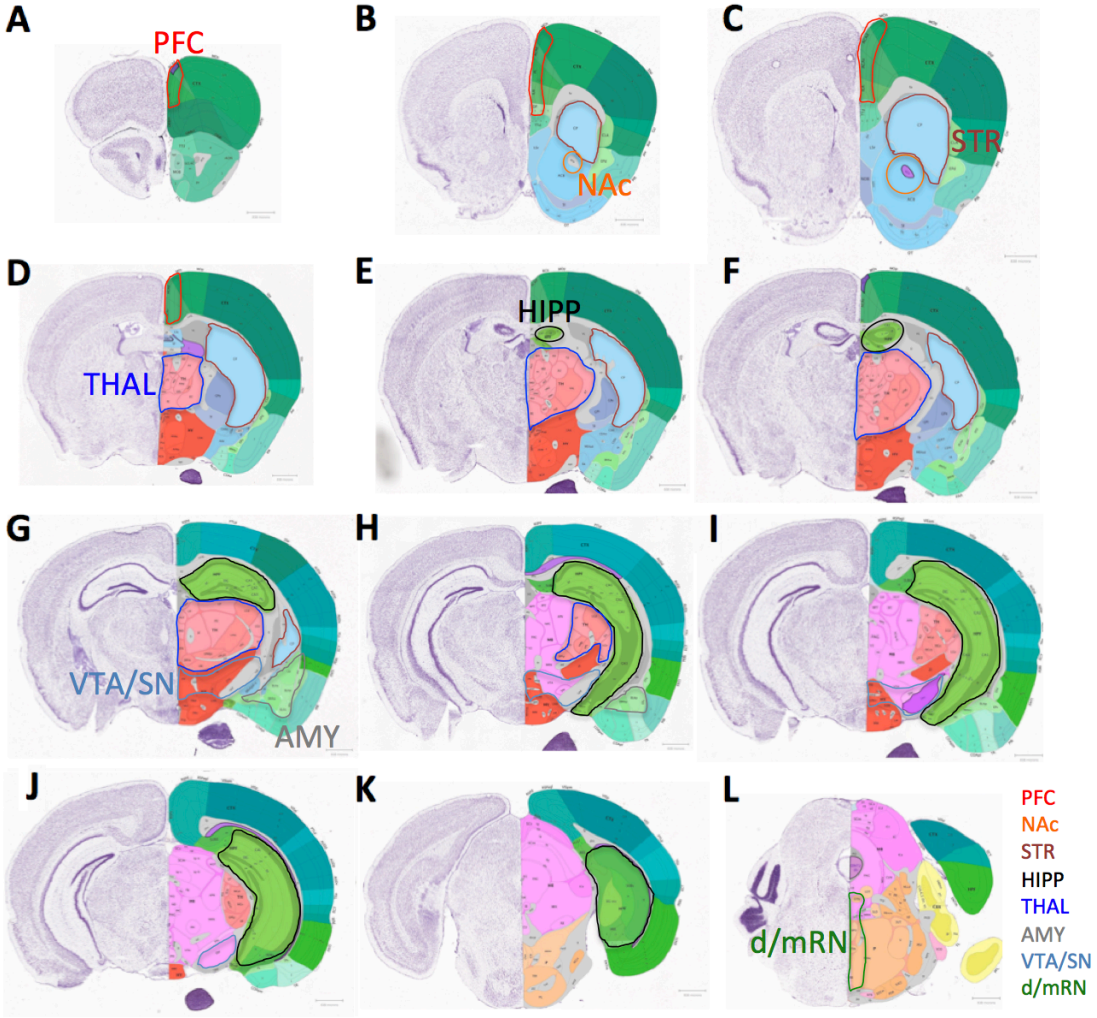

Figure 8 - Coronal slices of a mice brain with highlighted areas. Identification of brain structures without histological stains was guided using structural landmarks. The extraction of the PFC started with the recession of the olfactory bulbus and ended with the emergence of the HIPP, resulting in the complete extraction of the anterior cingulate area and including all but the most rostral parts of the prelimbic/infralimbic area (**A-D**). The NAc was identified by the prominence of the anterior commissure (ACO) of the olfactory limb in the presence of a clearly emerging prominence of the corpus callosum (CC). Tissue directly surrounding the ACO was removed and then cleared of the central ACO fibres (**C**). The HIPP was well defined by the CC and lacked a strong connection to the surrounding tissue due to the alveus surrounding it on the dorsal side. The HIPP was then extracted as a whole by pulling it gently out as one piece (**E-K**). The STR, comprising the putamen and the nucleus caudatus, is part of the basal ganglia and was highly visible in the unstained section by the prominence of white highly myelinated fiber tracts. Starting as early as these fibres, brain tissue with the strongly myelinated fibers, ventral to the CC was removed, excluding tissue of the pallium, NAc AMY, THAL and isocortex (**B-G**). The first parts of the THAL extracted where identified by the emergence of medial forebrain bundle and stopped together with the disappearance of the last STR tissue. This left out the more anterior and posterior regions of the THAL, which were hard to identify, but allowed a more accurate extraction of only THAL tissue (**D-G**). The AMY was identified using the external and amygdalar capsule, two structures that were extension of the CC and formed a triangle shape. Following the ventral side of the brain, a triangular segment of tissue was cut out. A ventral sliver of

this segment was then removed to avoid including olfactory areas, thus also excluding the piriform-amygdala area. Extraction of the AMY was finished with the recession of the amygdalar capsule (**E-H**). At the same time the amygdalar capsule receded, the VTA/SN was removed by cutting out a rectangular section of ventral brain tissue along the central axis as defined by the aqueduct. Depending on the exact depth of the slice, tissue surrounding the VTA/SN was then carefully removed. (**H-J**). The d/mRN where extracted, using the aqueduct and the emergence of the inferior colliculus as landmarks. A midsection along the mid of the brain was then cut out and surrounding tissue from the dorsal and ventral side removed. Due to the irregular shape and dispersion of the raphe nuclei, surrounding tissue of the pons and the midbrain could not be fully removed and are included in the sample (**L**). Source and Images: Allen Brain atlas (http://atlas.brain-map.org).

7.3 Brain extraction and blood sampeling

Table 8 - Materials used for brain extraction and blood sampeling.

Item	Manufacturer
Isofluran CP	CP-pharma
2-methylbutane	Sigma-Aldrich
Microvette® CB300	Sarstedt, Germany
Corticosterone rat/mouse ELISA, DEV9922	Demetitec Diagnostics GmbH, Kiel, Germany
Various tootls	Fine Science Tools GmBH, Heidelberg, Germany

7.4 RNA isolation, cDNA production and qRT-PCR

Table 9 - List of materials used for RNA isolation and cDNA production.

Item	Manufacturer
miRNeasy Mini Kit (50)	QIAGEN GmbH
DNase I Mix (DNase I, RDD-buffer)	QIAGEN GmbH
RNase-Free DNase Set (50)	QIAGEN GmbH
Trichloromethan/Chloroform	Carl Roth GmbH
QIAzol Lysis Reagent	QIAGEN GmbH
Ethanol absolute	AppliChem
QuantiTect Reverse Transcription Kit	QIAGEN GmbH
TE buffer	AppliChem
RNase free ddH2O	QIAGEN GmbH Equipment
Thermocycler	Biometra
SYBR Select Master Mix for CFX (Taq-Polymerase dNTPs SYBR green)	ThermoFisher Scientific
Centrifuge 5430	Eppendorf Vertrieb Deutschland GmbH
CFX384 Real-Time System	Bio-Rad

7.5 qRT-PCR primer list

Table 10 - List of primers used in the qRT-PCR analysis. Target: Target gene symbol and further information on exon targeted, when applicable, Gene: full name of the gene the primer is designed to recognise, Company: provider of primer, F: forward primer, R: reverse primer.

Targets	Gene	Company	Primer-Sequence
5HTt_F/R	5HT-transporter (Slc6a4)	Sigma	F: GCATCTGGAAAGGAGTCAAAA R: ACCAGCAGGACAGAAAGGAC
BDNF_ExIV_F/R	Brain derived neurotrophic factor, exon IV (*Bdnf*, exIV)	Sigma	F: GATCCGAGAGCTTTGTGTGG R: AACCATAGTAAGGAAAAGGATGGTC
BDNF_ExIX_F/R	Brain derived neurotrophic factor, exon IX (*Bdnf*, exIX)	Sigma	F: AAAACCATAAGGACGCGGAC R: TAGACATGTTTTGCGGCATCC
Crh_Mm_q_F/R	Corticotropin releasing hormone (*Crh*)	Sigma	F: AGGCATGCTGAGAGAAGTCC R: TGTTAGGGGGCGCTCTCTTC
Crhr1_v2_Mm_q_F/R	Corticotropin releasing hormone receptor 1 (*Crhr1*)	Sigma	F: CAGGATCAGCAGTGTGAGAGC R: CACCGTAGAAAAAGGCAGGG
Diras2_Mm_q_F/R	GTP-binding RAS-like 2 gene (*Diras2*)	Sigma	F: GAGCTGCGCCTGGAGACCTG R: CCGCCACCCGGTAGTCGGTTG
Dnmt3a_v1_Mm_q_F/R	DNA-methyl-transferase 3 (Dnmt3a)	Sigma Aldrich	F: ATTGATGAGCGCACAAGGGA R: GTGACATTGAGGCTCCCACA
Dnmt3a_v2_Mm_q_F/R	DNA-methyl-transferase 3a (*Dnmt3a*)	Sigma	F: GGGCGTTAGTGACAAGAGGG R: GCAGCAGACACTTCTTTGGC
Gabra1_Mm_q_F/R	GABA-A-Receptor (*Gabra1*)	Metabion International AG	F:CCCACTAAAATTCGGAAGC R:CTTCTGCTACAACCACTGAACG
Gabra1_Mm_q_F/R	GABA-A-Receptor (*Gabra1*)	RocheAssay Design Center	F: GCCCACTAAAATTCGGAAGC R: CTTCTGCTACAACCACTGAACG
Gad1/Gad67_Mm_q_F/R	Glutamic acid decarboxylase 1 (*Gad1*)	Metabion International AG	F: ATACAACCTTTGGCTGCATGT R: TTCCGGGACATGAGCAGT
Gad2/Gad65_Mm_q_F/R	Glutamic acid decarboxylase 2 (*Gad2*)	Metabion International AG	F: TGTAGCTGACATCTGCAAAAAGTA R: GGGACATCAGTAACCCTCCA
Gphn_Mm_q_F/R	Gephyrin (*Gphn*)	RocheAssay Design Center	F: TGATCTTCATGCTCAGATCCA R: GCAAATGTTGTTGGCAAGC
Gsk3a_v1_Mm_q_F/R	Glycogen synthase kinase 3a (*Gsk3a*)	Sigma	F: CTGGTGGCCATCAAGAAGGTT R: AAAGTACCGCAGCCTCACAAT
Gsk3a_v1_Mm_q_F/R	Glycogen synthase kinase 3a (*Gsk3a*)	NCBI primer blast	F: CTGGTGGCCATCAAGAAGGTT R: AAAGTACCGCAGCCTCACAAT

Gsk3b_v1_Mm_q_F/R	Glycogen synthase kinase 3b (*Gsk3b*)	Sigma	F: GCGGGACCCAAATGTCAAAC R: TGAATCCGAGCATGTGGAGG
Gsk3b_v1_Mm_q_F/R	Glycogensynthasekinase 3b (*Gsk3β*)	NCBI primer blast	F: GCGGGACCCAAATGTCAAAC R: TGAATCCGAGCATGTGGAGG
Itgb1_V7_Mm_q_F or R	Integrin beta-1 (*Itgb1*)	Metabion International AG	F: GACCCATTGCAAGGAGAAGGA R: ACGCCTGCTACAATTGGGAT
Itgb3_Mm_q_F or R	Integrin beta-3 (*Itgb3*)	Metabion International AG	F: TGGCACCGACAACCACTA R: AGTCATCAGCCCCAGAGATG
Maoa_F/R	Monoamine oxidase a (*Maoa*)	Sigma	F: TCGGGAGAATTTTACCCAAACCA R: AACTCTATCCCGGGGCTTCCA
Mc4r_v2_Mm_q_F/R	Melanocortin 4 receptor (*Mc4r*)	Sigma	F: ACAAGAACCTGCACTCACCC R: ATGACGATGGTTTCCGACCC
Mm_5HTr2a_v1_F/R	Serotonin receptor 2a (*Htr2a*)	Metabion-International AG	F: ACCCCCATTCACCATAGC R: CCGAAGACTGGGATTGGC
Mm_Htr1a_1_SG_F/R	Serotonin receptor 1a (*Htr1a*)	Sigma	F: AACCAGTTTTGTGTCCTCTCA R: AGCACCTAAATAATTTTCTTCTCTG
Mm_Htr1a_1_SG_F/R	Serotonin receptor 1a (*Htr1a*)	Qiagen (Quantitect primer assay)	Not reported from Qiagen
mTph2_pPCR_F/R	Tryptophan hydroxylase-2 (*Tph2*)	Metabion International AG	F: TGGGGATTTGATGCCTAG R: TGGGTTCTTTAGAGCATTTTTGTGT
Nr3c1_v1_Mm_q_F/R	Glucocorticoid receptor (*Nr3c1*)	Sigma	F: GGCCGCTCAGTGTTTTCTAATG R: GCTTCATCGGAGCACACCA
Oxtr_v1_Mm_q_F/R	Oxytocin receptor (*Oxtr*)	Sigma	F: TCTTCGTGCAGATGTGGAGC R: GCACGAGTTCGTGGAAGAGA
Oxtr_v1_Mm_q_F/R	Oxytocin receptor (*Oxtr*)	NCBI primer blast	F: TCTTCGTGCAGATGTGGAGC R: GCACGAGTTCGTGGAAGAGA
TRDMT1	tRNA (cytosine-5-)-methyltransferase (*Trdmt1*)	Sigma	F: GGAGCCTGTCCCCTCTATGTA R: TTGCTGCGATACGCTCTCTT
Vgat_Mm_q_F/R	Vesicular GABA transporter (*Vgat*)	Metabion International AG	F: ACGTGACAAATGCCATTCAG R: TGAGGAACAACCCCAGGTAG
Vgat_Mm_q_F/R	Vesicular GABA transporter (*Vgat*)	Roche Assay Design Center	F: ACGTGACAAATGCCATTCAG R: TGAGGAACAACCCCAGGTAG
Vglut1_Mm_q_F/R	Vesicular glutamate transporter 1 (*Vglut1*)	Metabion International AG	F: GTGCAATGACCAAGCACAAG R: AGATGACACCGCCGTAGTG
Vglut2_412_Mm_q_F/R	Vesicular glutamate transporter 2 (*Vglut2*)	Metabion International AG	F: CCTGTCATGGGATATGGA R: GGATACCAGCTAAGGGCA
Vglut3_612_Mm_q_F/R	Vesicular glutamate transporter 3 (*Vglut3*)	Metabion International AG	F: TGGGATGTGGAGTAAGTG R: TGCAAGAGGCATAGCAAC

Materials for mRNA sample preparation using *TruSeq*®

Table 11 - List of materials used in the TruSeq Stranded mRNA sample preparation guide from Illumina. All work was carried out by the RNA sequencing core unit, where all the remaining work was carried out (medical faculty, CU systems medicine, IMIB, Margarete Göbel and Konrad Förstner) according to the TruSeq Stranded mRNA sample preparation guide from illumina (Illumina 2013).

Item	Manufacturer
Illumina - Synthesize First Strand cDNA	
FirstStrandSynthesisActD	Illumina
CDP(cDNAPlate)Barcode Label	Illumina
Illumina - Synthesize Second Strand cDNA	
Optional)EndRepairControl (CTE)	Illumina
ResuspensionBuffer(RSB)	Illumina
SecondStrandMarkingMaster Mix(SMM)	Illumina
ALP(AdapterLigationPlate)BarcodeLabel	Illumina
Illumina - Adenylated 3'Ends	
Optional)A-TailingControl (CTA)	Illumina
A-TailingMix(ATL)	Illumina
ResuspensionBuffer(RSB)	Illumina
Illumina - Dual Indexing	
TruSeq SR Dual Index Sequencing Primer Box (For use with single-read flow cells)	Illumina
Illumina - Enriching DNA fragments	
PCRMasterMix(PMM)	Illumina
PCRPrimerCocktail(PPC)	Illumina
ResuspensionBuffer(RSB)	Illumina
Barcodelabels	Illumina
Illumina - RNA Sequencing	
Illumina 500 Next Generation Sequencer	Illumina

7.6 Software used

Table 12 - Software and statistical tools used in this thesis.

Statistical programs and packages		
CFX Manager 3.0	Bio-Rad	
NanoDrop ND-1000 3.7	Peqlab Biotechnologie GmbH	
Primer-BLAST	National Center for Biotechnology Information (NCBI)	
LinRegPCR	Heart Failure Research Center, Amsterdam	Version 2014.4
qBase+	Biogazelle NV, Zwijnaarde	Version 2.5
Graphpad Prism	GraphPad Software Inc., La Jolla	Version 6.04
R-Studio	RStudio, Inc.	Version 1.0.143
R-Package - ez	by Michael A. Lawrence	Version 4.4-0
R-Package - edgeR	Bioconductor	Version 3.20.9
R-Package - limma	Bioconductor	Version 3.34.9
R-Package - Glimma	Bioconductor	Version 1.6.0
R-Package - Mus.musculus	Bioconductor	Version 3.5.0

8 Abbreviation Index

Table 13 - Used abbreviations.

Behavioural tests

BM	Barnes maze
cFC	contextual fear condtionining
EPM	elevated plus maze
LDB	light-dark box
OF	open-field
OR	object-recognition

Brain regions and neuronal cell types

AMY	amygdala
CA1	cornu ammonis 1 (hippocampus region)
CI	cortical interneurons
d/m	dorsal/median (raphe)
d/mRN	dorsal raphe nucleus
DR	dorsal raphe
HIPP	hippocampus
Nac	nucleus accumbens
PFC	prefrontal cortex
RGC	radial glia cells
SN	substantia nigra
STR	striatum
THAL	thalamus

Disorders

ASD	autism spectrum disorder
NDD	neurodevelopmental disorder
OCD	Obsessive-compulsive disorder
SAD	seasonal affective disorder

Genes and Pathways

APN	adipocyte-secreted hormone adiponectin
GABA	gamma-butyric acid
GO	Gene Ontology
GPI	glycosylphosphatidylinositol (anchor)
KEGG	Kyoto Encyclopedia of Genes and Genomes
Tph2	tryptophan hydroxylase 2
UPR	unfolded protein response

General abbreviations

ADHD-C	ADHD with combined presentation of hyperactivity and inattention
ADHD-PH	ADHD with predominantly hyperactive presentation
ADHD-PI	ADHD with predominantly inattentive presentation
ADHD	attention deficit hyperactivity disorder
Cdh13	cadherin-13 / T-cadherin
***Cdh13*$^{+/-}$**	cadherin-13 heterozygous
***Cdh13*$^{-/-}$**	cadherin-13 homozygous knockout
***Cdh13*$^{+/+}$**	cadherin-13 wild-type
CAM	cell adhesion pathway, KEGG.4514
cDNA	Complementary deoxyribonucleic acid
CTRL	control
ELS	early-life stress
E	Embryonic stage (usally followed by a number representing days)
ER	endoplasmic reticulum

EC	endothelial cell(s)
E	environment
G	gene
GxE	gene by environment
GWAS	genome wide association studies
IRC	inter run calibrator
MS	maternal separation
MS	maternal separation
PN	postnatal stage (usually followed by a number, starting with birth at P0)
qRT-PCR	quantitativ (real-time) polymerase chain reaction
SNP	single nucleotide polymorphism
ZEMM	Zentrum für Experimentelle Molekulare Mediz

Statistical values and terms

ANOVA	analysis of variance analysis
df	degrees of freedom
F	Fischer Value
fdr	false discovery rate
N	number/sample size
p	probability value
η^2	eta square

Units

AE	amplification efficiency
c_q	quantification cycle
DI	discrimination index
h	hours
min	minutes
RT	room temperature
s	seconds

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Publications

Kiser DP, Popp S, Schmitt-Böhrer AG, Strekalova T, van den Hove DL, Lesch KP, Rivero O. EARLY-LIFE STRESS IMPAIRS DEVELOPMENTAL PROGRAMMING IN CADHERIN 13 (CDH13)-DEFICIENT MICE. Prog Neuropsychopharmacol Biol Psychiatry. 2018 Aug 28, doi: 10.1016/j.pnpbp.2018.08.010

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation “Gen x Umwelt-Interaktionen in Cdh13-defizienten Mäusen: CDH13 als ein Faktor für Umweltanpassung” eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertit 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 Unterschrift
Ort, Datum

Affidavit

I hearby confirm that my thesis entitled “Gene x Environment Interactions in Cdh13-deficient Mice: CDH13 as a Factor for Adaptation to the Environment” is the result of my own work. I did not receive any help or support from commercial consultants. All sources and 7 or materials applied are listed and specified in the thesis.

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

…… ……..
Würzburg Signature
Place, Date