Interneuron function in pathological memory processes – Relevance for posttraumatic stress disorder

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Declaration of Authorship

Hiermit erkläre ich, Iris Müller, dass ich die von mir eingereichte Dissertation zum Thema

"Interneuron function in pathological memory processes – Relevance for posttraumatic stress disorder"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und alle benutzten Quellen und Hilfsmittel vollständig angegeben habe.
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Abstract

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by Dipl.-Neurowiss. Iris MÜLLER

The experience of a trauma can lead to clinical conditions, like posttraumatic stress disorder (PTSD). On a neurobiological level insufficient activity of GABA, the major inhibitory neurotransmitter, has been implicated in the pathophysiology. PTSD is often conceptualized as a memory disorder, and thus extensive research employs classical fear conditioning and operant dual solution memory tasks.

In this context, I aimed at investigating the role of GAD65, the activity–dependent GABA–synthetic enzyme, and more specifically the role of the parvalbumin–positive subclass of GABAergic interneurons in pathological memory processes. I could show that heterozygous GAD65 knock out mice are protected from stress–induced memory disturbances. Together with the previously described delayed GABAergic maturation in these mice, a very successful compensatory molecular mechanism is indicated. Gene expression analysis in brain regions involved in (pathological) memory processes revealed a regionally and temporally focused expression profile in mutant mice.

Next, I extended the phenotype of homozygous GAD65 knock out mice by an operant conditioning task. Here mice could choose between a spatial– and a cue–guided learning strategy for reward localization. Indeed, unstressed GAD65 knock out mice resembled stressed wild type mice, by preferring a cue–guided strategy to a spatial strategy.

Finally, I found a context specific fear extinction deficit in mice with genetic enhancement of parvalbumin–positive interneuron function.

Together, I could show that GAD65 mediates memory formation in different learning paradigms. Moreover, genetic enhancement of parvalbumin–positive interneuron activity leads to fear memory persistence. All the described phenotypes bear relevance for the better understanding of posttraumatic stress disorder.

Zusammenfassung

Interneuron function in pathological memory processes – Relevance for posttraumatic stress disorder

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Die posttraumatische Belastungsstörung (PTBS), ist eine chronische psychiatrische Krankheit, die für Betroffene und Familie mit einem großen Leidensdruck verbunden ist. Sie wird durch lebensbedrohliche, traumatische Situationen, wie beispielsweise einem Autounfall, einer Vergewaltigung oder einer Naturkatastrophe ausgelöst.

Das Risiko im Laufe des Lebens ein solches Trauma zu erleiden liegt bei 90 %, jedoch kommt es nur bei 20–30 % der Opfer zum Ausbruch dieser Krankheit. Diese Diskrepanz hat zur intensiven Erforschung von Resilienz- beziehungsweise Vulnerabilitätsfaktoren geführt. Auf neurobiologischer Ebene wurde eine verminderte Aktivität des wichtigsten inhibitorischen Neurotransmitters Gamma-Aminobuttersäure (engl. gamma amino butyric acid; GABA) nachgewiesen. Dieser kontrolliert die Erregbarkeit verschiedener emotionsrelevanter Hirnregionen, wie zum Beispiel der Amygdala und des Hippokampus. In tierexperimentellen, sowie in humanen Studien wird PTBS oft als Gedächtnisstörung konzeptualisiert.

Für die Untersuchung pathologischer Gedächtnisprozesse im Tiermodell dienen zum einen klassische Furchtkonditionierungsexperimente, zum anderen operante Konditionierung. Trotz der sehr unterschiedlichen Ansätze, erlauben beide Methoden die Integration von einzelnen Hinweisreizen sowie dem kontextuellen Umfeld zu untersuchen. Bei der klassischen Furchtkonditionierung, wird ein anfangs neutraler Reiz, wie zum Beispiel ein Ton mit einem aversiven Reiz, wie einem elektrischen Schock gepaart.In moderaten Konditionierungsprotokollen wird der Reiz mit dem größten Vorhersagewert die größte Furchtreaktion auslösen. In diesem Beispiel wäre das der Ton, der Kontext alleine, also die Kammer, in der der Schock präsentiert wurde, tritt in den Hintergrund und löst eine geringere Furchtreaktion aus.

Bei der operanten Konditionierung wird die Häufigkeit eines anfangs zufällig auftretenden Verhaltens erhöht, indem es an eine Belohnung, wie Futtergabe gekoppelt

wird. Eine Form dieser Konditionierung ist die dual-solution Lernaufgabe. Hierbei kann die Maus zwei mögliche Strategien anwenden um den Ort der Futtergabe zu erinnern. Zum einen kann sie sich an einem konkreten Hinweisreiz, der die Stelle signalisiert, orientieren, zum anderen, kann sie kontextuelle Reize aus dem Raum und deren Relation zueinander nutzen.

Ziel meiner Doktorarbeit war die Untersuchung inhibitorischer Interneurone in der Schlüsselreiz/Kontext Balance in den beiden oben beschriebenen Paradigmen, beziehungsweise in reinen Schlüsselreiz– und Kontextgedächtnisprozessen.

Dafür habe ich, unter anderem, die konstitutive GAD65 knock out Maus verwendet. GAD65 (engl. glutamic acid decarboxylase) ist an der Synapse lokalisiert und synthetisiert GABA aktivitätsabhängig. In meiner ersten Studie präsentierte ich juvenilen heterozygoten GAD65 knock out Mäusen und Wildtyp-Geschwistertieren verschiedene Stressoren. Eine umfangreiche Verhaltenstestbatterie im Erwachsenenalter ermöglichte die Untersuchung der Auswirkungen. Diese Mäuse weisen, anders als homozygote knock out Mäuse, *per se* keine Verhaltensauffälligkeiten auf. In meinen Untersuchungen zeigten die GAD65(+/–) Mäuse eine generelle Resilienz, sie wiesen keine stress-induzierte Reduktion des Explorationsverhaltens im Offenfeld auf so wie ihre Wildtyp-Geschwistertiere. Nach einer auditorischen Furchkonditionierung wiesen sie weiters eine Stressor-spezifische Resilienz auf. Wenn ein kurzes und sehr intensives Stressprotokoll präsentiert wurde, entwickelten die Mutanten keine Generalisierung auf den Hintergrundkontext, sowie keine Steigerung der Angst oder weitere Reduktion der Aktivität.

In einer anschließenden Faktoranalyse segregierten Angst–, und Aktivitätsmessungen vor der Furchtkonditionierung, klar von jenen nach der Konditionierung. Letztere erklärten zusammen mit der Kontextfurchtantwort den größten Teil der Varianz der Daten. Die beobachtete Resilienz war insbesondere überraschend, da diese Mäuse eine verzögerte Reifung des GABAergen Systems aufweisen und eine negative Korrelation zwischen GABAergem Tonus und Stressanfälligkeit in der Literatur oft beschrieben ist. Dieser Befund deutet auf sehr erfolgreiche Kompensationsmechanismen auf molekularer Ebene hin. Um das weiter zu untersuchen isolierte ich mittels Lasercapture Mikrodissektionen stressrelevante Gehirnareale in verschiedenen Reifungsstadien des GABAergen Systems in GAD65(+/–)– und GAD65(+/+) Mäusen. Mithilfe der quantitativen Polymerase-Kettenreaktion untersuchte ich die Expressionslevels verschiedener stresssensitiver Komponenten des

GABA ergen Systems. Hier waren die Befunde in zeitlicher und regionaler Hinsicht sehr heterogen, was auf hoch spezialisierte Kompensationsmechanismen hindeutet. Ein weiteres Experiment, die oben beschriebene dual-solution Lernaufgabe, führte ich mit homozygoten GAD65 knock out Mäusen durch. Diese weisen in Furchtkonditionierungsexperimenten tonabhängige Gedächtnisauffälligkeiten, wie Generalisierung der Furchtantwort auf einen ungepaarten Ton oder Defizite bei der Tonextinktion, auf. Auch in dieser Lernaufgabe präferierten GAD65(–/–) Mäuse eine Schlüsselreiz-Strategie, wohingegen Wildtyptiere eine räumliche Strategie verfolgten um den Ort der Futtergabe zu erinnern. Dieser Befund deutet darauf hin, dass GAD65 eine Rolle bei der Interaktion zwischen Striatum, Hippokampus und Amygdala, den Hirnregionen, die entscheidend an der Strategieselektion beteiligt sind, spielt.

Ziel meines vierten und letzten Experiments war die Untersuchung der Bildung und Extinktion verschiedener Furchtgedächtnis–Typen in einem neuen genetischen Mausmodell. Bei diesen Tieren führt die Expression einer gain-of-function Variante eines Glyzinrezeptors zur Erhöhung der Aktivität der Parvalbumin-positiven Interneuronenpopulation. Diese Subpopulation ist an der Generierung oszillatorischer Aktivität beteiligt, die einen Einfluss auf Lernen und Gedächtnis hat. Ich konnte zeigen, dass diese Mäuse ein kontext-spezifisches Extinktionsdefizit, bei weitgehend normaler Gedächtnisbildung und Tonextinktion aufweisen.

Zusammenfassend, konnte ich eine Rolle für GAD65 bei der Schlüsselreiz/Kontext Integration in verschiedenen Lernparadigmen zeigen und eine beeinträchtigte Furchtextinktion bei Mäusen mit gesteigerter Aktivität Parvalbumin-positiver Interneurone. Die beobachteten Verhaltensauffälligkeiten weisen Parallelen zu Symptomen der posttraumatischen Belastungsstörung auf und sind deshalb für das bessere Verständnis dieser Krankheit relevant.

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Abbreviations

ANOVA	**Analysis of variance**
BLA	**Basolateral amygdala**
BDNF	**brain derived neurotrophic factor**
bp	**Basepair**
CA	***cornu amonis***
camk2a	**Calcium/calmodulin-dependent protein kinase type 2α chain**
cDNA	**copy Desoxyribonucleic acid**
cm	**Centimeter**
CREB	**cAMP response element–binding protein**
CS	**Combined stress**
CS–	**conditioned stimulus minus (shock)**
CS+	**conditioned stimulus plus (shock)**
CR	**conditioned response**
ctr	**control**
dB	**Decibel**
dCT	**δ Critical threshold**
ddCT	**$\delta\delta$ Critical threshold**
DG	**dentate gyrus**
DMDC	**Dimethyldicarbonat**
E	**Extinction**
e.g.	**Example given**
EPM	**Elevated plus maze**
EtOh	**Ethanol**

FAM	**6-carboxy-fluorescine**
FC	**Fear conditioning**
Fig.	**Figure**
GABA	**γ-Aminobutyric acid**
$GABA_A$	**GABA receptor type A**
$GABA_B$	**GABA receptor type B**
Gabra1	**GABA receptor type A α1 subunit**
Gabra2	**GABA receptor type A α2 subunit**
GAD	**Glutamic acid decarboxylase**
GAPDH	**Glycerinaldehyde-3-phosphate dehydrogenase**
gDNA	**genomic Desoxyribonucleic acid**
GlyR	**Glycine receptor**
HPA	**hypothalamus–pituitary–adrenal**
i	**interval**
i.e.	***id est* engl. that is**
ICD–10	**International Classification of Diseases**
IS	**Isolation stress**
ISI	**Inter-stimulus interval**
ITI	**Inter-trial interval**
kHz	**Kilohertz**
LA	**Lateral amygdala**
LCM	**Laser capture microdissection**
LD	**Light/dark test**
LTD	**Long term depression**
LFP	**Local field potentials**
LSD	**Least significant difference**
mA	**Milliamper**
MGB	**Minor groov-binder**
min	**Minute**
μl	**Microliter**

μm	**Micrometer**
NFQ	**Non-fluorescent quencher**
n.s.	**not significant**
nt	**nucleotide**
OF	**Open field**
P	**Postnatal day**
PCR	**Polymerase chain reaction**
PEN	**Polyethylene naphtalate**
PFC	**prefrontal cortex**
PLL	**Poly-L-Lysine**
pm	***Post meridiem***
PTBS	**Posttraumatische Belastungsstörung**
PTSD	**Posttraumatic stress disorder**
Pval	**Parvalbumin**
qPCR	**Quantitative polymerase chain reaction**
R/E	**Retrieval/Extinction**
RNA	**Ribnucleic acid**
rpm	**Rounds per minute**
RT	**Room temperature**
s	**Seconds**
SEM	**Standard error of mean**
SNR	**Signal to noise ratio**
SoIn	**Social interaction**
SPL	**Sound pressure level**
SWR	**Sharp wave ripples**
T	**Training**
Tab.	**Table**
TS	**Tail suspension**
TWA	**Two-way analysis of variance**
U	**Units**
UNG	**uracil-N-glycosylase**

US	**Unconditioned stimulus**
UV	**Ultraviolet**
vs.	***Versus***
VS	**Variable stress**

To my family

Chapter 1

Introduction

1.1 Posttraumatic stress disorder

1.1.1 Background

Posttraumatic stress disorder (PTSD) is an anxiety disorder that can develop in the aftermath of a potential life–threatening experience. Such events include for example sexual or physical abuse, natural catastrophes or war. In the international classification of diseases (ICD)–10, PTSD is clustered together with other anxiety disorders under the superordinate "neurotic, stress-related and somatoform disorders". Core symptoms include intrusive memories (involuntary re–experiencing of the trauma), avoidance of situations similar to the trauma, social withdrawal, emotional numbing, and persistent hyperarousal. The experience of a concrete trauma is a precondition for the diagnosis of this disorder [1].
Of note, only about 20–30 % of people that experience a traumatic event, are prone to develop this disorder [2]. The majority of victims of a trauma show symptoms in the immediate aftermath, which decline with time as the individual recovers from the episode [3]. This discrepancy between trauma experience and disease development has led to the extensive research on genetic and environmental resilience– and vulnerability factors. In the course of this, stress was identified as a prime risk factor for the development of psychiatric disorders later in life. Stress has particularly severe consequences, when experienced early in life and does not only lead to PTSD, but also to personality disorders or depression [4].

Thus, animal research has established a large variety of stress paradigms to model PTSD in rodents (see section 1.1.4).

1.1.2 Neuroanatomical basis of PTSD

Human and rodent studies suggest disturbances in the functional integrity of the amygdala, the hippocampus, and the medial prefrontal cortex (PFC) and their interaction with each other to be implicated in the pathophysiology of this disorder. The amygdala, located deep in the medial temporal lobe [5] is prominently involved in the behavioral manifestation of fear and anxiety[1] [7, 8] and has been shown to be overactive in various anxiety– and stress–related disorders [9]. In humans, structural studies revealed more inconsistent results than functional studies did. In PTSD–patients increases in amygdalar volumes [10] have been reported, as well as decreases [11] and no changes [12, 13]. In functional imaging studies a hyperactivation in response to trauma–related [14, 15] and –unrelated [16–18] stimuli is frequently observed. Similarly, severe stress experiences elicit hypertrophy in the rodent amygdala [19–22].
The hippocampus on the other hand is known for processing complex temporal and contextual information [23], and disturbed context representation can lead to fear generalization, a critical feature of PTSD [24]. A large body of evidence reports reduced hippocampal volumes in PTSD–patients [11, 13, 25, 26], which, however, might rather be a pre–existing risk factor than a consequence of the trauma or the disease [27]. Moreover, apart from structural changes, also reduced hippocampal activity has been observed [28]. In rodents, stress induces dendritic atrophy in the hippocampus [19] and apoptosis [29, 30]. Amygdala and hippocampus are highly interconnected and projections from the amygdala to the ventral hippocampus have been implicated in the generation of anxiety-related behavior in rodents [31]. Moreover, both regions sychnronize firing during different stages of fear memory retrieval ([32, 33].
Medial prefrontal regions are, apart from their prominent role in cognitive functions [34], also implicated in emotional regulation [35] and fear extinction [36, 37]. The latter is disturbed in PTSD–patients [38, 39] and activation in medial frontal regions is diminished in patients after presentation of trauma–related [14], as well as trauma–unrelated [17] cues. This is also accompanied by volume loss [40] and

[1] Anxiety refers to basal conditions and is, in contrast to fear, not triggered by distinct stimuli. Thus, it presents with slower on– and off–set dynamics [6].

corresponds to changes in the dendritic architecture in rodents [41]. Interaction and synchronization between these three regions is pivotal to emotional processing during fear and anxiety states [32, 33, 42, 43].
On a systemic level, it has been postulated that in the pathogenesis of PTSD the medial prefrontal cortex and the hippocampus exert insufficient inhibition over a hyperactive amygdala [44]. This hypothesis is for example supported by the inverse correlation of cerebral blood flow in the amygdala and medial frontal regions during recollection of traumatic memories [14].

1.1.3 Conceptualizing PTSD with learning paradigms

Once a stressful or dangerous situation is experienced, it is beneficial to avoid such potentially harmful situations in the future. This is only possible, if an organism forms associations between characteristic stimuli of this particular situation, to predict danger. This process is learning, as a result of which memory is formed. In PTSD–patients however, traumatic memories differ from normal memories, for example by resistance to extinction or generalization [45]. Two forms of associative learning are classical and instrumental conditioning [46]. Both are modulated by prior (stress–) experiences, thus bearing relevance for modelling memory malfunctions in anxiety disorders, like PTSD. Moreover, both paradigms require proper functioning of the amygdala and the hippocampus.

1.1.3.1 Fear conditioning

Fear conditioning is relevant for PTSD research, since pathological memory processes like the abovementioned extinction deficits and fear generalization can be modelled with this paradigm [47].
The classical fear conditioning concept was developed from reflex conditioning, first described by Pawlow (1927) [48]. Here, an initially neutral stimulus (e.g. a tone) is repeatedly paired with an aversive stimulus (e.g. electric foot shock; unconditioned stimulus, US), so that the neutral stimulus becomes a signal for the US. This so called conditioned stimulus (CS) is now capable of eliciting a conditioned response (CR) similar to the unconditioned response induced by the US. In rodents, freezing is the most prominent indicator of fear, which is measured by

complete absence of movement other than respiration [49]. Under moderate training conditions the fear response is specific to the stimulus most predictive of the US [50]. The association between CS and US is formed in the lateral amygdala (LA), where fibers carrying information for both stimuli, converge on the same neurons [51]. Information about the conditioning context is predominantly processed in the basolateral amygdala (BLA), mediated by its close connection to the hippocampus [5]. Finally, all information converges on the central nucleus of the amygdala, from where it is then forward to the periphery to generate a behavioral response [52] (Fig. 1.1).

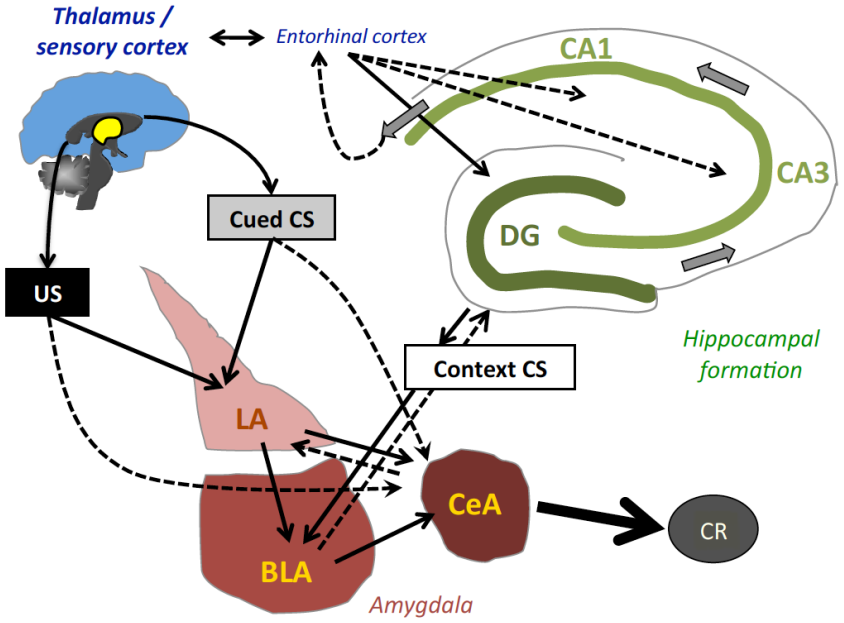

FIGURE 1.1: Schematic illustration of fear memory formation: Information about the foot shock and the tone converge in the lateral amygdala. Contextual information is processed by the hippocampus and the basolateral amygdala. Finally, all information is forwarded to the central amygdala to generate fear behavior. US: unconditioned stimulus, CS: conditioned stimulus, DG: dentate gyrus, CA: cornu amonis, LA: lateral amygdala, BLA: basolateral amygdala, CeA: central amygdala, CR: conditioned reaction. modified after [52]

If the US is presented without being paired to a discrete sensory stimulus the context of the conditioning chamber becomes the sole predictor of the US. In this pure contextual fear conditioning paradigm, the hippocampus (together with the BLA) becomes the key structure for memory encoding ([53, 54]. Information

about complex contextual stimuli and their relation to each other enters the hippocampal formation through the entorhinal cortex. It is then processed within the hippocampal subfields dentate gyrus (DG), *cornu amonis* (CA) 3 to 1 [55].
In addition to the subfields, a functional specialization of dorsal and ventral hippocampus has emerged concerning the formation of fear memories. Thus, the ventral hippocampus appears to mediate the storage of pure contextual memories (also called "foreground context memory"), whereas the dorsal hippocampus is critical for the memory accompanying contextual features when a discrete CS is applied ("background context memory") [56]. With increasing number of tone/shock pairings or shock intensities, generalization to similar tones and to the background context can occur [50, 57]. Apart from modulation of the fear conditioning paradigm itself, also prior stress experience leads to a similar cue/context imbalance [58].
Upon repeated presentation of the CS (being it a salient cue or the training context) without reinforcement by the US freezing levels will decline. This process is called extinction and implies a new learning process. Here, the PFC comes to interact with the hippocampus and the amygdala to suppress fear [59].

1.1.3.2 Operant conditioning

In operant conditioning, the frequency of an initially random behavior, like lever pressing, is increased or decreased by coupling this behavior with the presentation of either a reward (like a food pellet) or a punishment (like an electric shock), respectively [60].
The anatomical core structure in operant conditioning is the dorsal lateral striatum, where sensory and motor information from the neocortex and information about movement sequences from midline thalamic nuclei and *substancia nigra pars compacta* converge. This information is further sent to the globus pallidus and the *substantia nigra pars reticulata*, regions of higher order motor functions [61].
The operant conditioning paradigm can be used to study the interaction of multiple memory systems in ambiguous experimental setups, as in so called dual–solution tasks. Here, the location of a target can be remembered with the help of distal cues and their relation to each other (spatial strategy), or via a distinct cue that is proximal to the target. The spatial strategy depends on functional integrity of the hippocampus while the cue–guided strategy is mediated by the dorsal stritatum [62]. The relative use of these two strategies is highly influenced by the activity

state of the amygdala, in a manner that increased activation biases towards a cue–based strategy [63–66]. The influence of the amygdala on both memory systems is accomplished by on one hand its direct interaction with the hippocampus and on the other hand by its indirect interaction with the striatum via *substantia nigra*, ventral tegmental area and *nucleus accumbens* [61].
Several theories containing multiple memory system interaction have been formulated to explain certain aspects of memory disturbances in PTSD. They converge in the increased weight of certain distinct cues (e.g. the noise of slamming a door, reminiscent of gun fire in a war zone) at the expense of contextual information (e.g. the safe setting of home) [67, 68], leading to inappropriate behavior.

1.1.4 Animal models of PTSD

Studies in humans are mostly correlative and do not allow for a clear dissociation between predisposing factors and features that develop in response to the trauma. Therefore, various animal models for PTSD have been established. In general, animal models of PTSD can be subdivided in three categories: models applying high intensity electric foot shocks, models with social stress (e.g. predator scent exposure) and models that use single prolonged stress exposure (e.g. restraint, platform exposure) [69]. These models have to meet validity criteria, such as chronicity or correlation between symptoms and stress severity (a detailed review is given in [45]. Such stress regimens are particularly potent of inducing long-lasting and severe disturbances, when presented in stress-sensitive time windows, like juvenility [70]. Important parameters e.g. the duration of stress and its intensity determine the outcome of the experience, with brief and strong stressors producing anxiety symptoms [70–72] and chronic, mild stressors inducing depression-like features ([73–75].

1.1.4.1 Stress during development

During development, the brain regions that regulate the stress response of an organism are under maturation [76], thus, early life stress has been shown to have profound effects on the pathogenesis of psychiatric disorders ([77–79].The first two postnatal weeks in rodents, considered as a stress hyporesponsive period, are characterized by a diminished responsiveness to external stressors. However,

this responsiveness particularly depends on the characteristics of a stressor. Brief episodes of maternal separation are beneficial for future stress coping and cognitive performance, whereas prolonged maternal separation has opposite effects [80].
In the present thesis, stress paradigms targeting a juvenile period of young adolescence were used to address mechanisms involved in PTSD–related behavior. Adolescence in rodents circumscribes the time between postnatal days (P)23-P61 [81]., This developmental stage is characterized by sexual maturation, progressive independence from the parents and orientation towards peers [82]. At this age the hypothalamus pituitary axis (HPA) axis is fully developed, but brain regions including the medial prefrontal cortex, the hippocampus and the amygdala that modulate the HPA-response are still under maturation [82]. In line, the corticosterone response to an acute stressor rises and peaks similarly in juvenile and adult rodents, but the shutdown is delayed in juveniles, indicating ineffective silencing by forebrain regions [83]. Brief intensive episodes of stress at juvenility, particularly when combined with a second stressor in adulthood, lead to a wide range of disturbances ([70, 84]. Increases in anxiety [85–87] and impairments in cognitive performance, like novel object recognition [85] or spatial learning [71], are two examples. Particularly when learning takes place in a setting, similar to the trauma, performance is diminished [88]. Likewise, hippocampal brain derived neurotrophic factor (BDNF) levels are reduced after combined juvenile and adult stress, suggesting impaired hippocampal plasticity [70]. However, aversive learning is enhanced after brief, intensive stress experiences. Single prolonged stress leads to exaggerated fear responses [86, 89], background context generalization [85] and disruption of extinction ([90, 91]. Of note, stress-induced disabilities are not rigid or insensitive to further manipulations, since many of them can be reversed by environmental enrichment [86, 92] or physical exercise [93].
Apart from acute and intense stress, chronic mild stress paradigms, like social isolation, also induce anxiety [94], hyperactivity [95, 96], cognitive deficits [97, 98] and alterations in social behavior [99]. Also in this paradigm disturbances can, at least in part, be reversed by environmental enrichment. In rats, also re-socialization after prolonged isolation can rescue some of the social deficits [100]. However, prolonged social isolation was also shown to induce PTSD-like memory-disturbances, such as exaggerated fear response to the cue [101], fear generalization [58], and extinction deficits [102].
In the presence of the multifaceted effects of either stress paradigm, the importance of a comprehensive behavioral profiling is highlighted. This does not only

allow the identification of the affected behavioral domains, but also investigation of possible interactions.

1.2 The GABAergic system

γ-amino butyric acid (GABA) is the major and most widely distributed inhibitory neurotransmitter in the mammalian brain [103]. The above mentioned key regions of (pathological) memory formation and stress regulation, the amygdala and the hippocampus, are under tight GABAergic control, although GABAergic interneurons account for only 10–20 % of the neurons in these regions [104, 105].
PTSD is a multifaceted disorder that involves disturbances of many different transmitter systems, including the GABAergic system [69]. Yet, human studies addressing the role of GABA in PTSD development have remained controversial. For example, low GABA-levels in the immediate aftermath of a trauma [106] or even 1 year later [107] positively correlate with PTSD development. In contrast, pharmacologically increased inhibition after a trauma also positively correlates with disease onset [108], or had no effect [109]. These discrepancies may be related to the complexity of the disorder as well as the multitude of GABAergic local circuit neurons and their different functions in the central nervous system. Thus research using animal models is required to provide better insight into the specific GABAergic mechanisms in traumatic stress-induced pathology.

1.2.1 GABA synthesis

GABA is synthesized through decarboxylation of glutamate by two isozymes of glutamic acid decarboxylase, GAD65 and GAD67. These two enzymes, which are named according to their respective molecular weights, are encoded by separate genes and underlie differential modes of regulation [110, 111]. Although these enzymes are typically co–expressed in GABAergic neurons, total levels and ratios differ greatly between the brain regions and species [112, 113]. Contribution of GAD65 to total GAD in the frontal cortex and hippocampus is approximately 80 % in the rat and 50 % in the mouse [113]. Expression of GAD65 in the central amygdala is higher than in the lateral and basolateral amygdala [112].
These isozymes produce GABA for different cellular purposes. Evidence suggests that GAD65 produces GABA in nerve terminals for the neurotransmitter pool

to provide rapid release in an activity-dependent manner: Several findings support this hypothesis: Firstly, it is primarily found at the synapse [111], secondly, saturation with the co-factor pyridoxal phosphate is low [111, 114] and finally, membrane association to GABA vesicles is reversible [115–117]. GAD67 in contrast, is localized in the cytoplasm and synthesizes GABA for metabolic needs of the cell [110, 111]. However, this functional distinction is by no means strict, as the isozymes can form heteromers [118] and as in GAD65 knock out, GAD67 at least to some extend can produce GABA for vesicular release [119].

1.2.2 GABA metabolism

After receptor binding GABA is cleared from the synaptic cleft either by neurons or glia cells. In glia cells, GABA is converted to succinic semialdehyde by GABA transaminase and is then further oxidized to succinic acid. Succinic acid is transformed to glutamate and further to glutamine, which is finally transported to the nerve terminal. Here, glutaminase converts glutamine to glutamate, which is decarboxylated to GABA by glutamic acid decarboxylase.
GABA that is transported back to the presynaptic neuron either undergoes the same cycle or is immediately recycled into synaptic vesicles, where it is ready for future release (fig. 1.2, [120]). The majority of GABA taken up from the synaptic cleft is used to restore the vesicular pool, and only a small fraction enters the metabolic cycle [121].

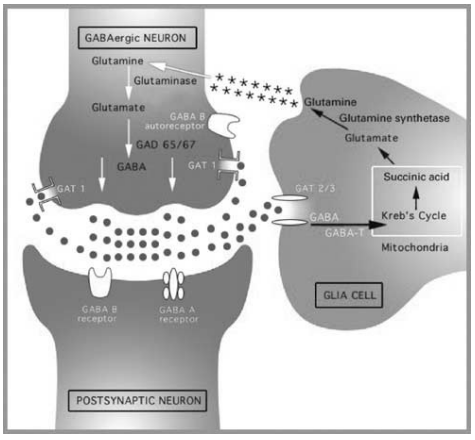

FIGURE 1.2: Illustration of the GABA metabolism. After release GABA is either taken back up from the presynaptic neuron or a glia cell, where it is converted to glutamine and presented to the neuron [120].

1.2.3 GABA receptors

When released into the synaptic cleft GABA can bind to $GABA_A$– or $GABA_B$ receptors. $GABA_A$ receptors are ionotropic and quickly hyperpolarise the postsynaptic cell whereas metabotropic $GABA_B$ receptors exert a slower G-protein coupled response. The latter is mainly localized at presynaptic sites and provides inhibition via the suppression of calcium conductance [120].
The $GABA_A$-receptor is a pentameric chloride channel that is predominantly composed of 2 α, 2 β and 1 γ subunits [122, 123]. In total, 6 α, 3 β, 3 γ, 1 δ, 1 ϵ, 1 θ, 1 π and 3 ρ subunits exist [123]. Depending on its subunit composition the $GABA_A$ receptor can reside at the synapse or at extrasynaptic sites of the postsynaptic neuron, thus mediating phasic and tonic inhibition, respectively [124]. This assignment is not strict, for example, α1 and α2 subunits are found at synaptic $GABA_A$-receptors [125], but the α1 subunit is also expressed extrasynaptically on the parvalbuminergic (Pvalb+) subclass of GABAergic cells in the DG [126]. This subunit is present in the majority of all $GABA_A$ receptors in the brain [127], particularly in the hippocampus its expression is higher in DG and CA1, and weaker in CA3. The α2 subunit expression is highest in the DG and CA3. In the amygdala, the expression of the α2 subunit is higher than that of α1 [128]. Together, regulation of the GABAergic system is regionally specialized to allow optimal response towards a variety of environmental challenges.

1.2.4 Development of the GABAergic system

GABA is unique among the neurotransmitters since it performs a developmental switch from initially excitatory to later inhibitory. This switch is mediated by a change of intracellular chloride concentrations from high, early in development to low, later in development, brought about by differential expression levels of chloride transporters [129]. It is assumed that the GABA switch towards an inhibitory action is completed around the second postnatal week of the rodent development. In general, GABAergic activity arises before glutamatergic and develops towards a more precise and faster GABAergic activity at the synapse that is mediated by changes in receptor subunit composition and GABA uptake [130].
GAD65 and GAD67 also display specific developmental functions, as GAD67 is critical in prenatal development [131], whereas GAD65 determines GABA levels in postnatal maturation [132–134]. However, the GABAergic system does not

develop uniformly across the brain, but in a regionally specialized manner (e.g. [135–138]. In the rat hippocampus $GABA_A$-receptor $\alpha 1$ subunit expression massively increases at postnatal stage, whereas the $\alpha 2$ subunit is detectable already at embryonic stage and slightly decreases after P12 [137]. Both GAD65 and GAD67 significantly increase between P5 and P10 in this region [138]. In the rat amygdala, $GABA_A$ receptor mediated GABA currents rise until P28, and here also the $\alpha 1$ subunit levels increase during postnatal development [139]. Moreover, it was shown in null mutant mice that GAD65 mediates a rise in amygdalar, hippocampal and neocortical GABA levels between the first and second postnatal month [134].

A prominent example for GABA mediated developmental plasticity is the maintained critical time window for ocular dominance development in GAD65 knock out mice [140]. In wild type mice, occlusion of one eye during the sensitive period (P23-33) results in loss of vision in this eye. In GAD65 knock out mice, this plasticity can be induced independently of age, if GABAergic activity is increased by application of diazepam [140]. Moreover, application of a $GABA_A$ $\alpha 1$, $\alpha 2$ and $\alpha 3$ selective ligand can open this sensitive window prematurely [141]. Interestingly, the onset of this critical period coincides with the first appearance of a specific interneuron subpopulation, namely parvalbumin-positive cells [142].

1.2.5 The GABAergic system and stress

Evidence suggests that in the adult, a regionally and cell type specific regulation of GABAergic functions occurs at the level of gene expression. The comparison of effects of acute and chronic stress on GAD expression [143] suggests a highly specific and differential expression regulation of both isozymes in the amygdala, bed nucleus of the stria terminalis, DG and CA3, as well as various hypothalamic subregions. Further, GAD expression is highly dependent on the nature of the stressor experienced, and the species–specific stress response. It was shown that expression of GAD67 is reduced in the mouse amygdala after chronic restraint stress [144]. However, another study, in rats, found no effect of restraint, but reduced GAD65 in the hippocampus and reduced GAD67 in the amygdala after chronic daily injection of the stress hormone, corticosterone ([145]. Moreover, GAD65 and GAD67 are differentially regulated in the dorsal hippocampus and the amygdala following conditioned fear stress [112, 146].

Apart from GAD isozymes, the GABA receptor subunits also respond to environmental challenges and correlate with the behavioral manifestation of anxiety. $GABA_A$ receptor $\alpha 1$ expression is decreased in emotion-relevant brain regions of the less anxious C57BL/6 strain compared to the more anxious BALB/c strain [147]. Social stress induces an increase in $\alpha 1$ and 2 subunits in the cortex, but failed to do so in the hippocampus and expression changes lasted for at least 3 days and returned to baseline after 7 days [148]. Another 14–day long stress protocol was capable of reducing $\alpha 1$ in the hippocampus, an effect that was not yet apparent after 7 days of stress [149]. A genetic mouse model, displaying anxiety, also shows reduced expression of $\alpha 1$ and 2 subunits in temporal and frontal regions, despite unaltered GAD expression [150].
Thus, controlling expression levels of GABAergic factors appears to provide a mechanism for fine-tuning of inhibitory function in response to different stressful experiences.

1.2.5.1 The GABAergic system and the variable stress paradigm

The variable stress paradigm encompasses presentation of different stressors on few consecutive days. Such stressors are for instance, exposure to bright light or restraint. They are of rather short duration, very intensive, uncontrollable and unpredictable, thus mimicking human traumata, like a car accident or a terrorist attack.
Apart from GAD itself, which is lastingly decreased throughout the rat amygdala following a 7–day unpredictable peripubertal stress protocol [151], the $GABA_A$ receptor subunit composition displays highly plastic regulation in response to this paradigm alone, as well as in combination with a second challenge in adulthood [152, 153]. After combined juvenile and adult stress experience, an immature-like $GABA_A$ receptor subunit profile is reinstated, i.e. a decreased $\alpha 1$ to $\alpha 2$ and $\alpha 1$ to $\alpha 3$ expression ratio. This is true for both amygdala and hippocampus [152]. Accordingly, these rats are less sensitive to the $GABA_A$ receptor agonist brotizolam [152]. In the prefrontal cortex, the $\alpha 2$ subunit is increased after adult stress experience, but reduced if juvenile stress is preceded. However, this is true for social adulthood stressors, but not for restraint stress. The $\alpha 5$ subunit responds only to adulthood treatment, irrespective of the stressor type. In the amygdala, $\alpha 2$ and $\alpha 5$ subunits are increased upon juvenile stress alone. Adult stress alone also increases α 2 and $\alpha 5$ levels, whereas levels are reduced when combined with juvenile

stress. $\alpha 3$ is downregulated after social stress regardless of prior stress experience [153]. In summary, this stress paradigm leads to region specific and long-lasting alterations of $GABA_A$ receptor subunits that are differentially influenced by later stress experience and stressor quality.

1.2.5.2 The GABAergic system and the social isolation stress paradigm

Social isolation differs fundamentally from the variable stress protocols, since it usually lasts longer, is less intensive and factors like uncontrollability and unpredictability are not experienced. Gilabert-Juan et al. (2012) showed that rats reared in social isolation results in increased GAD67 levels in the amygdala, without changing GAD65 levels [154]. In another study, social isolation in adulthood was accompanied by a downregulation of hippocampal and amygdalar allopregnanolone [58], a positive allosteric modulator of GABA at the $GABA_A$ receptor [155]. Moreover, social isolation leads to increases in the $GABA_A$ receptor $\alpha 4$ subunit, without altering $\alpha 1$ [156]. Taken together, a role for GAD and $GABA_A$ receptor subunit regulation in stress coping is generally evident, although the changes are highly dependent on the brain region and characteristics of the stressor.

1.2.6 GABAergic interneurons

1.2.6.1 Interneuronal subpopulations and stress

GABAergic interneurons are not a uniform cell population, but display great diversity [104]. In the hippocampal CA1 subregion alone, 21 different types of interneurons have been identified [157]. They can be classified into subgroups based on morphology, expression of molecular markers (e.g. somatostatin, neuropeptide Y, cholecystokinin, parvalbumin) or function. However, this classification is not strict, since overlap exists [158]. A distinct role for most interneuonal subpopulations has been identified for the regulation of fear and anxiety ([159–164].

However, the most abundant GABAergic interneurons are the parvalbumin positive interneurons, which are classified electrophysiologically as fast spiking interneurons and morphologically said to be classical basket cells [165]. These interneurons target soma and proximal dendrites or the initial segments of axons

[166]. Their activity in different brain regions including the hippocampus is negatively influenced by stress, since the number of Pvalb+ cells is reduced by different stress paradigms [167, 168], as well as in a high-anxiety rat strain [169].
Moreover, a role for this class of interneurons in learning and memory processes is evident. Knock down of GAD67 selectively in Pvalb+ cells leads to reduced GABA release and fear extinction deficits [170] and contextual fear conditioning is accompanied by high Pvalb and GAD67 expression [171]. After contextual fear extinction, amygdalar GAD67 and Pvalb levels were increased only around those principal cells that had been active during fear conditioning and silenced during extinction, but not around neurons that were active during both states [172]. In cued fear conditioning, the role of Pvalb+ interneurons is more complex. During a tone/shock pairing they contribute to principal cell disinhibition in the BLA via distinct microcircuits. During tone presentation, Pvalb+ cells are excited and inhibit principal cell targeting somatostatin positive cells. During shock presentation they reduce their inhibition on principal cells directly, both finally allows excitation of BLA projection neurons and memory formation [173].

1.2.6.2 Interneuronal subpopulations and oscillations

Probably one of the most important features of GABAergic interneurons is their ability to shape the oscillatory activity in the brain. Since interneurons receive input from multiple excitatory neurons and in turn also contact multiple excitatory neurons, they are well suited to synchronize network activity [174]. Oscillations are rhythmic and coordinated activity patterns, which promote the transmission and integration of information within neurons [175]. Depending on the physiological state of the animal, different types of oscillatory patterns occur that can be captured by recording local field potentials (LFP) during different behaviors *in vivo* [176, 177]. For instance, in the hippocampus, nested θ–γ oscillations (5–10 Hz vs. 30–100 Hz) emerge during alert activity such as attention–requiring learning paradigms [176, 178, 179]. In line, altered γ oscillations have been associated with impaired working memory [180, 181]. Γ oscillations can be modulated by theta activity [104] and their coupling increases upon context dependent reward learning [182].
In the hippocampus, during quiescent behavior and slow wave sleep, another network activity pattern, sharp waves, appears [176, 177]. These LFP transients that

are superimposed by local ripple oscillations of about 200 Hz also occur spontaneously *in vitro* [183–185]. These events utilize not more than 18 % of pyramidal neurons [186]. They originate from hippocampal CA3 and propagate to CA1 [187, 188]. Sharp wave-ripples (SWRs) have a central role in memory consolidation processes by providing a temporal window in which compressed off–line cell reactivation and information transfer to the neocortex may occur [177, 189, 190]. Indeed, they are augmented after learning [191, 192] and their selective suppression impairs spatial memory [193, 194]. Of note, this process is also plastic and dynamic, since suppression of learning induced SWRs leads to a compensatory increase in SWRs after interference is released in a radial maze [195]. This phenomenon is specific for SWRs after learning, because a similar recovery does not occur when SWRs are suppressed after spatial exploration [195]. Moreover, presenting a stimulus during [196], but not after [197] ripple-activity accelerates learning success in rabbits, highlighting the increased capacity of information processing in this state. In the same hippocampus-dependent eye blink conditioning task ripple synchronized extinction training was impaired [196].
Specifically, studies on optogenetic modulation of Pvalb+ cell activity during SWRs both *in vitro* and *in vivo* elucidated an indispensible role of this particular interneuron type on SWR generation and cell-reactivation during SWRs [198, 199]. Despite the well-described role of hippocampal SWRs in spatial learning, literature concerning contextual fear conditioning in relation to modulation of specific interneuron population is sparse.

1.3 Genetic mouse models of stress–related psychiatric disorders

1.3.1 The GAD65 knock out mouse

As illustrated above, a balance between distinct cues and the surrounding environment, as well as intramodal specificity are essential for normal memory formation and the GABAergic system is massively regulated by stress experiences of different kinds. Previous studies have established a role of GAD65 in memory precision. GAD65 (–/–) mice display a generalized fear response to a neutral tone in an auditory cued fear conditioning task [146], a phenomen characteristic of anxiety

disorders, like posttraumatic stress disorder [57, 200–202]. It occurs in wild type mice after intensive training [50, 146] or after a moderate training with coincident viral overexpression of cAMP response element-binding protein (CREB) in the auditory thalamus [203]. Shaban et al. (2006) reported auditory generalization upon intensive training as well as reduced presynaptic inhibition of thalamic fibers terminating in the amygdala of mice deficient for the presynaptic $GABA_B$(1a) receptor [204]. In GAD65(–/–) mice this generalized fear response was accompanied by reduced θ synchronization between the amygdala and dorsal hippocampus [146], and amygdalar c-Fos expression is increased after fear retrieval [205], indicating a deficit in amygdalo–hippocampal communication and amygdalar inhibition.
Moreover, GAD65(–/–) mice exhibit a cue–specific extinction deficit [206], thus, presenting another hallmark of pathological fear. This was accompanied by sustained LA–CA1 θ synchronization. In wild type mice this synchronization declines together with freezing as extinction progresses [206]. The fact that generalization to the shock context, context fear extinction deficits, as well as differences in other hippocampus–dependent tasks were not observed [146, 206, 207], suggests overall intact intrinsic hippocampal processing. Additionally, GAD65(–/–) mice show an exaggerated expression of conditioned fear, i.e. increased escape attempts at the expense of freezing,[208] resembling panic disorder [209]. Application of yohimbine, a panicogenic drug, elicits a similar behavior [210], like local injections of a $GABA_A$ receptor antagonist or GAD inhibitor into the periaquaeductal grey, the region from where fear expression is transmitted to the periphery ([211, 212]. This shift in fear expression is particularly evident, when training and retrieval take place in the first half of their active phase [205]. A negative correlation between GAD65 and locomotor activity has been described previously [213, 214], but unstimulated hyperlocomotion is not observed in GAD65 mutant mice [207, 215]), or failed to reach significance [134] unless circadian fluctuations are considered [205]. Moreover, GAD65(–/–) mice show less time immobile in the forced swim test [216], which might reflect their generally increased psychomotor activity upon stress experience rather than reduced depression–like behavior. This view is supported by studies that report a decrease rather than an increase of GAD65 in depression models [73, 145, 217].
Finally, GAD65(–/–) mice exhibit alterations in social behavior, namely, reduced attacks in the male intruder test. Of note, this was also the only parameter in which heterozygous mice differed significantly from wild type mice [134]. In all other tests, GAD65(+/–) mice were indistinguishable from GAD65(+/+) mice

[134, 208]. This is particularly interesting, since they present with a two–month delay in the maturation of the GABAergic system in emotion–relevant brain areas. At juvenile age all three genotypes display low GABA levels, in young adulthood (2 months) wild type mice have developed high GABA levels. This state is reached by GAD65(+/–) mice only at 4 months of age. GAD65(–/–) mice maintain low GABA levels throughout development [134]. This discrepancy between the molecular and the behavioral phenotype, suggests very successful coping mechanisms in these mice.

1.3.2 A mouse model with genetic enhancement of parvalbuminergic neuron function

This new mouse model was originally designed to investigate epilepsy–related alterations in brain physiology. It expresses, in a cell–type specific manner, a gain of function variant of a glycine receptor that is found in hippocampectomies of epileptic patients [218]. This receptor variant facilitates vesicle association to the cell membrane and thus transmitter release. If expressed in glutamatergic neurons under the control of a calcium calmodulin α 2 (Camk2a) promotor, the network excitability is increased and γ–oscillations are impaired. This is accompanied by cognitive dysfunctions and impaired associative learning [219]. If expressed exclusively in Pvalb+ interneurons, the network excitability of these mice is reduced and increases in anxiety are observed [219]. Interestingly, they also present with increased hippocampal sharp wave ripple incidence and frequencies and facilitated CA3–CA1 network interaction (Fig. 1.3, unpublished observation Calsican G.), making it tempting to speculate about alterations in fear learning and extinction given the above mentioned role of SWRs in memory formation.

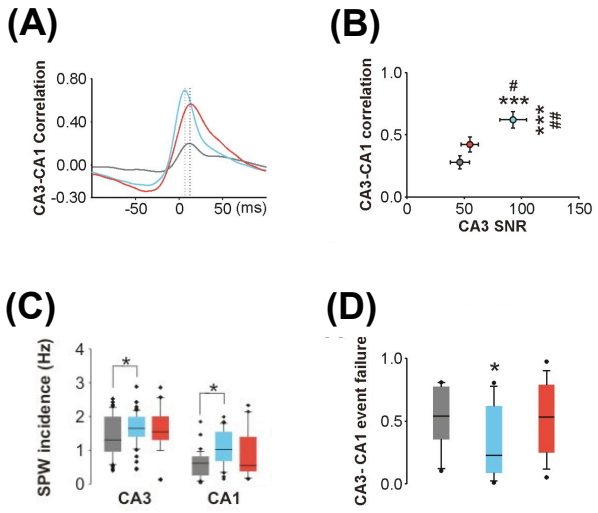

FIGURE 1.3: Recent findings suggest facilitated CA3–CA1 communication and increased sharp wave ripple frequencies in a new genetic mouse model with increased functioning of Pvalb+ cells (blue) and glutamatergic cells (red), respectively. (A) CA3–CA1 correlation is highest mice with functional increase of Pvalb+ cells. (B) Mean values of CA3–CA1 correlation of sharp waves (y axis) plotted against mean values of signal to noise ratio (SNR, x axis) for each genotype. Note that functional increase of Pvalb+ cells results in a significant increase of SNR in CA3 as well as CA3–CA1 correlation of SPWs. (C) Incidence of sharp wave ripples is also increased in these mice. (D) The ratio of failures of SPW propagation from CA3 to CA1 was decreased as well. *$p < 0.05$, ***$p < 0.001$ compared to control mice (grey), $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to mice with functional enhancement of glutamatergic neurons. (unpublished observation G. Caliskan)

1.4 Aims

As illustrated above, memory disturbances like generalization of traumatic memory components or extinction deficits are frequently reported phenomena in stress–induced psychiatric disorders, like PTSD and animal models thereof. GABA exerts tight control over emotion– and memory–relevant brain regions, like the hippocampus and the amygdala, and the parvalbumin–positive subclass of GABAergic interneurons are prominently involved in shaping the oscillatory activity of the hippocampus.

GAD65(+/–) mice display a delayed maturation of the GABAergic system, but no psychiatric features on a behavioral level. So the first question I asked was: Does this genetic vulnerability interact with environmental stress experiences? And if so, does this lead to a psychiatric phenotype or does it even trigger adaptive resilience towards additional adversities? To answer this question I applied different stress paradigms, ranging from brief and intensive to chronic and rather mild, to juvenile GAD65 heterozygous knock out mice and wild type littermates. Adult mice then underwent a behavioral test battery including auditory fear conditioning, covering all behavioral domains classically affected after stress experience.

The abovementioned discrepancy between the molecular and the behavioral phenotype suggests successful compensatory mechanisms. In my second study, I investigated molecular correlates of the delayed GABAergic maturation in GAD65(+/–) mice. To this end, I used laser capture microdissections to isolate emotion-relevant brain regions and quantitative polymerase chain reaction (PCR) to quantify expression levels of GABA–related genes at three critical stages of development.

Homozygous GAD65 knock out mice display cue–specific memory abnormalities in aversive classical fear conditioning experiments. In my third study, I aimed at expanding the role of GAD65 in cue–processing to a reward–based, operant dual–solution learning task. Here, mice could choose between a spatial– and a cue–guided strategy for reward localization. The role of GAD65 in hippocampus and amygdala interaction is well established, but its influence in the hippocampus–amygdala–striatum trajectory the main key players in this learning paradigm, is less clear.

Parvealbuminergic interneurons are pivotally involved in the generation of hippocampal sharp wave ripples, and these hypersynchronous discharges are thought

to replay newly acquired memory and transport it to the neocortex. In my fourth study, I used a new genetic mouse model with enhanced parvalbuminergic functioning to address the question: Do the *a priori* increased incidence and frequency of sharp wave ripples that these mice display influence memory formation and extinction? To this end, I performed contextual and cued fear conditioning and extinction. All my experiments aimed at the study of GABAergic mechanisms in (pathological) memory formation and extinction with relevance to PTSD.

Chapter 2

Materials and Methods

2.1 General remarks

Animal housing and experiments were in accordance with the European and German regulations for animal experiments and approved by the Landesverwaltungsamt Sachsen–Anhalt (2-939, 2-1177, 2-887). For the first three experiments, I used male GAD65 constitutive knock out mice developed by Asada et al. (1996) [207] and wild type litter mates that were bred on a C57BL/6N (Tac) background (M and B Taconic, Ejby, Denmark). For study 1 and 2 mice were obtained from GAD65(+/+) x GAD65(+/–) breeding pairs, for study 3 offspring from GAD65(+/–) x GAD65(+/–) parents were used. Mice were genotyped using an allelespecific PCR (for details see appendix A.1). In our animal facility, mice were kept in litter mate groups of 2–6 in type 2 long cages (Techniplast Deutschland GmbH, Hohenpeissenberg, Germany and Bioscape, Castrop-Rauxel, Germany) and had *ad libitum* access to food and water, if not otherwise stated. They were kept on an inverted 12 h light/dark cycle with light onset at 7:15 pm. Experiments took place in the active phase, i.e. the dark phase. For study 1 and 2 pubs were weaned at P21, for study 3 at P28 according to the standard protocol in our animal facility. Throughout all experiments, I was blind to the genotype and the group affiliation. Experimental apparatuses were cleaned with 70 % ethanol (EtOH) after each mouse.

2.2 Interaction of GAD65 haplodeficiency and juvenile stress (study 1)

2.2.1 Paradigm

Male heterozygous mice and wild type littermates were randomly divided into one of four groups (N = 8–14):

- Variable stress (VS): Mice received a variable stress protocol from P24–26 adopted from Tsoory et al. (2008) [72]. On P24 mice were immobilized for 30 min using a 20 ml plastic tube (length: 9 cm, diameter: 2 cm; Braun Melsungen, Germany), with holes at the front allowing animals to breathe freely. On the following day mice were exposed three times for 30 min, at 1 h intervals, to a bright light (400 lux) on a circular, 105 cm elevated platform (diameter: 14.5 cm; Greiner, Frickenhausen, Germany). And on P26 mice had to swim for 15 min in 24 +/-2 °C warm water in a bucket of 16 cm diameter. Restraint and forced swimming took place under red light conditions (< 5 lux). Animals were returned to their home cage and group housed until P107, then they were separated 5 days before behavioral testing started.
- Isolation stress (IS): Mice were isolated at P24 and kept single in standard home cages until the end of behavioral tests.
- Combined variable and isolation stress (CS): Mice were exposed to the variable stressors and adjacent social isolation as described above.
- Control (ctr): Mice were left undisturbed in littermate groups of 2–6 until P107, then they were separated in preparation of the behavioral testing.

An overview of stress regimen and behavioral tests in relation to GABAergic maturation is given in figure 2.1.

2.2.2 Behavioral tests

Behavioral testing commenced on P112, when naive heterozygous mice had reached wild type GABA-levels [134]. All animals underwent every test in the order listed

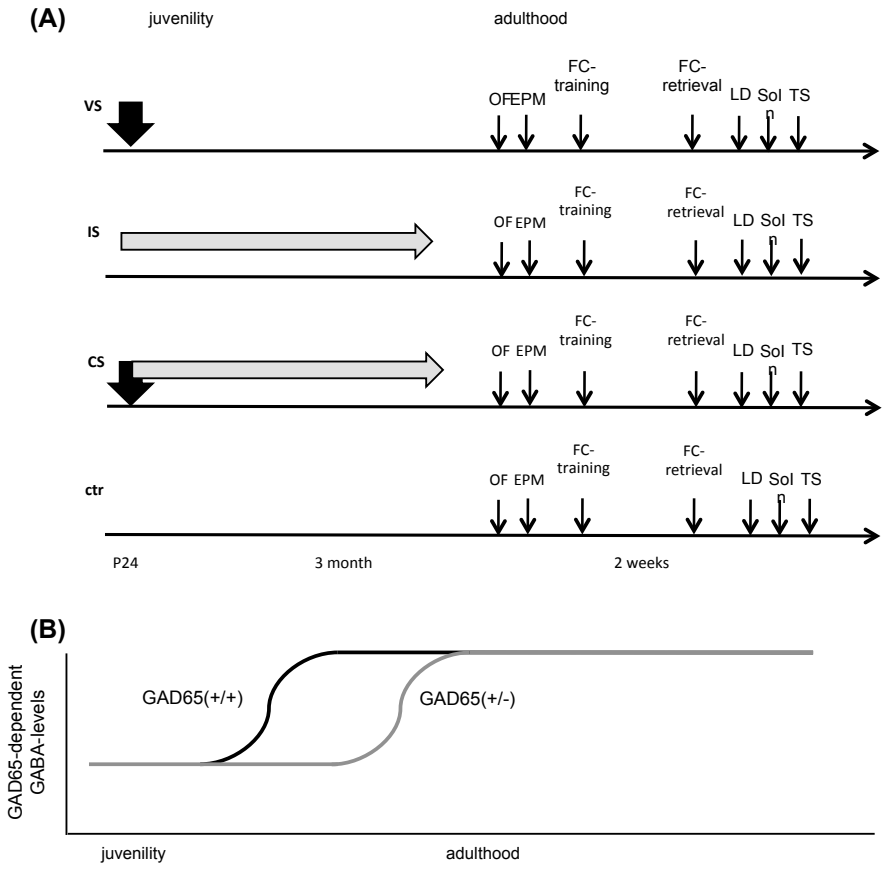

FIGURE 2.1: (A) Overview of stress regimen and the behavioral test battery in relation to (B) the postnatal maturation of the GABAergic system. VS: variable stress (black arrow), IS: isolation stress (grey arrow), CS: combined variable and isolation stress, ctr: control, OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SoIn: social interaction, TS: tail suspension.

below with one test per day. Order of the tests was chosen according to stress level of the tests and behavioral relevance for PTSD–like features, thus employing anxiety tests before and after fear conditioning.

2.2.2.1 Open field

In the open field (OF) test mice were placed in the center of a square arena (50 x 50 cm) and allowed to explore the new environment for 20 min in red light. Time in the center (25 x 25 cm) was recorded to assess anxiety and the distance the mice moved was tracked as a parameter of activity (ANY-maze^{TM} Video Tracking System, version 4.50, Stoelting Co., Wood Dale, USA).

2.2.2.2 Elevated plus maze

Mice were tested on the elevated plus maze (EPM) for 5 min under low light conditions (10 lux). Entries to open and closed arms were recorded as measures of anxiety (% open arm entries) and activity (total arm entries) using the ANY-maze^{TM} Video Tracking System [220, 221].

2.2.2.3 Fear conditioning

The fear conditioning apparatus consisted of a rectangular acrylic chamber with a metallic grid floor for shock delivery (TSE, Bad Homburg, Germany). The protocol used was established by Laxmie et al. (2003) and induces a specific fear response to the conditioned stimulus under normal circumstances [50]. Mice were confronted with 4 adaptation sessions on 2 days each containing 4 tone presentations (conditioned stimulus (CS)–: 2.5 kHz, 10 s, 80 dB SPL, ISI 20 s) followed by a training session on the next day with 3 tone/shock pairings (CS+: 9 s, 10 kHz, 80 dB SPL, 0.4 mA, 1 s; ISI 20 s). Two weeks later a retrieval in the neutral context (4 x CS–, 4 x CS+, 10 s, ISI 20 s) and one day later in the shock context (context) were performed. In all of these, stimulus presentations were preceded by a 2 min stimulus-free interval at the beginning and followed by the same at the end of the session. Thus 2 min intervals of CS–, CS+ and context were analyzed for freezing (complete immobility except for respiratory movements) and automatically recorded online as indicator for learning by a photo beam system (TSE, Bad Homburg, Germany).

2.2.2.4 Light/dark test

Animals were placed in the light compartment (100 lux) of a standard light/dark test and their behavior was recorded for 5 min using a photo beam system (TSE, Bad Homburg, Germany). The activity (movements at a velocity of more than 3 cm/s) in the light and dark compartments was recorded as a measurement of anxiety (% activity in the light) and activity (cumulative activity in light and dark [50, 216].

2.2.2.5 Social interaction

Social interaction was tested in a standard 3–compartment chamber (40 x 20 cm) with one circular containment tube (8 cm in diameter and with holes spaced 1 cm apart) in the outer compartments. In a 5min adaptation session mice were allowed to explore the empty chamber. Then a young male mouse was put in either cylinder and interactions with the tubes were manually scored. Time interacting with the partner mouse was normalized to total time exploring both tubes [222].

2.2.2.6 Tail suspension

Mice were suspended on a cylinder for 5 min with a tape wrapped around the tail. Time immobile was recorded manually as a measure for depression–like behavior [223].

2.2.3 Statistical analysis

Two–way ANOVAs (genotype and stress group) were performed followed by Fisher's LSD tests for *post–hoc* comparisons, if significant group effects or significant interaction effects were obtained. In cases of only significant group effects, LSD–tests were performed only within one genotype. In cases of only significant genotype effects, two–tailed t–tests were carried out. To avoid false positive effects and to ensure data homogeneity, outliers were identified using the Dean and Dixon test and excluded from analysis for the respective test. In addition I performed a factor analysis with Varimax rotation on all parameters evaluated in the test battery. Factor extraction was validated with a Quartimax rotation. To test genotype and stress group effects on whole factors, parameters were z–transformed to allow merging of measures with different units. Parameters that loaded high (above 0.5) on the same factor were averaged to form the indices, if necessary they were multiplied by (–1) before averaging for achieving the same polarity. The α-level was set to 0.05. Analysis was performed using SPSS 22 (IBM Deutschland GmbH, Ehningen, Germany).

2.3 Alterations in gene expression across development in GAD65 haplodeficient mice (study 2)

2.3.1 Tissue collection

Naive group housed GAD65 heterozygous and wild type control mice were sacrificed at three different developmental stages (P24, P56, P112). Animals were decapitated under isoflurane (Baxter GmbH, Unterschleissheim Germany) anesthesia, brains were transferred to tissue freezing medium (Leica Microsystems Nussloch GmbH; Germany) and snap-frozen in 2–methylbutane (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), cooled in liquid nitrogen. The whole procedure was carried out within 2 minutes to avoid RNA degradation. Brains were stored at -80 °C until laser capture microdissections.

2.3.2 Laser capture microdissections - preparations

2.3.2.1 Coating of polyethylene naphtalate slides

Glass slides with a polyethylene naphtalate membrane (PEN, Carl Zeiss, Jena, Germany) were used to improve laser cutting and tissue catapulting. For tissue adherence to the membrane, slides were coated with 0.05 % Poly-L-Lysine (PLL, Sigma-Aldrich, Seelze, Germany) for 30min at room temperature (RT) after membranes had been activated by irradiation for 30 min under ultraviolet (UV)–light. Excess PLL was then washed 3 x 5 min in 200 ml double–distilled water. Slides were air–dried over night. To minimize RNAse–contamination, slides were soaked in RNAse–Zap spray (Life Technologies, Darmstadt, Germany) for 2 min at RT and then washed 5 x 5 min in 200 ml dimethyl-dicarbonate (DMDC, Sigma-Aldrich, Seelze, Germany)–treated double–distilled water.

2.3.2.2 Cryosectioning

One day before cryosectioning, brains were transferred from –80 °C to –20 °C. Before tissue cutting, the cryostat (CM 1950, Leica, Nussloch, Germany) was

sterilized with UV–light for 30 min. During this time brains covered in aluminum foil were allowed to climatize to the cryostat (chamber temperature: –18 °C, block temperature: –16 °C). 20 μm thick coronal slices (bregma: –1.80) were collected on PLL–coated PEN slides. Every second slice was collected. Object slides were placed on a warming plate (40 °C) after every section and for additional 2 min after the last section.

2.3.2.3 Cresyl violet staining

For better identification of brain regions, slices were stained with cresyl violet immediately after cryosectioning. For this, slices were first fixed with –21 °C cold 70 % EtOH for 1 min and then stained in a 4 °C cold 1 % cresyl violet solution (dissolved in in 50 % EtOH) for 1 min. For dehydration, slices were transferred to 70 % EtOH for 2 min and then to 96 % EtOH for 2 min (both at 4 °C). To achieve RNAse–minimized conditions DMDC–treated double–distilled water was used to prepare solutions and glassware was baked for 3 h at 180 °C. Finally, sections were air–dried for 2–3 min on a warming plate, before laser capture micordissections were carried out.

2.3.3 Laser capture microdissections

For collection of dorsal hippocampal and amygdalar subregions I used the PALM MicroBeam laser capture microdissection system (Carl Zeiss Microscopy GmbH, Göttingen, Germany). In brief, a microscope and a camera were connected to a computer and regions of interest were defined on the digital image. A software controlled laser beam cut out these regions and catapulted the tissue into the adhesive cap of a capture device (Carl Zeiss Microscopy GmbH, Göttingen, Germany) above the slide [224, 225]. This way, approximately 1, 200, 000 μm^2 of DG, CA3, CA1 and BLA (bregma: –1.80) from both hemispheres were collected and lysed in 350 μl RLT lysis buffer (RNeasy Plus Micro kit, Qiagen, Hilden, Germany) with 0.01 % β mercaptoethanol (Serva Electrophoresis GmbH, Heidelberg, Germany). Subfields were not divided into layers with the exception of the DG, here the hilus was collected separately from the granule/molecular layer, because previous work indicated a specific role for the hilus in the cue/context balance in auditory fear conditioning experiments (Raza et al., in preparation). After a 30 min incubation

period at 4 °C samples were centrifuged for 5 min at 12 000 rpm to spin down lysates from the adhesive caps. Samples were stored at -80 °C until further processing. For an exemplary illustration of the BLA, before and after laser capture microdissections see figure 2.2

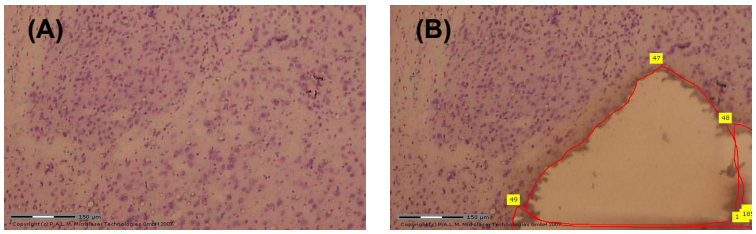

FIGURE 2.2: BLA (A) before and (B) after lasercapture microdissections.

2.3.4 RNA–Isolation

RNA-isolation was performed via a spin column system according to manufacturer's instructions with the RNeasy Plus Micro kit (Qiagen, Hilden, Germany). Lysates were transferred to gDNA eliminator columns and centrifuged for 30 s at 10 200 rpm to remove genomic DNA from the lysate. The flow through was then mixed with 350 μl 70 % EtOH for adjustment of binding conditions, transferred to MinElute Spin columns and centrifuged for 30 s at 10 200 rpm. The RNA was now bound to the column and washed in three different steps. Firstly, 700 μl RW1 buffer was added to the column and centrifuged for 30 s at 10 200 rpm. Secondly, 500 μl RPE buffer was added and again centrifuged for 30 s at 10 200 rpm. Finally, 500 μl 80 % EtOH was added to the column and centrifuged for 2 min at 10 200 rpm. After each washing step the flow through was discarded. To dry the membrane of the columns, they were centrifuged for 5 min at full speed with open lids. To finally elute the RNA, the columns were placed in new Eppendorf tubes and 14 μl RNAse–free water was added to the center of the membrane. After 1 min incubation at RT, columns were centrifuged for 1 min at full speed to obtain 12 μl of elute containing the RNA that was then stored at –80°C until reverse transcription.

2.3.5 Reverse transcription

For reverse transcription the Sensiscript kit from Qiagen (Hilden, Germany) was used. A master mix, additionally containing oligonucleotides and random decamers (each 50 μM, Life technologies, Darmstadt, Germany), and an RNAse inhibitor (SuperaseIN, 20 U/μl, Life technologies, Darmstadt, Germany) was prepared according to table 2.1. 16 μl master mix were distributed to 50 μl microfuge PCR tubes (Life Technologies GmbH, Darmstadt, Germany). 4 μl of RNA was added, briefly vortexed and centrifuged (<5 s). To control for possible contamination of genomic DNA, 16 μl mastermix without reverse transcriptase were prepared and 0.5 μl of 8 random RNA samples were added. The cDNA synthesis was conducted at 37 °C for one hour. Afterwards the reverse transcription product was diluted 1:5 in nuclease-free water and stored at –20 °C until further use for qPCR.

Table 2.1: Master mix for reverse transcription of RNA samples.

8 µl	RNAse-free water
2 µl	10x RT buffer
2 µl	dNTP Mix (5 mM each dNTP)
1 µl	Oligo-dTprimer (50 µM)
1 µl	Random Decamers (50 µM)
1 µl	SuperaseIN (20 U/µl)
1 µl	Reverse Transcriptase

2.3.6 Quantitative PCR

Expression of 4 stress–regulated GABAergic markers, GAD65, GAD67, $GABA_A$ receptor subunits α1 and 2 (Gabra1 and Gabra2) (assays obtained from Life Technologies, Darmstadt, Germany; for details on the assays see table A.4 in appendix) was measured in each isolated brain region. For qPCR a master mix according to table 2.2 was prepared and 8 μl were distributed to a 96 well plate (Life Technologies, Darmstadt, Germany). 2 μl sample DNA was added to the mastermix. Samples were measured in triplicates in the thermocycling profile shown in table 2.3. The house-keeping gene Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH, Life Technologies, Darmstadt, Germany) was used as endogenous control. All qPCRs were performed in a multiplex manner, except for GAD65, which

was measured as singleplex (with 2.5 μl H^2O instead of 2 μl). All qPCR–runs were performed with the ABI Prism Step One real time PCR apparatus (Life Technologies, Darmstadt Germany) and controlled with the Step One v2 software package. For each sample the delta critical threshold (dCT) was calculated by subtracting the CT (GAPDH) from CT (target gene). This was averaged over the triplicates to achieve mean dCT values. By further calculating the mean ddCT the heterozygous mice were normalized to wild type levels of the respective age group using the formular: ddCT (target) = mean dCT (target) - mean dCT (control group) [226]. The principle of qPCR is explained in appendix A.2.

Table 2.2: Master mix for qPCR with the housekeeping gene GAPDH as the internal control.

5 µl	Gene expression master mix
2 µl	Aqua dest.
0.5 µl	Assay 1 (“target”-FAM)
0.5 µl	Assay 2 (GAPDH-VIC)

Table 2.3: Thermocycling profile for qPCR.UNG: uracil-N-glycosylase

Phase	**Duration**	**Temperature**	**Number of repetitions**
Decontamination with UNG	2 min	50 °C	1
Initial denaturation	10 min	95 °C	1
Denaturation	15 s	95 °C	50
Elongation	1 min	60 °C	50

2.3.7 Statistical analysis

Two-tailed t-tests were carried out between genotypes with an α-level of 0.05. Analysis was done using SPSS 22 (IBM Deutschland GmbH, Ehningen, Germany).

2.4 Learning strategy selection in homozygous GAD65 knock out mice (study 3)

2.4.1 Paradigm

Adult male GAD65(+/+) (N = 10) and GAD65(–/–) mice (N = 9) were single caged 5 days before the experiment started. Moreover, they were starved to 85 % of initial bodyweight and accustomed to the food reward (Kellogg's Choco Krispies, Kellogg Company, USA). When the food reward was presented first in the home cage, the latency to first contact was measured as indicator for neophobia. The experiment took place in a 50 x 50 cm open field arena under low light conditions (10 lux).

In order to reduce basal anxiety, mice were familiarized to the empty open field for 20 minutes 1 hour before training started. For the dual–solution memory task, a food reward (Kellogg's Choco Krispies, Kellogg Company, USA) was presented in a cell culture dish (Greiner Bio-One GmbH, Frickenhausen,Germany) on positions 1–4 (see fig. 2.3). Only the food reward on one position was accessible, the covers of the dishes on the remaining positions had holes to prevent orientation via odor cues. The arena was equipped with 1 of 4 different pictures on each wall serving as distal cues to allow a hippocampus–dependent place strategy. To enable a striatum–dependent cue–guided strategy a wooden toy block next to the food reward served as the proximal cue. Training contained 6 trials á 4 minutes with 15 minutes inter–trial interval, during which mice remained in their home cages. To prevent an egocentric learning strategy mice were placed at alternating starting points. These positions were equidistant from the reward and the parameter "latency to eat" indicated learning. Of note, the baited position was balanced between mice to avoid a systematic bias caused by the setup itself, but the position of the toy block in the retrieval was always diagonal to that in the training.

The setup for the retrieval on the next day was similar, with the exception that dishes were empty and the toy block was placed at a new position. Mice were classified as "cue-", "place-", or "non-learners" based on the first position they searched for the food reward. 15 minutes thereafter, the retrieval was repeated to investigate strategy stability. Mice were grouped into "same strategy", "strategy switch" regardless of switching from place to cue or *vice versa*, or "other", when

none of the above applied. Figure 2.3 displays an overview of the experimental set up.

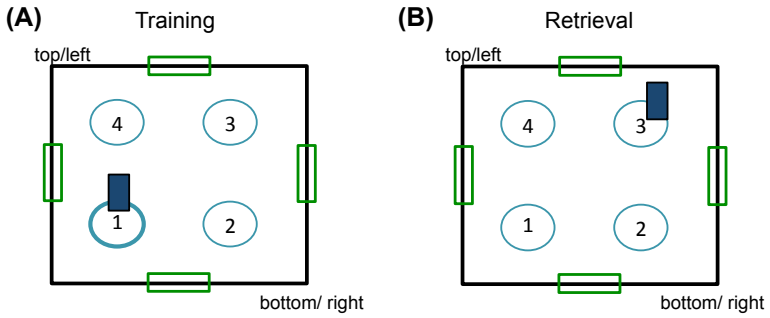

FIGURE 2.3: Setup overview of the dual solution memory task. (A) In the training, petri dishes at positions 1–4 were equipped with a food reward to avoid orientation via odor–cues. Only at one position the food reward was accessible. To enable a cue–based strategy a wooden toy block (blue rectangle) was placed right next to this reward. Moreover, pictures on the walls (green lined rectangles) allowed for a spatial strategy. (B) In the retrieval, the toy block was placed at a new position, and the position the mice visited first was the basis for classification. Here, all dishes were empty.

2.4.2 Statistical analysis

For "latency to eat", "distance" and "center time" two–tailed t–tests between genotypes were carried out. For the learning curve a repeated measures ANOVA with "trial" and "genotype" as independent variables was performed, if applicable Fisher's LSD test for *post–hoc* comparisons was applied. To compare the relative portions of learner type between the genotypes χ^2–tests were carried out. For this, percentages were calculated to account for the slightly unequal sample size. The α–level was set to 0.05 and SPSS 22 (IBM Deutschland GmbH, Ehningen, Germany) was used for analysis.

2.5 Memory formation and extinction depending on neural network homeostasis (study 4)

2.5.1 Paradigm

Adult, male Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ (N = 7), Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ (N = 9), and Hprt$^{3L\alpha185L+/0}$ (N = 10) mice were transported from the Max Delbrück Center for Molecular Medicine in Berlin (RNA Editing and Hyperexcitability Disorders, Jun. Prof. Jochen Meier) to our animal facility. In this Cre-loxP system Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ - and Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mice express the gain of function GlyR α3L^{185L} variant under the control of a parvalbumin– and Camk2a promoter respectively. Hprt$^{3L\alpha185L+/0}$ mice serve as the control group, since they are Cre–negative. This glycine receptor variant facilitates transmitter release and thereby increases the functional weight of the expressing cell type in the general network excitability [219].

At first mice underwent a hot plate test to reveal possible differences in pain threshold. For this, mice were put on a 55 °C warm plate enclosed by a transparent acrylic cylinder (11 cm in diameter) and latency to lick or shake hind paws was measured as indicator of pain. Mice were removed from the hot plate immediately after first occurrence of this behavior or after a maximal duration of 30 s to avoid tissue damage [227].

After a 3–week aclimatization period, during which mice were single caged and handled, fear conditioning started. To monitor learning and extinction curves, 3 consecutive days of contextual fear conditioning were followed by 5 days of extinction with one session per day. One training session contained a single electric food shock (1 s, 0.4 mA) preceded by 2 minutes without stimulus presentation and terminated by 30 seconds without stimulus presentation. An extinction session consisted of a 10 minute exposure to the fear conditioning chamber.

Auditory cued fear conditioning was carried out in a new batch of mice that was transported and familiarized to our animal facility like the first batch. To minimize the response to the shock chamber itself, mice were presented with 4 adaptation sessions (2 on 2 consecutive days á 6 minutes), in which no stimulus was presented. On the next day training comprised 3 tone/shock pairings (tone: 9 s, 10 kHz, 80 dB SPL; foot shock: 1 s, 0.4 mA) flanked by 2 minutes in the beginning and the end without stimulus presentation. Extinction training started

one day later and encompassed 20 tone presentations (10 s, 10 kHz, 80 dB SPL, ISI: 20 s) in a neutral context (a clean cage with fresh bedding similar to their home cages). Again, each session was flanked by 2 blank minutes in the beginning and the end. In total 5 extinction sessions were presented, one on each day. Based on the results from the preceding experiment, only Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ (N = 10) and Hprt$^{3L\alpha185L+/0}$ mice (N = 9) were used for the cued fear conditioning experiment.

2.5.2 Statistical analysis

For the hot plate test a one–way ANOVA was used. To dissect memory retrieval from adaptation that might occur in the course of the 10 minute extinction session, 2 minute intervals were analyzed and the first 2 minutes of each session were used for the between session extinction analysis. two–way repeated measures ANOVA was performed with "genotype" and "session" as the fixed factors. For *post–hoc* comparisons Fisher's LSD test was employed. The threshold for statistical significance was set to 0.05 and analysis was done with the help of SPSS 22 (IBM Deutschland GmbH, Ehningen, Germany).

Chapter 3

Results

3.1 Interaction of GAD65 haplodeficiency and juvenile stress (study 1)

3.1.1 Open field

For the distance traveled in the open field, a significant effect of genotype [TWA, $F(1, 80) = 8.463$, $p = 0.005$] and stress group became apparent [$F(3, 80) = 2.85$, $p = 0.043$]. In wild type mice each of the stressors induced a significant reduction in activity [variable stress (VS): $p = 0.038$, isolation stress (IS): $p = 0.006$, combined stress (CS): $p = 0.045$] compared to controls. These changes generally failed to reach significance inGAD65(+/–) mice and socially isolated GAD65(+/–) differed significantly from the corresponding group of GAD65(+/+) mice ($p = 0.006$). On the other hand, no significant effect on center exploration of the open field could be observed (Fig. 3.1).

3.1.2 Elevated plus maze

Concerning open arm exploration, significant genotype [$F(1, 75) = 4.562$, $p = 0.03$] and stress group effects [$F(3, 75) = 2.815$, $p = 0.045$] as well as genotype x stress group interaction [$F(3, 75) = 3.813$, $p = 0.013$] were observed. In GAD65(+/+) mice, enhancement of open arm exploration was observed in the VS–group ($p=0.046$) and the IS–group ($p = 0.001$), compared to control. Strikingly, the increase was

abolished in the combined CS–group (p = 0.006, compared to IS). In GAD65(+/–) mice, the IS–group failed to increase open arm exploration (p = 0.046, compared to VS) and differed from the behavior of the corresponding GAD65(+/+) mice (p = 0.001). No significant effects of genotype or treatment were evident with respect to total arm entries (Fig. 3.1).

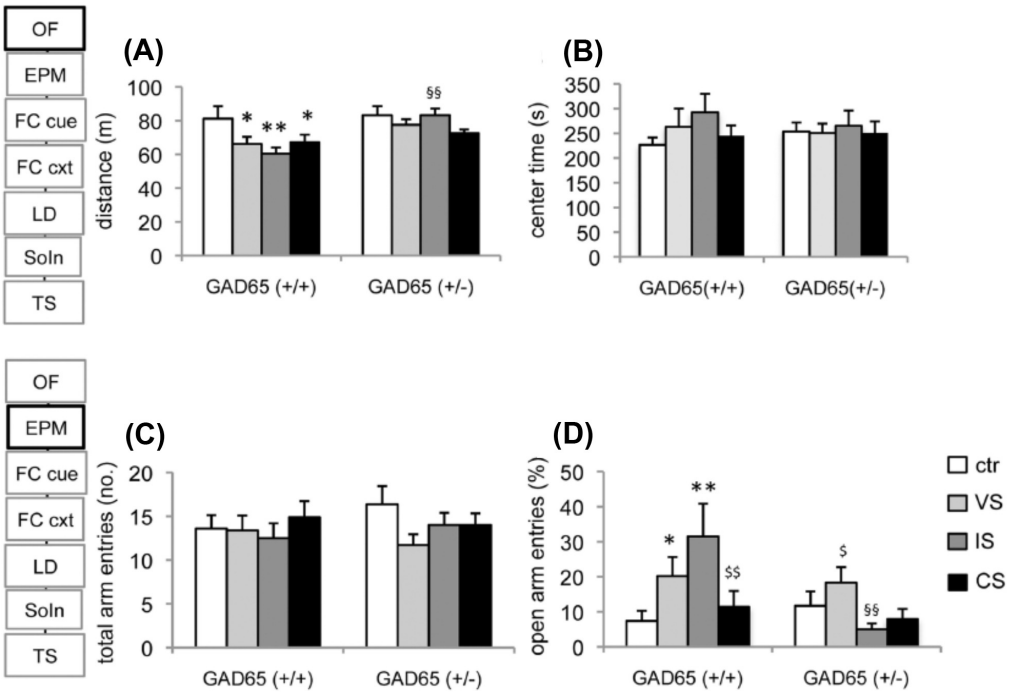

FIGURE 3.1: Open field and elevated plus maze. (A) All three stress procedures led to a significant reduction in activity in the open field in GAD65(+/+) but not in GAD65(+/–) mice in the OF. Socially isolated wild type mice differed significantly from their heterozygous littermates of the same group. (B) No behavioral alterations were observed with respect to the time spent in the center. (C) In the EPM no differences were observed in total arm entries in either genotype or stress group. (D) Heterogeneous results were obtained in the percentage of open arm entries. In GAD65(+/+) mice VS and IS increased open arm entries, but mice confronted with a combination of both stressors were indistinguishable from controls. In GAD65(+/–) mice social isolation reduced open arm exploration that reached significance when VS and IS mice were compared. ctr: unstressed control, VS: variable stress, IS: isolation stress, CS: combined variable and isolation stress; OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SoIn: social interaction test, TS: tail suspension. Data are mean + SEM. *$p < 0.05$, **$p < 0.01$ compared to ctr of the same genotype, $^{\S\S}p < 0.01$ compared to GAD65(+/+) of the same stress group, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ compared to IS of the same genotype.

3.1.3 Fear conditioning

The data reveal a significant stimulus (CS–, CS+, context) effect on memory retrieval [$F(1.775, 131.319) = 86.8$, $p < 0.001$], a significant main effect for stressor type [$F(3, 74) = 4, 698$, $p = 0.005$] and a significant interaction between memory type and stress group [$F(5.324, 131.319) = 3.786$, $p = 0.003$].

3.1.3.1 Within–group comparisons

In GAD65(+/+) mice, freezing to the CS+ was significantly higher than freezing to the CS– in all experimental groups (control: $p = 0.001$; VS: $p = 0.03$; IS and CS: $p < 0.001$). Freezing to the context was only elevated of CS– levels in the stress groups (VS: $p = 0.032$; IS: $p = 0.001$ and CS: $p < 0.001$), but not in the control group. Only the latter group displayed significantly increased freezing levels, when CS+ was compared to the context ($p = 0.001$).
In GAD65(+/–) mice, freezing to the CS+ was significantly higher than freezing to the CS– in all experimental groups (control: $p = 0.001$; VS, IS and CS: $p < 0.001$). Freezing to the context compared to the CS– was only elevated in groups IS ($p = 0.005$) and CS ($p = 0.001$), but neither in control mice nor in the VS– group. Freezing levels to the context compared to the CS+ reached significance in the VS ($p = 0.01$) and the VS+IS ($p = 0.016$) groups.

3.1.3.2 Between–group comparisons

In GAD65(+/+) mice all three stressors induced a contextual generalization compared to controls (control vs. VS: $p = 0.033$, control vs. IS: $p = 0.004$, control vs. CS: $p = 0.002$). In GAD65(+/–) however a significant difference was obtained only between the VS- and the IS–group ($p = 0.043$). The response to the CS+ and CS–, in contrast was largely similar between genotypes: In GAD65(+/+) mice, the IS–group and the CS–group showed increased freezing levels ($p = 0.009$ and $p = 0.049$, respectively) compared to VS. In GAD65(+/–) mice a similar increase was observed in these groups (IS vs. control: $p = 0.021$; CS vs. control: $p = 0.009$; CS vs. VS: $p = 0.049$). No significant difference was seen between groups concerning the response to the CS– (Fig. 3.2).

3.1.4 Light/dark test

A significant effect on the activity in the light compartment was observed for stress group [$F(3, 78) = 13.44$, $p < 0.001$], but no effect of genotype [$F(1, 78) = 0.303$, $p = 0.584$] or genotype x group interaction was observed [$F(3, 78) = 1.729$, $p = 0.168$]. *Post hoc* comparisons in GAD65(+/+) mice revealed significantly less activity in the light of the CS–group than of the control-group ($p = 0.003$). GAD65(+/–) mice displayed reduced activity in the light in the IS– and the CS–group ($p < 0.001$, compared to both control– and VS–groups). Moreover, an effect of stressor type was observed on the total activity in this test system [$F(3, 80) = 7.013$, $p < 0.001$]. A gradient was observed in GAD65(+/+) mice across stress groups, with a significant reduction of total activity in the CS–group ($p = 0.003$). GAD65(+/–) mice showed a reduced activity in the IS–group ($p = 0.056$ to control, $p = 0.013$ to VS) and in the CS–group ($p = 0.01$; $p = 0.002$) (Fig. 3.2).

3.1.5 Social interaction

Two-Way analysis of variance revealed a significant effect for the stress group [$F(3, 72) = 3.159$, $p = 0.03$], but no effect of genotype [$F(1, 72) = 0.175$, $p = 0.677$] or genotype x stressor interaction [$F(3, 72) = 0.51$, $p = 0.676$]. *Post hoc* comparisons failed to reach significance (Fig. 3.3).

3.1.6 Tail suspension

A significant stress group effect was also observed concerning immobility in the tail suspension test [Two-way ANOVA, $F(3, 73) = 4.43$, $p = 0.006$]. Pairwise comparison revealed a preferential effect on the social isolation group ($p = 0.003$ vs. control) in GAD65(+/–) mice (Fig. 3.3).

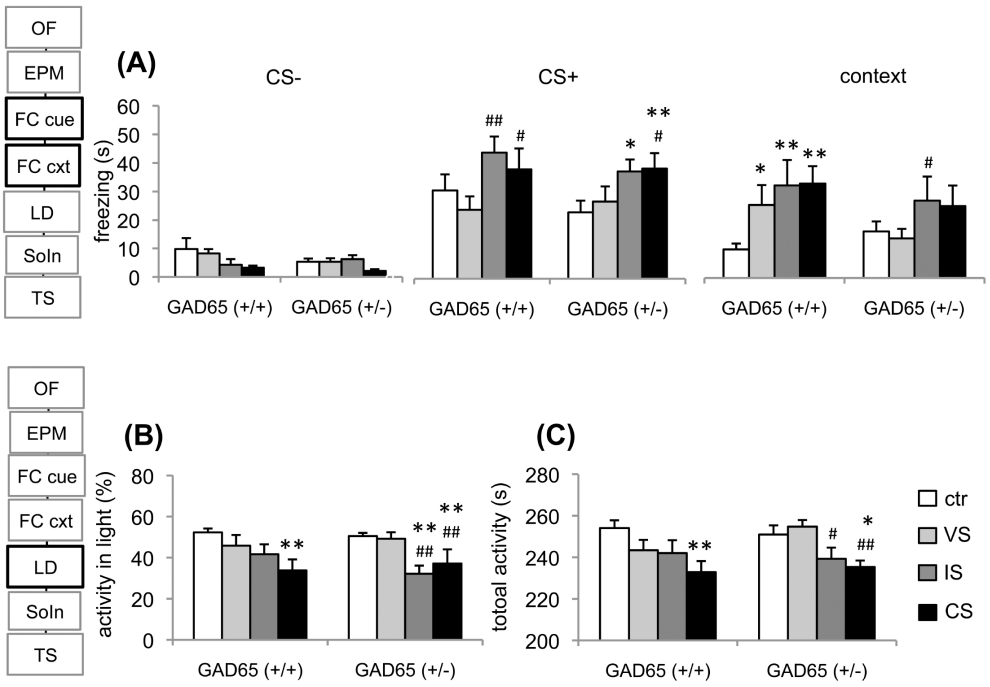

FIGURE 3.2: Conditioned fear and light/dark avoidance test. (A) Cued fear memory did not generalize to a neutral tone (CS–) in response to stress experience or genotype. Social isolation increased freezing to the conditioned tone (CS+) in both genotypes, reaching statistical significance when wild type IS and CS mice were compared to VS mice. IS and CS heterozygous mice differed significantly from the ctr–group and the CS–group also differed from VS mice. In GAD65(+/+) mice all three stress protocols lead to contextual fear generalization. In contrast, only the IS-group differed from the VS-group in GAD65 (+/–) mice. (B) In GAD65 wild type mice the three stress protocols lead to a gradual decrease in their activity in the light in the light/dark avoidance test, reaching significance in the CS–group. In GAD65(+/–) mice stressors that contained protracted social isolation significantly decreased activity in the light compartment compared to ctr and VS–mice. The latter are indistinguishable from unstressed controls. (C) A similar pattern arose with respect to total activity, with wild type mice showing a gradual reduction with stress severity and heterozygous mice displaying a strong dependency on the stressor type. ctr: unstressed control, VS: variable stress, IS: isolation stress, CS: combined variable and isolation stress; OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SoIn: social interaction test, TS: tail suspension. Data are mean + SEM. *p < 0.05, **p < 0.01 compared to ctr of same genotype, $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 compared to VS of the same genotype.

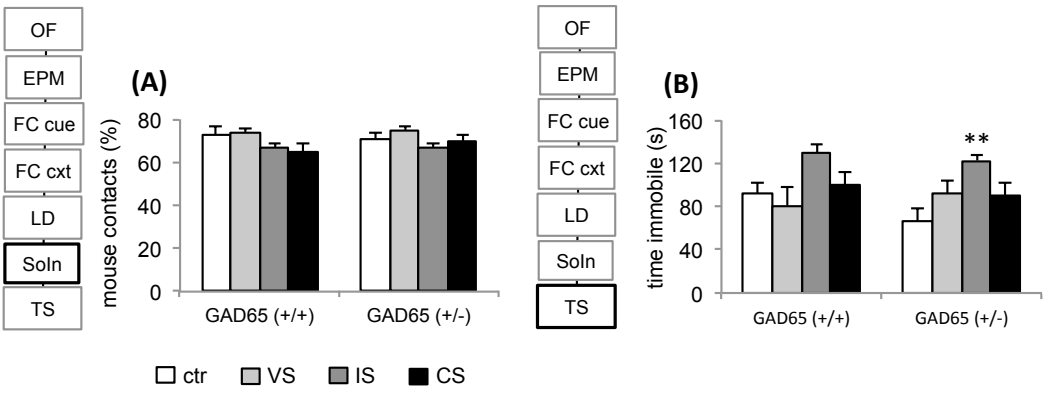

FIGURE 3.3: Social interaction and tail suspension test. (A) *Post hoc* comparisons for social interactions did not reveal group differences. (B) In both genotypes social isolation produced the strongest effects on depression–related behavior, reaching significance in GAD65(+/–) mice. ctr: unstressed control, VS: variable stress, IS: isolation stress, CS: combined variable and isolation stress; OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SoIn: social interaction test, TS: tail suspension. Data are mean + SEM. **p $<$ 0.01 compared to ctr.

3.1.7 Factor analysis

Factor analysis with Varimax rotation extracted five independent factors that together accounted for 70 % of total behavioral variance (Tab. A.5 in appendix). Factor separation was validated by repeating the factor analysis with Quartimax rotation (Tab. A.6 in appendix). An overview of the factor loadings after Varimax rotation of each parameter with the latent factor can be taken from table 3.1.

3.1.7.1 Factor 1: generalized contextual fear and anxiety

The first extracted factor accounts for 17.91 % of total variance (see Tab. A.5 in appendix) and is composed of contextual fear memory (freezing), anxiety (% activity in the light) and total activity in the LD–test (tab. 3.1). Z–transformed "activity in the light" and "total activity" values were multiplied by (–1) before being combined with contextual freezing, such that increased z would reflect increased fear/anxiety. A significant group effect [$F(3, 80) = 13.998$, $p < 0.001$], but no effect of genotype [$F(1, 80) = 0.44$, $p = 0.509$] or genotype x stressor interaction effects [$F(3, 80) = 2.08$, $p = 0.109$] were obtained. Thus *post hoc* LSD–tests were carried out individually for each genotype. For GAD65(+/+) mice, all three stress groups showed significantly increased scores compared to controls (VS: $p = 0.028$,

TABLE 3.1: Factor loadings of the analyzed parameters for every factor extracted by factor analysis. The first factor is represented by context freezing and post-conditioning activity- and anxiety measures. Pre-conditioning activity and anxiety were shown to be independent from each other (factors 2 and 3). Social interaction and depression measures loaded on the same factor (factor 4). Cue–related fear memory displayed high correlations to the fifth factor. Factor loadings above 0.5 are considered high loadings and highlighted in bold. Extraction Method: Principal Component Analysis. Rotation Method: Varimax with Kaiser Normalization, rotation converged in 8 iterations. OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SI: social interaction test, TS: tail suspension.

VARIMAX-rotation	Rotated component matrix				
	1	2	3	4	5
OF: distance (m)	0.093	**0.867**	−0.114	−0.078	−0.053
OF: center time (s)	−0.129	0.011	**0.807**	−0.083	0.004
EPM: total arm entries	0.059	**0.833**	0.026	−0.061	0.083
EPM: % open arm entries	0.39	−0.058	**0.656**	0.212	−0.117
FC: shock context_freezing (s)	**−0.506**	−0.242	0.424	0.266	0.07
FC: CS− freezing (s)	0.028	0.089	−0.127	0.08	**0.874**
FC: CS+ freezing (s)	−0.42	−0.23	0.324	−0.072	**0.515**
LD: % activity in light	**0.865**	−0.124	−0.039	−0.202	0.097
LD: total activity (s)	**0.735**	0.327	0.164	0.102	−0.111
SI: % time of mouse contacts	−0.034	0.052	−0.038	**−0.817**	−0.285
TS: time immobile (s)	−0.236	−0.112	0.027	**0.679**	−0.365

IS: $p = 0.006$, CS: $p < 0.001$). For GAD65(+/–) mice, however, the VS–group was indistinguishable from controls ($p = 0.653$), while the other two stress groups showed increased scores compared to both control– and VS–groups (IS vs. control: $p = 0.001$, CS vs. control: $p = 0.001$, IS vs. VS: $p < 0.001$, CS vs. VS: $p < 0.001$) (Fig. 3.4). This analysis confirms the gradually increased response depending on the stress intensity in wild type mice and a bisectioning of the response in heterozygous mice depending on social interaction with littermates.

3.1.7.2 Factor 2: pre–conditioning activity

The second extracted factor comprised measures for activity collected in the open field and elevated plus maze before the fear conditioning in adulthood. A significant effect was observed only for the genotype [$F(1, 81) = 4.303$, $p = 0.041$], whereas the stressors led to a uniform, but not significant reduction in activity in

GAD65(+/+) mice. Only social isolation induced a significant effect [t(15) = –2.227, p = 0.042], reducing activity in wild type mice, but not in heterozygotes (Fig. 3.4).

3.1.7.3 Factor 3: pre–conditioning anxiety

The third extracted factor comprised anxiety–related behaviors collected in the open field (center time) and elevated plus maze (open arm entries) before the fear conditioning in adulthood. The overall effects in this factor are in line with the observations in the elevated plus maze, with a significant effect of stress group [F(3, 79) = 3.592, p = 0.014]. *Post hoc* comparisons revealed a significant difference from control mice for the VS– (p = 0.04) and the IS–group (p = 0.01) of wild type mice. Moreover, GAD65(+/+) IS–mice differed from CS–mice (p = 0.043) (Fig. 3.4).

3.1.7.4 Factor 4: depression-like behavior

The fourth extracted factor comprised measures of social withdrawal and depression in the social interaction and tail suspension tasks. A significant group effect was observed [F(3, 80) = 5.586, p = 0.002]. In GAD65(+/+) mice the IS group with an increased depression score differed significantly from controls (p = 0.026) and VS–mice (p = 0.013). The CS–mice showed less of an increase, but still differed from VS (p = 0.036). GAD65(+/–) mice showed similar trends, but failed to reach significance (not shown).

3.1.7.5 Factor 5: cued fear memory

The fifth extracted factor includes both cue–specific and generalized auditory fear memory. The combined factor revealed no statistically significant impact of genotype or stress exposure [genotype: F(1, 77) = 2.938, p = 0.091; stressor type: F(3, 77) = 0.607, p = 0.613; genotype x stressor type: F(3, 77) = 1.083, p = 0.361] (not shown).

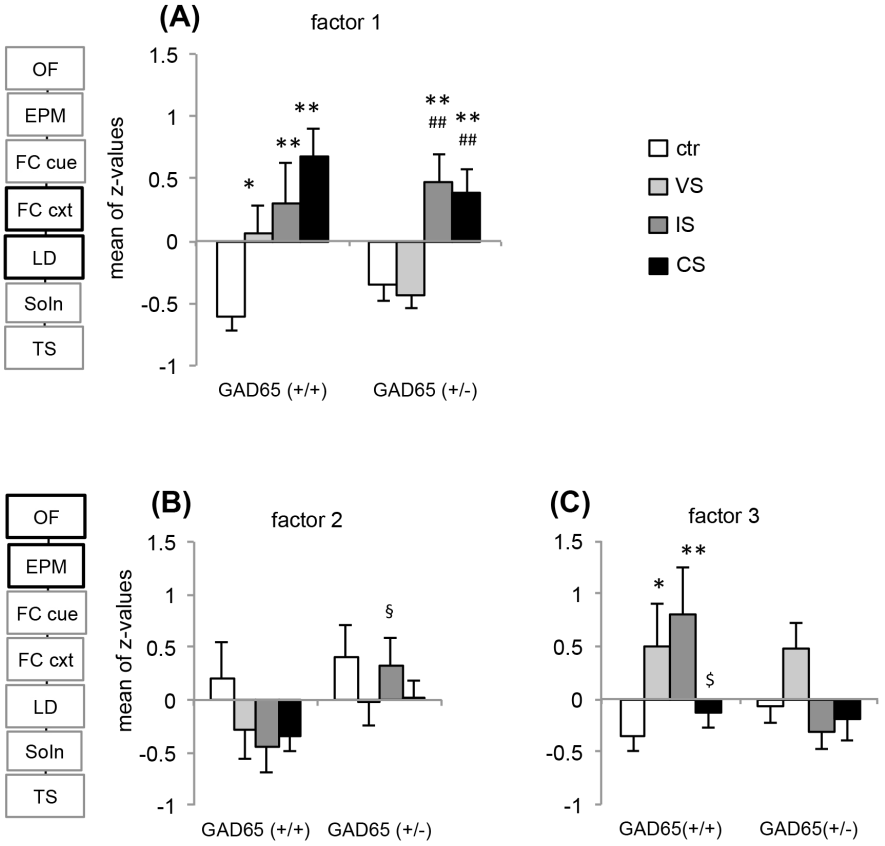

FIGURE 3.4: Identified behavioral domains. (A) The strongest factor extracted "generalized fear and anxiety", containing contextual fear memory, post–conditioning anxiety and activity was differentially affected by the genotype and stress experience. In GAD65(+/+) mice any of the stress protocols induced PTSD–related symptoms. Heterozygous VS–mice fully recovered from juvenile stress experience and show increased behavioral responsiveness only after IS or CS. (B) The second extracted factor "preconditioning activity" combined distance (OF) and total arm entries (EPM) and revealed a social isolation–induced reduction in activity in wild type compared to heterozygous mice. (C) The third extracted factor, "preconditioning anxiety" combined center time (OF) and % open arm entries (EPM) and confirmed the different responsiveness of GAD65(+/+) mice to each of the stressors alone and a combination of both. ctr: unstressed control, VS: variable stress, IS: isolation stress, CS: combined variable and isolation stress; OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SoIn: social interaction test, TS: tail suspension. *$p < 0.05$, **$p < 0.01$ compared to ctr of the same genotype, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to VS of the same genotype, $^{\S}p < 0.05$ compared to GAD65(+/+) of the same stress group, $^{\$}p < 0.05$ compared to IS of the same genotype.

3.2 Alterations in gene expression across development in GAD65 haplodeficient mice (study 2)

3.2.1 Gene expression differences at P24

T-tests between genotypes were carried out and statistical details are presented in tables A.7 – A.21 in the appendix. At juvenile age significant genotype differences appeared only in the DG and hilus. GAD67 in dentate granule/molecular layer were significantly upregulated (p = 0.030) and hilar GAD65 and $GABA_A$ receptor $\alpha 1$ subunit (Gabra1) were reduced (p = 0.001 for GAD65 and p = 0.008 for Gabra1) compared to wild type littermates. Moreover, GAD67 was tendentially reduced in the BLA (p = 0.072) (Tab. 3.2).

TABLE 3.2: Gene expression differences at P24 of GAD65(+/–) mice compared to wild type littermates of the same age. n.s.: not significant; black arrows indicate significant in- or decreases; grey arrows indicate only tendencies (i.e. $p < 0.1$)

P24	BLA	DG	Hilus	CA3	CA1
GAD65	n.s.	n.s.	↓(p=0.001)	n.s.	n.s.
GAD67	↓(p=0.072)	↑(p=0.030)	n.s.	n.s.	n.s.
Gabra1	n.s.	n.s.	↓(p=0.008)	n.s.	n.s.
Gabra2	n.s.	n.s.	n.s.	n.s.	n.s.

GAD65(+/-) mice compared to GAD65(+/+) mice of the same age

3.2.2 Gene expression differences at P56

At P56 GAD65 was reduced in the amygdala (p = 0.026) and a tendency thereof arose in the hilus (p = 0.060). GAD67–mRNA levels instead were increased in the hilus (p = 0.036) (Tab. 3.3).

TABLE 3.3: Gene expression differences at P56 of GAD65(+/–) mice compared to wild type littermates of the same age. n.s.: not significant; black arrows indicate significant in- or decreases; grey arrows indicate only tendencies (i.e. $p < 0.1$)

P56	BLA	DG	Hilus	CA3	CA1
GAD65	↓(p=0.026)	n.s.	↓(p=0.060)	n.s.	n.s.
GAD67	n.s.	n.s.	↑(p=0.036)	n.s.	n.s.
Gabra1	n.s.	n.s.	n.s.	n.s.	n.s.
Gabra2	n.s.	n.s.	n.s.	n.s.	n.s.

GAD65(+/-) mice compared to GAD65(+/+) mice of the same age

3.2.3 Gene expression differences at P112

In later adulthood, effects were more prominent and widespread. A GAD65–deficit developed in all hippocampal subregions except for the DG (hilus: $p = 0.0007$, CA3: $p = 0.005$, CA1: $p = 0.002$). Gabra1 was downregulated in the BLA ($p = 0.043$) and the DG ($p = 0.027$). For $GABA_A$ receptor $\alpha 2$ subunit (Gabra2) only a tendency for a reduction was observed in the DG ($p = 0.059$) and the CA3 ($p = 0.092$) (Tab. 3.4).

TABLE 3.4: Gene expression differences at P112 of GAD65(+/–) mice compared to wild type littermates of the same age. n.s.: not significant; black arrows indicate significant in- or decreases; grey arrows indicate only tendencies (i.e. $p < 0.1$)

P112	BLA	DG	Hilus	CA3	CA1
GAD65	n.s.	n.s.	↓(p=0.0007)	↓(p=0.005)	↓(p=0.002)
GAD67	n.s.	n.s.	n.s.	n.s.	n.s.
Gabra1	↓(p=0.043)	↓(p=0.027)	n.s.	n.s.	n.s.
Gabra2	n.s.	↓(p=0.059)	n.s.	↓(p=0.092)	n.s.

GAD65(+/-) mice compared to GAD65(+/+) mice of the same age

3.3 Learning strategy selection in homozygous GAD65 knock out mice (study 3)

3.3.1 Baseline and learning curve

Both genotypes displayed equal latencies to first contact to the unfamiliar food reward (t(17) = –0.125, p = 0.902), as well as distance traveled (t(17) = –0.035, p = 0.0972) in the OF and equal time in the center (t(17) = –0.693, p = 0.498 (Fig. 3.5).

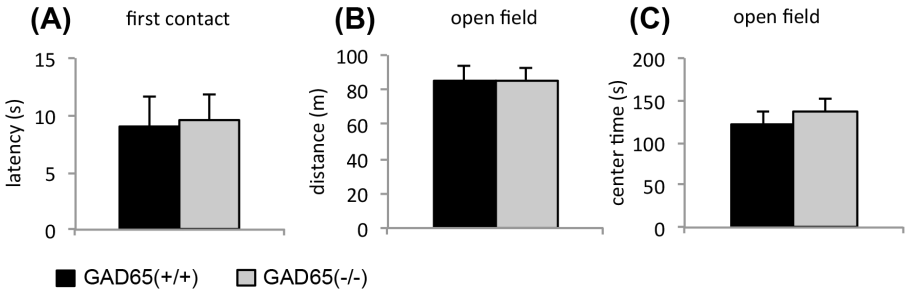

FIGURE 3.5: Latency to first contact the food reward and open field. (A) When the food reward was presented for the first time, both genotypes displayed similar latencies to explore it. Likewise, baseline (B) activity and (C) anxiety were comparable between genotypes, before training started. Data are mean + SEM

Repeated–measures ANOVA for latency to eat revealed a significant effect for trial [Greenhouse Geisser: F(2.789, 44.62) = 38.808, p < 0.001], but not for genotype [F(1, 16) = 2.078, p = 0.169] or for a trial x genotype interaction [F(2.789, 44.62) = 0.159, p = 0.912]. *Post hoc* analysis indicated faster learning in wild type mice (Fisher's LSD: trial 1 vs. trial 2, 3, 4, 5, 6: p < 0.001, trial 2 vs. trial 3: p = 0.015, trial 2 vs. trial 4: p = 0.021, trial 2 vs. trial 5: p = 0.012, trial 2 vs. trial 6: p = 0.001) than in GAD65(–/–) knock out mice (trial 1 vs. trial 2: p = 0.008, trial 1 vs. trial 3, 4, 5, 6: p < 0.001, trial 2 vs. trial 6: p = 0.031) (Fig. 3.6).

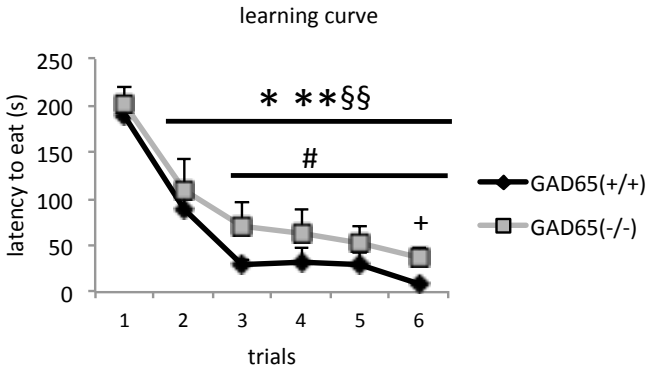

FIGURE 3.6: Learning curve. Both genotypes acquire the task in the course of the training. However, the learning curve in GAD65(+/+) mice is slightly steeper than in GAD65(–/–) mice. *$p < 0.05$ GAD65(+/+) compared to trial 1, $^{\#}p < 0.05$ GAD65(+/+) compared to trial 2, $^{\S}p < 0.05$ GAD65(–/–) compared to trial 1, $^{+}p < 0.05$ GAD65(–/–) compared to trial 2.

3.3.2 Distribution of learner type and cognitive flexibility

χ^2–analysis for first position visited in the first retrieval revealed that the distribution of strategy employment differed significantly between genotypes [χ^2 (2) = 101.000; $p < 0.001$]. 80 % of wild type mice used the, under the present conditions expected [64] place strategy to memorize the position of the food reward, and only 20 % employed a cue–based strategy. In contrast, the majority of GAD65(–/–) mice were classified as cue–learners (44.44 %), followed by place learners (33.33 %) and a portion of mice that did not first visit either of the two positions (non-learners, 22.22 %) (Fig. 3.7).

When returned to the arena 15 minutes later, χ^2–analysis confirmed a significant difference in separation into the three categories between genotypes [$\chi^2(2) = 60.500$, $p < 0.001$]. Among wild type mice 70 % used the same category, 20 % switched to the respective other strategy and 10 % fell into the "other" category. For GAD65(–/–) mice this portions were 44.44 %, 33.33 % and 22.22% (Fig. 3.8)

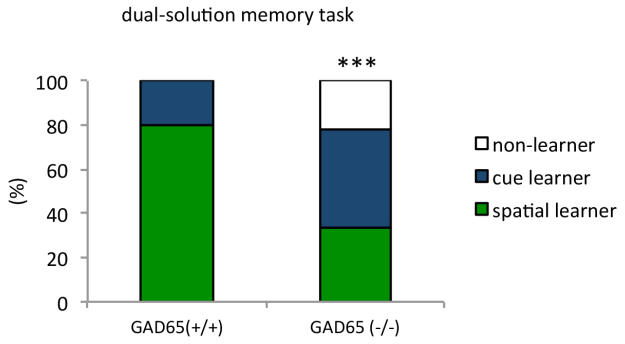

FIGURE 3.7: Classification of strategy employment in the first retrieval. The proportion of mice using a cue- or a spatial strategy differed significantly between the genotypes. GAD65(+/+) mice preferentially used a spatial strategy. In contrast, the majority of GAD65(–/–) mice employed a cue-based strategy. ***p < 0.001

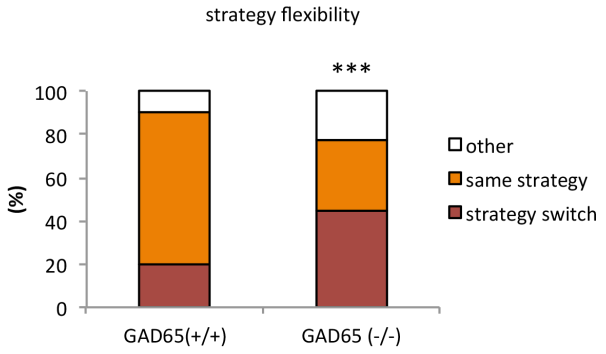

FIGURE 3.8: Cognitive flexibility in the second retrieval. Unlike expectations the proportion of mice that switched from one to the other strategy between the first and the second retrieval was reduced in GAD65(+/+) mice compared to GAD65(–/–) mice. ***p < 0.001

3.4 Memory formation and extinction depending on neural network homeostasis (study 4)

3.4.1 Baseline

Initially, a comparable pain threshold in all three genotypes was confirmed in the hot plate test [F(2, 23) = 0.346, p = 0.711] (Fig. 3.9 A). One-way ANOVA for % activity in the first 2 min of the first training session reached significance [F(2, 23) = 8.839, p = 0.001] and pair-wise comparisons rendered Hprt$^{3L\alpha185L+/0}$;

Pvalb$^{Cre+/-}$ mice more active than the other two genotypes at baseline (Fisher's LSD–test: Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$: p = 0.001, Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$: p = 0.001) (Fig. 3.9 B). However, pre–training incidence of freezing duration and number of freezing bouts at T1 were not different between genotypes, ruling out *a priori* differences in these behavioural parameters (Fig. 3.10).

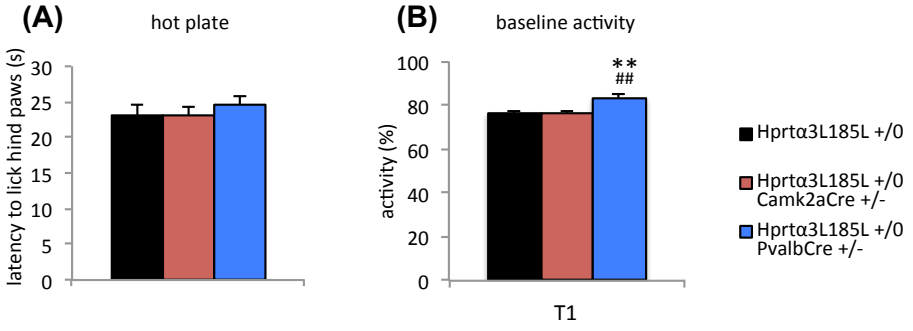

FIGURE 3.9: Hot plate and baseline activity. (A) Pain thresholds were comparable between genotypes. (B) Activity at T1 was increased in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice compared to the other groups. T1 = training 1. **p < 0.01 Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$, ##p < 0.01 Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$.

3.4.2 Contextual fear conditioning and extinction

3.4.2.1 Freezing duration

To test fear memory performance, the development of freezing duration during the first 2 min of each training and extinction session was compared between genotypes. In the repeated measures ANOVA a significant session effect [Greenhouse-Geisser: F(4.652, 106.985) = 58.714, p < 0.001] and an interaction of session and genotype [F(9.303, 106.985) = 3.161, p = 0.002] became evident, but no significant main effect of genotype [F(2, 23) = 1.491, p = 0.246] arose. *Post hoc* comparisons revealed an increase of freezing duration in all three genotypes during training (T1 vs. T3: Hprt$^{3L\alpha185L+/0}$: p < 0.001; Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$: p < 0.001, Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$: p < 0.001) and a decrease in all groups during extinction (R/E1 vs. E5: Hprt$^{3L\alpha185L+/0}$: p = 0.001; Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$: p < 0.001; Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$: p < 0.001). However, genotype differences were evident during both training and extinction session. Although Hprt$^{3L\alpha185L+/0}$;

Pvalb$^{Cre+/-}$ mice showed reduced freezing on the second training day compared to Hprt$^{3L\alpha185L+/0}$ control mice ($p = 0.018$), after completion of training on R/E1 performance was comparable between genotypes. During subsequent extinction sessions, Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice displayed higher freezing levels compared to both Hprt$^{3L\alpha185L+/0}$ controls (E2: $p = 0.028$, E3: $p = 0.026$) and Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mutants (E2: $p = 0.002$; E4: $p = 0.017$) (Fig. 3.10A).

3.4.2.2 Freezing bouts

Altered performance of Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice was also evident in the number of freezing bouts. Here, a significant session effect [repeated measures ANOVA, Greenhouse-Geisser: $F(5.36, 123) = 58.708$, $p < 0.001$] and a significant session x genotype interaction [$F(10.7, 123) = 4.313$, $p < 0.001$] but no significant genotype effect [$F(2, 23) = 2.534$, $p = 0.101$] appeared. Training significantly increased the number of freezing bouts in all genotypes (T1 vs. T3 in all groups: $p < 0.001$). Freezing bouts then decreased over the course of extinction in Hprt$^{3L\alpha185L+/0}$ and Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mice, reaching significance in E3 and E4 for the Hprt$^{3L\alpha185L+/0}$ group (R/E1 vs. E3: $p = 0.014$, R/E1 vs. E4: $p = 0.031$) and in E2–E5 for Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mice (R/E1 vs. E2: $p = 0.003$, R/E1 vs. E3: $p = 0.018$, R/E1 vs. E4: $p = 0.003$, R/E1 vs. E5: $p = 0.008$). However, a significant reduction was not observed in the Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice, demonstrating a deficit in fear extinction in this genotype. Comparison of freezing bouts between genotypes indeed confirmed a reduction on T2 ($p = 0.034$), and an increase on R/E1–E5 ($p = 0.024$) compared to Hprt$^{3L\alpha185L+/0}$ controls as well as on E2–E5 compared the other genotypes (Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice vs. Hprt$^{3L\alpha185L+/0}$ mice: E2: $p = 0.001$, E3: $p = 0.001$, E4: $p = 0.026$, E5: $p = 0.043$; Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice vs. Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mice: E2: $p < 0.001$, E3: $p = 0.012$, E4: $p = 0.022$, E5: $p = 0.015$) (Fig. 3.10B). The development of the fear response during a single extinction training is evaluated by a bin-by-bin analysis within each session in appendix A.6.

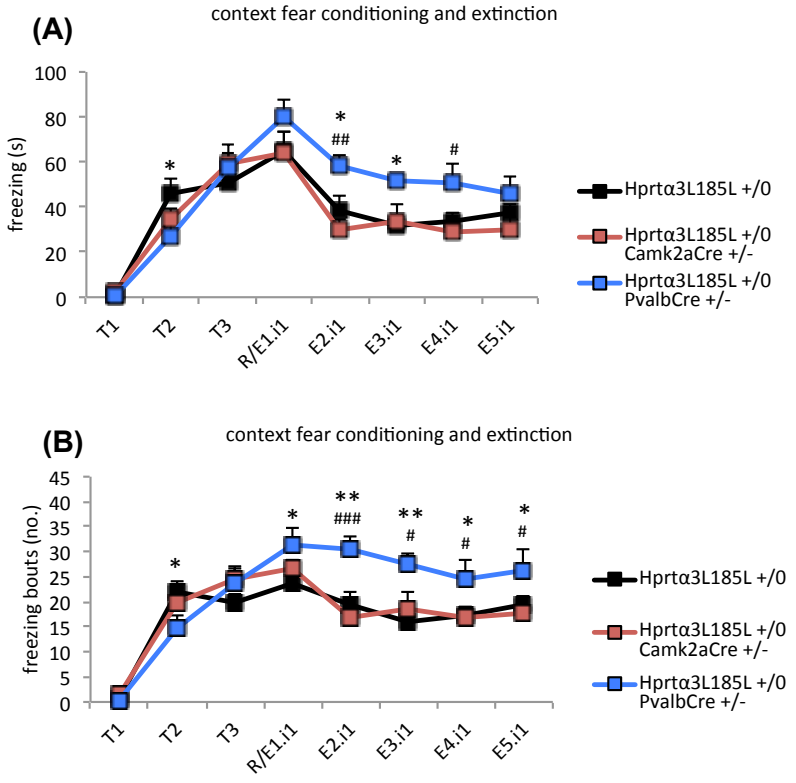

FIGURE 3.10: Learning and extinction curve of context fear conditioning. (A) Freezing duration and (B) number of freezing bouts rose during fear training and declined during extinction. This decline was diminished in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice. T1–3 = training 1–3, R/E1 = retrieval/extinction 1, E2–E5 = extinction 2–5, i1 = interval 1. *p < 0.05, **p < 0.01 Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$, #p < 0.05, ##p < 0.01, ###p < 0.001 Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ vs. Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$

3.4.3 Cue fear extinction

Mice responded similar to the first shock context presentation before training (freezing duration: Hprt$^{3L\alpha185L+/0}$ mice: 2.67 s (0.73), Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 3.30 s (0.83), t-test: t(17) = –0.568, p = 0.577; freezing bouts: Hprt$^{3L\alpha185L+/0}$ mice: 1.56 (0.44), Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 2.7 (0.65), t-test: t(17) = –1.42, p = 0.174), as well as immediately thereafter (freezing duration: Hprt$^{3L\alpha185L+/0}$ mice: 63 s (7.72), Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 78.5 s (7.16) t-test: t(17) = –1.473, p = 0.159; freezing bouts: Hprt$^{3L\alpha185L+/0}$ mice: 26.67 (1.67), Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 21.9 (2.32), t-test: t(17) = 1.634, p = 0.121), and to the first presentation of the neutral context before extinction (freezing duration: Hprt$^{3L\alpha185L+/0}$ mice: 20.45 s (8.85), Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 11 s (2.78), t-test: t(17) = 0.705, p = 0.49; freezing bouts: Hprt$^{3L\alpha185L+/0}$ mice: 10 (3.5),

Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice: 7.3 (1.81), t-test: t(17) = –0.41, p = 0.687). Both genotypes displayed a significant decline of freezing over the course of the five extinction sessions (repeated measurement ANOVA for freezing duration: session effect: F(4, 68) = 5.973, p < 0.001, session x genotype effect: F(4, 68) = 0.949, p = 0.441, genotype effect: F(1, 17) = 0.024, p = 0.878; repeated measures ANOVA for number of freezing bouts: session effect: F(4, 68) = 4.503, p = 0.003, session x genotype effect: F(4, 68) = 0.52, p = 0.722, genotype: F(1, 17) = 0.058, p = 0.813) (Fig. 3.11).

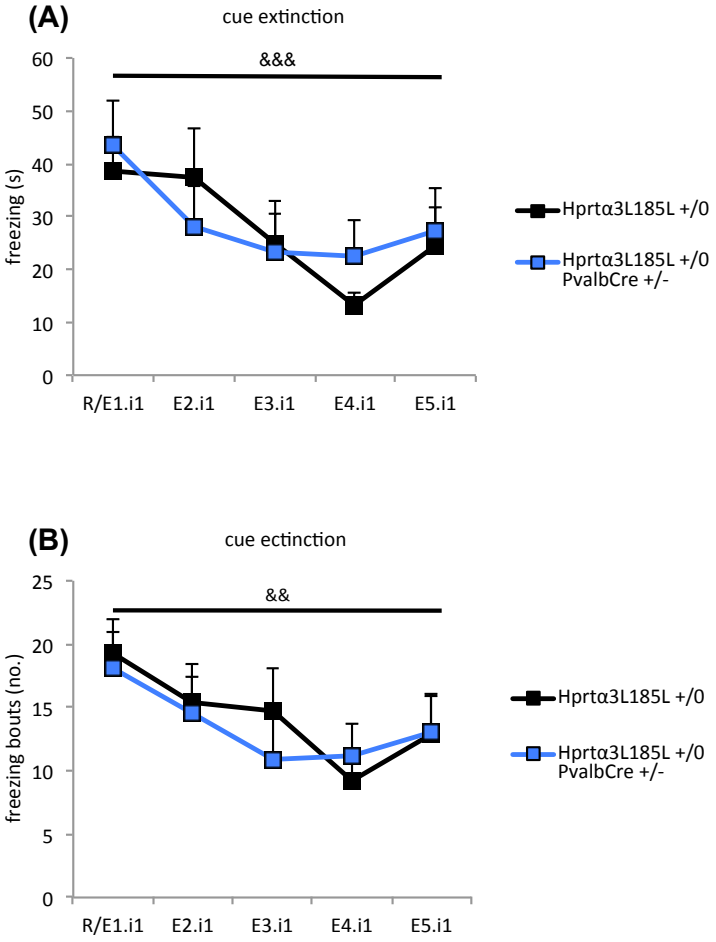

FIGURE 3.11: Extinction curve of cued fear conditioning. (A) Freezing duration and (B) number of freezing bouts declined similarly during extinction training. R/E1 = retrieval/extinction 1, E2–E5 = extinction 2–5, i1 = interval 1. $^{\&\&}p < 0.01$ session effect

Chapter 4

Discussion

4.1 Interaction of GAD65 haplodeficiency and juvenile stress

In the first and main study of my thesis, I investigated the interaction of different juvenile stress regimen and the maturation of the GABAergic system in GAD65 heterozygous knock out and wild type mice. The outcome was evaluated in a behavioral test battery comprising all behavioral domains typically affected by traumatic stress. Auditory fear conditioning served as a second hit in adulthood and elicited a protective effect of GAD65 haplodeficiency as the main finding.
Severe stress impacts an organism on multiple levels, and has consequences on the biological, behavioral, cognitive, and emotional state. Similarly complex is the clinical picture of resulting psychiatric disorders, like PTSD. Symptoms include memory– and mood disturbances, anxiety and social withdrawal [1]. Moreover, one or the other symptom cluster is more or less pronounced in the individual patient. To determine the mutant's response to traumatic stress I, did not only apply different behavioral tests, covering the majority of clinically relevant behavioral domains, but I also performed a factor analysis to condense the information gained. This analysis extracted five independent factors that together accounted for more than 70 % of the total behavioral variability. They have been validated by another extraction method (see appendix A.3) and are in line with confirmed behavioral measures. For instance, open arm entries in the elevated plus maze and time spent in the center of an open field are generally accepted to reflect

unconditioned anxiety. Both parameters loaded onto the same factor, called "pre–conditioning anxiety". Stressor– and genotype effects were investigated using both, the single parameters of each test and the combined indices calculated from the extracted factors. Both approaches confirmed significant genotype differences in PTSD–relevant behaviors.

The strongest behavioral component extracted from the data (factor 1), comprised background context fear and post–conditioning anxiety and exploratory activity. Disturbances in these parameters have been described in other rodent models of PTSD as well [58, 70, 221, 228, 229]. Moreover, stress–induced unconditioned anxiety and conditioned fear co–occur in the same animal [101]. Of note, only anxiety– and activity measurements collected after the second challenge in adulthood loaded onto the first factor. This supports other findings, according to which a combination of juvenile and adult trauma is most potent to mimic behavioral manifestations of PTSD [70, 84].

Also, a gradual increase in this factor depending on stress severity arose, thus fulfilling an important quality criterion of rodent PTSD models [45]. In accordance with previous studies [134, 146], unstressed control mice of both genotypes were indistinguishable from each other, thus ruling out *a priori* differences. However, within–genotype comparison revealed that GAD65(+/–) mice recovered from the variable stress regimen, since they behaved like unstressed control mice. In opposition to that wild type mice were equally affected by all stress regimen. This is in sharp contrast to the increased susceptibility for PTSD–like behavior of homozygous GAD65 mutants, which display increased anxiety, cue–specific fear generalization, and extinction deficits [134, 146, 206]. Moreover, in GAD65(–/–) mice fear expression in response to the cue is shifted towards flight attempts at the expense of freezing [205, 208]. However, the contextually generalized fear in the present study clearly segregated from cued fear memory and was not related to altered flight responses (see A.4 in appendix). Thus, the protective effect of GAD65 haplodeficiency appears to be fundamentally different from the phenotype of homozygous mutants.

It is likely that the delayed GABAergic maturation in GAD65(+/–) mice [134] may interfere with adaptive changes of this system that are induced by the variable juvenile stress [152, 230]. However, resilience was not observable in the paradigms preventing social contacts. One possible explanation for this discrepancy is the different duration of these two protocols. VS is completed before the GABA–deficit develops, but the social isolation lasts throughout postnatal GABA–development.

From a different perspective, one can argue that social interactions are necessary for GAD65 haplodeficiency to exert its protective effect. Although pre–existing differences in emotionality, like depression and anxiety between heterozygous GAD65 knock out mice and wild type littermates do not exist, mutants exert significantly fewer attacks and slightly increased grooming behavior in the male intruder aggression test [134]. This indicates changes in sociability that might translate into a different response to social isolation and augmented benefit from social contacts after trauma experience.

However, with respect to pre–conditioning assessments, a protective effect of GAD65 haplodeficiency arose also upon social isolation. In socially isolated wild type mice, but not in mutant mice exploration behavior in the open field decreased compared to unstressed mice, resembling previous studies in rats [71]. However, in mice isolation housing rather results in hyperactivity [95, 96] or no changes compared to group–housed animals [102, 231]. Moreover, increased exploration of open arms occurred in the elevated plus maze in my experiments, which may reflect an increased arousal response as has previously been observed in chronically stressed C57BL/6 mice [232] and stress–sensitive mutant mice [216]. In this line of argumentation, the results from the EPM may rather be interpreted as altered expression of anxiety than as a reduction thereof. Thus, social isolation stress in wild type mice altered activity and anxiety levels already before the adult fear conditioning. A similar effect did not appear in GAD65(+/–) mice. Interestingly, the combination of both stress protocols alleviated the hyperarousal response of wild types, indicating a process of adaptive resilience, similar to beneficial effects of brief maternal separation [233, 234].

Thus, by using the present paradigms I was able to induce alterations in both, associative (fear conditioning–related) and non–associative behavioral domains (pre–conditioning anxiety and activity), fulfilling another quality criterion of rodent models of PTSD [45]. Moreover, the response variations observed here and the segregation into different factors are in coherence with the independence of context generalization and unconditioned hyperarousal described in another PTSD–model [202].

Furthermore, depression–like and social numbing behavior (factor 4) were proven to be independent from the first three extracted factors. Although depression and PTSD often co–occur in the aftermath of a trauma [235], both disorders develop independently from each other and are related to different risk factors [236, 237]. In the present study, socially isolated wild type mice, but not mice confronted with

the variable stress protocol showed higher levels in the combined score, indicative of moderate depression–like changes. Similarly, Tsoory et al. (2007) showed that brief and severe stressors are more associated with an anxiety cluster than with a depression cluster [238]. Social isolation, however, induces both depression and anxiety, but via different mechanisms [239]. In the chronic mild stress–paradigm, a frequently used model of depression, hippocampal GABA and GAD65 levels are diminished immediately after [217] as well as weeks after [73] stress termination. However, GAD65 mutation in the present experiments had no effect on this extracted factor. Likewise a depression–relevant phenotype was not observed in naive mutant mice [134], suggesting a selective involvement of GAD65 heterozygocity in fear/anxiety, but not depression–related mechanisms in the present stress models. It is generally accepted that intact GABAergic functioning is pivotal to stress coping and impairments in this system are involved in the development of stress–related disorders (for review see [240]). Nevertheless, the underlying mechanisms are still insufficiently understood and research has revealed contrasting results. Most human studies report a negative correlation between the GABAergic tone and symptom severity [106, 107], but Girard et al. (2007) identified pharmacological elevation of GABA–levels by benzodiazepine application as a risk factor for PTSD development in in–patients of an intensive care unit [108]. In rodents, social isolation [58], variable stress [241] as well as fear conditioning [112, 242] induce changes in different GABAergic factors. Previously, the GAD65 knock out mouse has been established as a model for PTSD with behavioral disturbances in the fear– and anxiety cluster [146, 206, 208]. In contrast, Tasan et al. (2011) found increased GAD65 as well as GAD67 levels in the amygdala of a high anxiety mouse strain [243].

Thus, the presented data highlight the ambivalent and complex role of GAD65 by demonstrating a protective effect of GAD65 haplodeficiency towards the development of PTSD–like behavioral disturbances induced by juvenile stress and/or social isolation.

4.2 Alterations in gene expression across development in GAD65 haplodeficient mice

In the second experiment, I investigated potential expression changes of stress–responsive GABAergic genes in GAD65(+/–)– and GAD65(+/+) mice at three

different stages of postnatal GABAergic development (immature, immature in GAD65(+/–) mice / mature in GAD65(+/+) mice and mature). To this end, I used laser capture microdissection that enables high spatial resolution thereby allowing investigation of intraregional compensatory regulation.

In GAD65(+/–) mice the maturation of the GABAergic system is 2 months delayed. At the age of 2 months (P56) they have juvenile–like low GABA levels in emotion–relevant brain regions, like the amygdala and the hypothalamus [134]. The development in the hippocampus has not yet been investigated in these mice. However, they behave like wild type littermates, when anxiety or fear is assessed at this stage [134, 146, 208]. Moreover, heterozygous mutant mice even develop resilience to juvenile stress experience (study 1). Considering the importance of the GABAergic system for the regulation of anxiety levels [240] and stress coping [244, 245], this is surprising and suggests powerful molecular compensation.

In my experiment, however, relatively few and regionally and temporally focused differences emerged. At the intermediate time point the previously observed amygdalar GABA–deficit in heterozygous GAD65 mutant mice [134] is reflected by reduced GAD65 expression levels. This was not compensated by alteration of synaptic $GABA_A$–receptor subunits. $\alpha 1$ and $\alpha 2$ subunit regulation in the amygdala and the hippocampus is sensitive to stress experiences [152, 153]. It is thus, reasonable to expect an altered expression of these two genes in GAD65(+/–) mice to prevent an anxiety– or fear–related phenotype, but this was not observed in any brain region analyzed, at least on mRNA levels.

Moreover, since $\alpha 1$ is indispensable for GABA sensitivity [246] and is, together with the $\alpha 2$ subunit prominently expressed at synapses [247], one would expect an upregulation of this receptor subunit, to efficiently use the low amount of GABA present in the system. The lack thereof suggests other compensatory mechanisms. One such regulatory attempt could be the upregulation of the vesicular GABA–transporter, and the membrane–bound GABA–transporter to accelerate GABA availability for future release. The compensatory potential of the vesicular GABA-transporter in GAD65 knock out mice has already been shown [119].

Furthermore, the fact that GAD65 produces GABA mainly for synaptic release does not necessarily mean that the GABA–deficit present in GAD65(+/–) mice at this age is restricted to the synaptic GABA–pool. It is possible that GABA from the metabolic pool, for which GAD67 is mainly responsible [110, 111], is recruited to the synaptic pool. This might explain the upregulation of GAD67 in the hilus in the presence of tendentially reduced GAD65.

However, it is tempting to speculate about an elongated stress-sensitive phase in GAD65 heterozygous knock out mice, similar to the extended time window for ocular dominance development in homozygous mutant mice [248]. Thus future studies should investigate the consequences of stress presentation at this time point.
Differences emerged already in juvenility, a time point in which GABA–levels were similar between genotypes [134], indicative of adaptive processes already before the described GABA–deficit develops. Heterozygous mutant mice expressed less GAD65 and $GABA_A$-receptor $\alpha 1$ subunit in the hilus at juvenile age, the time when stressors where presented in study 1. In another genetic mouse model mild stress presentation led to a diminished expression of the $\alpha 1$ subunit in wild type mice, but had no effect in mutant mice that already present with lower levels *a priori* [150]. In this context one possible explanation for the observed resilience in my first experiment could be that a less advantageous starting condition in GAD65(+/–) mice turns out to be beneficial in the presence of challenge, because less deflections from homeostasis occur. Another interesting hypothesis regarding the formation of resilience and vulnerability is the match/mismatch hypothesis that was actually formulated to explain the discrepancy between stress experiences and later depression development. Here, Schmidt (2010) suggests a three–way interaction of genetic predisposition, early and adult environment. Early stress does not necessarily lead psychiatric conditions upon further adversities, they can also equip the individual (with the right genetic conditions) with stress coping abilities from which the individual can benefit when confronted with later challenges. Thereby, early and later challenges form a "match" [249]. In the context of my own work, the combination of the seemingly genetic disadvantage (together with seemingly disadvantageous molecular alterations) and juvenile environmental stress could interact to trigger adaptive resilience to overcome challenges in adulthood, like fear conditioning stress.
Specifically, juvenile stressed mutant mice were protected from background context generalization after cued fear conditioning and the hilus is particularly important in this process (Raza et al. in preparation). This suggests that molecular adaptations particularly in this region contribute to the observed resilience.
Moreover, interactions of the GABAergic with other neurotransmitter- or neuropeptide systems to prevent a phenotypic outcome have to be considered. GAD65(+/–) mice present with reduced aggressivity [134] and stress resilience is diminished, at least considering post-conditioning parameters, when contact to littermates is prevented. This suggests the oxytocinergic system, the prime mediator of social

behavior [250, 251], as a possible interaction partner. If so, mutant mice might benefit more from social interactions with conspecifics than their wild type littermates. Oxytocin has already been shown to increase extracellular GABA-levels in the hippocampus [252] and oxytocin inhibits stress reactions via GABAergic stimulation [253].
Finally, a GAD65–deficit developed most prominent in the last time point of assessment. At this age amygdalar and hypothalamic GABA-levels are comparable to wild type levels [134], but GABA has so far not been measured in the hippocampus. If GAD65 and GABA develop parallel, like in the amygdala at the intermediate time point, reduced hippocampal GABA levels at this late time point are possible as well. This would indicate that not only the development of the GABA-system is delayed in mutant mice, but takes place in a regionally and temporally more focused manner than previously expected. Hippocampal GAD65 expression declines with age [245]. The generally reduced GAD65 levels in almost all hippocampal subregions indicate, that not only the maturation of the GABAergic system is delayed, but GABAergic development throughout life is altered in these mice. Moreove, hippocampal and amygdalar $GABA_A$ $\alpha 1$ expression are reduced, a picture that arises also after stress [152] or in anxious mutant mice [150]. It would thus be interesting to investigate anxiety-related behavior at this late time point.
Taken together, the observed effects were less abundant than expected suggesting that by far not all compensatory mechanisms were revealed here.

4.3 Learning strategy selection in homozygous GAD65 knock out mice

Homozygous GAD65 knock out mice display increased weight towards distinct cues in memory formation, like tone generalization and extinction-deficits in auditory cued fear learning [146, 206]. Of note, these studies are aversive classical conditioning experiments. In the third study, I extended the phenotype of this mouse model by reward-based operant conditioning. And also in this very different experimental setting, GAD65(–/–) mice favor a cue-strategy, in contrast to their wild type litter mates that pursued a place strategy in most of the cases.
Since extensive training induces a shift from initial spatial to habit-learning [254], I used a protocol that would emphasize a place strategy in control mice [64]. In a

similar paradigm a bias towards the cue was observed after acute [255] or chronic stress [64] stress presentation. Also trait anxiety [66] and pharmacological amygdala activation [65] lead to cue-strategy employment. GAD65 knock out mice display heightened cFos expression in the amygdala after fear memory retrieval compared to wild type mice [205]. Together with the abovementioned characteristics of these mice, a hyperactive amygdala is likely to contribute to the observed behavior. The lack of difference in time spent in the center in the initial open field session (and thus the absence of an anxious phenotype under the present conditions) argues against an exclusive role for the amygdala in this paradigm. It cannot be ruled out that GAD65–deficiency in the hippocampus and/or the dorsal striatum, the key mediators of place and cue–based strategy use [62], respectively, are necessary or sufficient for the observed phenotype.

The lack of place–strategy use can stem from increased preference for the cue as well as from a deficit in spatial memory tasks as such. However, abnormalities in hippocampus–dependent memory have not been reported so far in GAD65 knock out mice. They perform like wild type control mice in a Morris water maze task [207] and contextual fear conditioning and extinction [206]. Although there is evidence for a correlation between water and dry mazes in a within–subject design [256], it should not be deduced from one to the other in the case of GAD65 knock out mice for two reasons: GAD65(–/–) mice display reduced floating in the forced swim test and a tendency of hyperactivity in other tests [134, 215], which could confound results in the water maze regardless of pure spatial abilities. Secondly, water is aversive to mice and GAD65(–/–) mice react to even mild stress differently than control mice [257].

In the present experiment no baseline differences between genotypes could be observed. They were indistinguishable with respect to anxiety and activity measures in the open field. Stork et al (2000) reported increased anxiety in the light/dark test, but only a tendency therefor in the open field test. Also activity is only tendentially increased [134], if circadian fluctuations are not considered [205]. The utter lack thereof in my experiment could be caused by handling and habituation to my person prior to the experiment [258]. However, it is still possible that the open field presentation itself influenced latter learning. Akirav and Richter–Levin (1999) showed that amygdala kindling 1 h before perforant path stimulation reduced hippocampal plasticity [259] and the open field as a relatively mild stressor might be enough to trigger similar processes in a sensitive system [260].

Latency to approach the food reward when first presented was similar, like latency

to eat in the first training trial. Thus, the flatter learning curve in mutant mice is likely not confounded by neophobia or reluctance of eating in an unfamiliar environment. Rather, differences in task acquisition are suggested. Both genotypes are able to learn the task as can be seen by the similar learning curves, but the learning curve of wild type mice declines faster than that of mutants mice. This contrasts the literature in two respects: First, a faster food localization in cue learners, as reported earlier in rats could not be observed [261, 262]. Second, longer escape latencies in a dual-solution hole board appear after stress, but only in mice that pursue a place-strategy, mice that employ a cue–strategy rescue this deficit and reach unstressed levels [255].
Finally, the increased proportion of mutant mice switching from one to the other possible strategy in the second retrieval, contradicts the amygdala as main contributor to the phenotype, since stress generally impairs cognitive flexibility [263–265]. Rather I suggest a disinhibited medial prefrontal cortex to contribute to the observed increased flexibility. Likewise, infusion of the $GABA_A$-agonist muscimol into frontal cortical regions impairs cognitive flexibility [266, 267]. Future studies are needed to investigate a possible interaction between cognitive flexibility and initial strategy selection.
In summary, I could show increased weight for a cue in GAD65(–/–) mice also in an reward-based, operant, dual-solution memory task. This suggests a role for GAD65 in hippocampus–striatum–amygdala interaction, despite its role in the well known amygdala–hippocampus interaction in fear learning.

4.4 Memory formation and extinction depending on neural network homeostasis

Finally, I performed cue– and contextual fear conditioning and extinction in mice with genetically enhanced functioning of Pvalb+ and glutamatergic neurons. Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$-mice were selectively impaired in contextual fear extinction, despite an overall normal contextual fear acquisition and cued fear learning and extinction. This takes place in the presence of altered hippocampal physiology. Recently, we observed *in vitro* an increased incidence and frequency of sharp wave ripples and an increased signal to noise ration in the ventral hippocampus of Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$-mice (Caliscan G., unpublished observation).
Extinction deficits and anxiety often co–occur [268–270] and Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$

mice present with an anxiety phenotype [219]. However, anxiety as a confounding factor of the observed extinction resistance is rather unlikely for several reasons. First, baseline freezing was not different between genotypes. Second, contextual fear acquisition was not augmented in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice, and third, cued fear memory formation and extinction was comparable between genotypes. All these parameters should be affected, if increased amygdala–mediated anxiety would confound the observed phenotype [101, 206, 271]. Moreover, differences in pain sensitivity or sensory perception as a potential bias can be ruled out as well, since no differences arose in an initial hot plate test.

Exploratory activity was even increased in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice at first context exposure. This might be related to the deficits in long term depression (LTD) these mice display [219], since LTD is important for encoding of new environments [272]. Moreover, deficits in LTD have been related to deficits in contextual fear memory formation [273, 274], which might also explain the slightly delayed fear acquisition. A major contribution of LTD disturbances to fear memory persistence is rather unlikely, because such deficits have been associated with facilitated, and not with impaired extinction learning [275, 276].

Thus, hippocampal SWRs are a more likely candidate to mediate fear persistence. A role for them in memory formation is evident [196, 197]. It is long been accepted that SWRs replay and transport newly acquired information to the neocortex (reviewed in [186]) and increased magnitude and density of hippocampal SWRs have been described following memory retrieval [191]. According to a current concept, a retrieval of a memory trace renders it labile again and an updated version is stored, a process called reconsolidation. Here, no decline in fear memory strength takes place and behavioral fear expression remains unaltered [277] or even increases [221]. Reconsolidation typically takes place after one or few brief sessions of reactivation, whereas extinction requires more and longer un–reinforced trials [277]. Upon confrontation with the fear associated context extinction and reconsolidation processes compete with each other [278]. It may be assumed that in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice reconsolidation outcompetes extinction, since hippocampal replay of consolidated memory is constitutively facilitated in these mice.

Of note, fear expression changes during a single extinction session (A.6 in appendix). Freezing declined over the course of each extinction training and reached control levels at the end of each session, but mutants were unable to consolidate and retain this new information until the next day. SWRs also occur during slow

wave sleep, and this is essential for memory formation [176, 192]. It is thus possible that not only the altered activity state of the hippocampus *per se* contributes to fear memory persistence, but also increased overnight reconsolidation during sleep.

Apart from the hippocampus, the amygdala and prefrontal cortex also play key roles in fear memory extinction [172, 279, 280]. The amygdala and the medial prefrontal cortex are particularly activated during fear memory extinction, whereas the hippocampus and the amygdala increase their activity upon contextual fear memory reconsolidation [277]. This, together with the lack of impairment in clearly amygdala and PFC dependent tasks, namely cue fear memory formation and extinction, supports the altered hippocampal physiology in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice as a correlate for the behavioral phenotype.

In a recent study, auditory cued fear extinction is disturbed by a selective knock down of GAD67 in Pvalb+ neurons in the PFC [170]. Another study identified enhanced activity of parvalbuminergic neurons in the BLA as neural substrate of contextual extinction [172]. Both findings contrast my results, since they reveal a positive correlation between parvalbuminergic activity and successful extinction. A possible explanation for this discrepancy could be that gain of function of perisomatic inhibitory interneurons does not affect all brain regions to the same extend, depending on the weight and function of these neurons in the particular network. For example glycine binding is very low in the amygdala [281], but has significant effects on synaptic transmission in the hippocampus [282, 283].

Together, Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice display a selective contextual fear extinction deficit that is most likely related to an oscillatory hippocampal state promoting fear memory stability. Resistance to extinction is a phenomenon often observed in anxiety disorders, like PTSD and animal models thereof [206, 284]. Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice may thus be of value for investigating underlying mechanisms of therapy–resistant fear.

Chapter 5

General discussion

5.1 Conclusion

I used heterozygous GAD65 knock out mice in combination with different stress protocols and homozygous GAD65 knock out mice without prior stress to investigate PTSD–related memory disturbances in different learning paradigms. I found an unexpected resilience in heterozygous mice. When juvenile mice experience severe and brief stress, wild type mice developed a generalized fear response to the background context in auditory cued fear conditioning. GAD65(+/–) mice, in contrast were protected against this and also did not show increased anxiety or decreased activity like wild type mice did. This was surprising, given the delayed maturation of the GABAergic system and the key role of GABA in pathological memory formation. I showed that the described delay in rise of GABA–levels is accompanied by developmentally variable and regionally focused regulation of GABA–related genes in emotion–relevant brain regions.

Furthermore, unstressed, homozygous GAD65 knock out mice preferentially employed a cue–guided strategy for reward localization, when they can chose between a cue– and a spatial strategy. In contrast, wild type mice mainly use a spatial strategy indicating an involvement of GAD65 in amygdalar-hippocampal-striatal interaction, the key mediators of strategy selection.

Finally, I used a mutant mouse with enhanced activity of parvalbimun-positive interneurons, a subpopulation of GABAergic interneurons, particularly important for shaping the oscillatory state of the hippocampus. Here, I could show an extinction deficit for only hippocampus–dependent context fear memory, despite an

overall normal fear acquisition and cue–extinction.
Taken together, I used genetic mouse models with altered GABAergic functioning to investigate different learning processes relevant to posttraumatic stress disorder. I could show an involvement of GAD65 in the cue/context balance in different paradigms and a role for parvalbuminergic interneurons in contextual fear memory extinction.

5.2 Outlook

I could show that GAD65 is involved in the cue/context integration in different learning paradigms and that the activity of parvalbuminergic interneurons contributes to resistance to contextual fear extinction.
In my first study, I could show that the delayed maturation of the GABAergic system is in fact beneficial for recovery from acute, severe stress in juvenility. In a future study, I would like to present the different stress paradigms in early adulthood, the time when the GABA–deficit develops [134] and GAD65–levels are reduced in the amygdala (study 2). Moreover, it would be interesting to investigate other possible juvenile features in these mice. Although they do not present with a strong phenotype *per se*, it is still possible that characteristics of young mice can be triggered. Typical hallmarks include the anxiogenic effect of the antidepressant fluoxetine [285] or the delayed shut down of the HPA–response after acute stress [83]. Such experiments are particularly tempting because GAD65(+/–) mice do not display alterations in anxiety or fear *per se* and thus provide a comparable baseline. Another hallmark of young rodents is the potential of fear memory erasure, i.e. context–independent extinction [286–288]. Thus, renewal (recovery of extinguished fear induced by a context shift), spontaneous recovery (rescue of extinguished fear without further manipulation, just by the passage of time) and reinstatement (recovery of extinguished fear by presentation of the US alone) [59] would be interesting paradigms in this context.
If juvenile–like behavioral features can be triggered in adult GAD65(+/–) mice, it will be interesting to see whether it is reflected also on the molecular level. For example, the development of erasure–resistant memories coincides with the maturation of perineuronal nets in the amygdala [288] which are enriched around parvalbuminergic interneurons [289] and both parvalbumin [142] and perineuronal nets [290, 291] are related to developmental plasticity during juvenility–adulthood

transition. Measuring parvalbumin and genes, representative of juvenile and mature perineuronal nets in the mutant amygdala, would therefore be interesting.

In the presence of a drastic reduction in GABA levels despite undisturbed anxiety and increased stress resilience, one would expect massive regulation of anxiety–relevant and GABA-related genes. But the changes I observed, particularly in young adulthood were less abundant and pronounced. Therefore, the search for further genes that are regulated is required. For example, an upregulation of the vesicular GABA-transporter or the membrane-bound GABA-transporter to efficiently use the less abundant GABA is imaginable. A compensatory upregulation of the vesicular GABA-transporter in homozygous GAD65 knock out mice has already been shown [119]. Moreover, it could be worth measuring the expression of α4- and δ-subunits of the $GABA_A$-receptor, which is increased in juvenile hippocampi. Both subunits have been associated with the impaired spatial learning abilities characteristic of juvenility [292] and could protect from background context generalization after stress experience.

In my next study, I found a shift towards a striatum–based cue–guided learning strategy at the expense of a hippocampus–dependent spatial strategy in homozygous GAD65 knock out mice. The first additional experiment should be a comparison of abilities to learn a pure spatial and a pure cue–based task as such. To do so, the same experiment could be repeated without the cue or with the cue at random and alternating positions in the maze. Next, increasing the sample size would allow the comparison of parametric data, like latency to reach the target and velocity, between the particular learner types. This could be interesting, since a strategy switch can rescue performance and allow for equally efficient retrieval [255].

Finally, I found a context–specific extinction deficit in mice with increased functioning of parvalbuminergic cells. This might be correlated to the increased incidence and frequency of hippocampal sharp wave ripples. To provide a prove for a causal relation between sharp wave ripples and memory persistency, the behavioral effect of blocking SWRs by hippocampal commissure stimulation [193] in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice could be observed. Since Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice present with increased SWR–incidence and –frequency *a priori*, this approach would also allow to block SWRs at specific phases of learning, such as acquisition, consolidation and post–training sleep. Finally, if the hypothesis that in mutant mice reconsolidation processes outcompete extinction holds

true, it is possible that behavioral reconsolidation drives SWRs more than extinction. To investigate this, hippocampal SWRs could be recorded after a short reactivation session and after a longer extinction session in wild type mice. Here, SWRs should be augmented after reconsolidation compared to extinction.

Appendix A

Appendix

A.1 GAD65 knock out mouse – genotyping

Tail biopsies (<0.5 cm) were taken shortly after mice had been weaned. The tissue was lysed with 125 μl PCR direct lysis buffer (Peqlab, Erlangen, Germany) and 3.75 μl Proteinase K (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Samples were incubated overnight at 55 °C and then heat inactivated for 45 min. at 85 °C. For genotyping a master mix according to table A.1 was prepared and 9 μl were distributed to PCR–tubes (Life Technologies GmbH, Darmstadt, Germany). The primer sequences are shown in table A.2. G65 S3 binds to a sequence present in both genotypes, whereas G65 A3 binds to a sequence present only in the wild type allele. Rnco3 binds to a sequence in the transgene. 1 μl sample DNA was finally added and PCR was conducted in a Thermocycler (Life Technologies GmbH, Darmstadt, Germany) using the thermocycling profile depicted in table A.3.
For genotype determination 9 μl PCR–product and 5 μl 100 bp marker (Thermo Scientific, Darmstadt, Germany) were transferred to a 2 % agarose gel. For gel preparation 2 g agarose pulver were dissolved in 100 ml 1x TAE-buffer by heating in a microwave. After cooling down, 7 μl ethidium bromid (0.5 mg/ml) were added and the fluid agarose was transferred into a gel preparation chamber. After hardening, the gel was placed into an electrophoresis chamber filled with 1x TAE buffer and the electrophoresis was carried out at 120 V until bands were separated. Under UV–light exposure the ethiduim bromid incorporated into the DNA fragments elicits a fluorescence signal. Detection of the DNA fragments was carried out with InGenius LHR gel documentation and analysis system (Syngene,

Cambridge, UK). The wild type fragment is 90 bp big, the knock out fragment is 160 bp big (Fig. A.1).

Table A.1: PCR master mix for genotyping of GAD65 knock out mice.

1 µl	10x Cl buffer (with magnesium)
1 µl	dNTPs
2 µl	Q-solution
0.3 µl	Primer G65 S3 (10 µM)
0.7 µl	Primer Rnco3 (10 µM)
2.94 µl	H_2O
0.06 µl	DREAM Taq polymerase (5 U/µl)

Table A.2: Primer sequences (5' to 3') for genotyping of GAD65 knock out mice.

Primer name	Sequence
G65 S3	GGG AAG CCA GCG GAG GGC GG (Forward)
Rnco3	GGC TGC TAA AGC GCA TGC TC (transgene)
G65 A3	CCC ATT TAC CTG TTG CGT GCA G (reverse)

Table A.3: Thermocycling program for genotyping of GAD65 knock out mice. Anneal.: Annealing, Elong.: Elongation.

Phase	**Duration**	**Temperature**	**Number of repetitions**
Initial denaturation	5 min	95 °C	1
Denaturation	20 s	94 °C	35
Anneal. + Elong.	90 s	68 °C	35
Final extension	7 s	72 °C	1
Storage	∞	4 °C	1

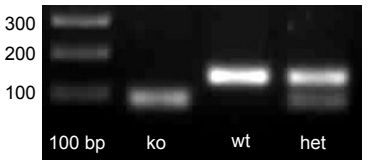

FIGURE A.1: Example of the PCR product after genotyping mice from the GAD65 knock out mouse line. The knock out- and wild type fragments are 160 bp and 90 bp big, respectively.

A.2 Qunatitative PCR – principle

Quantitative polymerase chain reaction follows the same principle as standard end–point PCR with repeated denaturation–annealing–elongation cycles. But unlike standard PCR, the amplification process is monitored after each cycle repetition leading to an exponential curve of the emitted signal. In the present experiment, I used TaqMan reagents (Life Technology, GmbH, Darmstadt, Germany) for detection. The assay for the target gene contains besides the gene–specific primer pair, a third oligonucleotide, the TaqMan MGB (minor groov-binder) probe. This also contains a fluorochrome (6-carboxy-fluorescine, FAM) and, in close proximity, a non-fluorescent quencher (NFQ). The assay for the housekeeping gene GAPDH contained another fluorescent dye (VIC) allowing simultaneous detection of both genes in a multiplex PCR. The probe will bind downstream to one of the primers and as elongation progresses the polymerase exerts its exonuclease activity and releases the quencher and the fluorochrome resulting in a fluorescence signal. Assay details are given in table A.4.

TABLE A.4: Assays used for detection of target genes. GAPDH was used as the internal control, it is labeled with the reporter dye VIC. The other assays are FAM–labeled. nt: nucleotide.

Target gene	Alias	Assay number	Probe exon location	Amplicon length (nt)
Gabra1	$GABA_A$ receptor, α1	Mm 004 39046-m1	9 - 10	98
Gabra2	$GABA_A$ receptor, α2	Mm 004 33435-m1	5 - 6	81
Gad2	GAD65	Mm 004 84623-m1	4 - 5	99
Gad1	GAD67	Mm 007 25661-s1	16	66
GAPDH	GAPDH	4352339E	3	107

A.3 Study 1 – supplement: factor analysis

Factor analysis is a method to condense the information gained from a large set of variables. It aims to explain a high degree of variability in the data with the help of few latent factors. Varimax rotation extracted five independent factors that together accounted for 70 % of total variability (Tab. A.5). Validity of factor extraction was confirmed with the Quartimax rotation method, since the same variables loaded high on the same factors as after Varimax rotation (tab. fig:quartimax).

TABLE A.5: Varimax rotation extracted five independent factors that together explained 70.079 % of total variance. Extraction method: Principal Component Analysis. V.: variance, Cum.: cumulative

Total Variance Explained

	Initial Eigenvalues			Extraction Sums of Squared Loadings			Rotation Sums of Squared Loadings		
Component	Total	% of V.	Cum. %	Total	% of V.	Cum. %	Total	% of V.	Cum. %
1	2.544	23.124	23.124	2.544	23.124	23.124	1.958	17.803	17.803
2	1.539	13.994	37.119	1.539	13.994	37.119	1.706	15.514	33.317
3	1.277	11.611	48.729	1.277	11.611	48.729	1.428	12.979	46.295
4	1.246	11.331	60.06	1.246	11.331	60.06	1.323	12.027	58.323
5	1.102	10.019	70.079	1.102	10.019	70.079	1.293	11.757	70.079
6	0.788	7.16	77.239						
7	0.736	6.693	83.931						
8	0.638	5.798	89.729						
9	0.513	4.662	94.391						
10	0.357	3.243	97.634						
11	0.26	2.366	100						

TABLE A.6: Factor extraction was validated by Quartimax rotation. Variables segregated into the same factors like after Varimax rotation. Factor loadings above 0.5 are bold. Extraction method: Principal Component analysis. Rotation method: Quartimax with Kaiser Normalization. Rotation converged in 7 iterations. OF: open field, EPM: elevated plus maze, FC: fear conditioning, LD: light/dark test, SI: social interaction, TS: tail suspension.

QUARTIMAX-rotation

	Rotated Component Matrix				
	1	2	3	4	5
OF: distance (m)	0.113	**0.866**	-0.11	-0.071	-0.05
OF: center time (s)	-0.155	0.012	**0.801**	-0.093	0.003
EPM: total arm entries	0.071	**0.833**	0.028	-0.056	0.085
EPM: % open arm entries	0.365	-0.066	**0.671**	0.211	-0.109
FC: shock context_freezing (s)	**-0.53**	-0.235	0.409	0.251	0.059
FC: CS- freezing (s)	0.015	0.087	-0.127	0.086	**0.873**
FC: CS+ freezing (s)	-0.442	-0.223	0.307	-0.082	**0.508**
LD: % activity in light	**0.865**	-0.137	-0.012	-0.186	0.114
LD: total activity (s)	**0.734**	0.315	0.191	0.115	-0.096
SI: % time of mouse contacts	-0.011	0.057	-0.046	**-0.818**	-0.282
TS: time immobile (s)	-0.244	-0.11	0.026	**0.672**	-0.373

A.4 Study 1 – supplement: fear expression of GAD65(+/–) in the background context

Homozygous GAD65 knock out mice present with an altered fear expression pattern [205, 208], i.e. increased flight attempts and reduced freezing. In my first study, I showed that the experience of variable stressors (VS) in juvenility induced increased freezing levels to the background context after auditory cued fear conditioning in wild type mice, but not in heterozygous GAD65 knock out mice. To prove that this non-increase in freezing was not caused by an increase in flight attempts and thus by a shift in fear expression, I analyzed the number of activity outbreaks (moving at a velocity faster than 20cm/s).

Indeed, a two–way ANOVA revealed a significant stress group effect ($F(3, 80) = 5.407$, $p = 0.002$), but no genotype– ($F(1,80) = 0.105$, $p = 0.747$) or interaction effect ($F(3, 80) = 0.256$, $p = 0.857$). Fisher's LSD tests for pairwise comparisons, carried out separate for each genotype, reached significance only for GAD65(+/+) mice. Unstressed control mice differed significantly from IS–mice ($p = 0.023$) and CS–mice ($p = 0.008$). An increase in activity outbreaks in heterozygous VS–mice did not occur ($p = 0.787$) (Fig. A.2).

Thus, the observed preservation from fear generalization to the background context in heterozygous VS–mice, can indeed be interpreted as resilience and does not reflect a shift in the fear expression pattern. Wild type mice displayed significantly reduced flight attempts after the experience of protracted social isolation, which resembles learned helplessness [293].

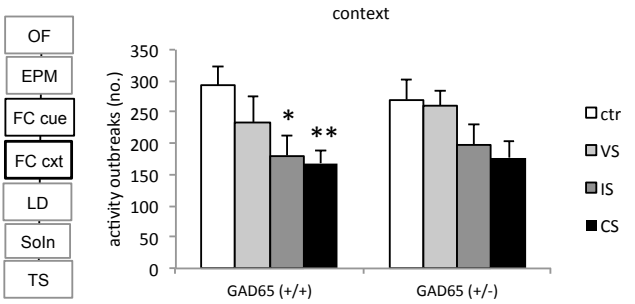

FIGURE A.2: Flight attempts in background context after auditory cued fear conditioning. Both genotypes displayed a gradual reduction in activity outbreaks that reached significance in wild type mice. In the VS–group of heterozygous mice an increase in activity outbreaks and thus a shift in the fear expression pattern did not occur. *$p < 0.05$, **$p < 0.01$ vs. ctr of same genotype.

A.5 Study 2 – supplement: statistical details

In the following, statistical details of the gene expression experiment are listed. The tables show averaged ddCT values of GAD65 (+/–) mice normalized to wild type levels of the same age group (Tab. A.7–A.21).

TABLE A.7: Gene expression levels in the BLA of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P24	**BLA**			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.046 +/-0.081	-0.309	0.763	n.s.
Gabra2	0.248 +/-0.154	-1.355	0.200	n.s.
GAD65	0.577 +/-0.380	-1.223	0.249	n.s.
GAD67	0.444 +/-0.194	-1.974	0.072	n.s.

Table A.8: Gene expression levels in the DG of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P24	DG			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.137 +/-0.122	-0.958	0.357	n.s.
Gabra2	-0.115 +/-0.144	0.552	0.591	n.s.
GAD65	0.796 +/-0.339	-1.308	0.218	n.s.
GAD67	-0.604 +/-0.175	2.528	0.030*	sign.

Table A.9: Gene expression levels in the hilus of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P24	hilus			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.290 +/-0.070	-3.418	0.008**	sign.
Gabra2	0.167 +/-0.425	-0.312	0.763	n.s.
GAD65	1.020 +/-0.168	-4.585	0.001**	sign.
GAD67	0.060 +/-0.029	-0.718	0.498	n.s.

Table A.10: Gene expression levels in the CA3 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P24	CA3			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.148 +/-0.204	0.060	0.953	n.s.
Gabra2	-0.397 +/-0.349	1.011	0.334	n.s.
GAD65	0.891 +/-0.211	-1.547	0.150	n.s.
GAD67	0.147 +/-0.201	-0.592	0.566	n.s.

Table A.11: Gene expression levels in the CA1 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P24	CA1			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.142 +/-0.125	0.892	0.390	n.s.
Gabra2	-0.162 +/-0.226	0.536	0.602	n.s.
GAD65	0.801 +/- 0.294	-1.185	0.259	n.s.
GAD67	0.063 +/-0.128	-0.272	0.790	n.s.

Table A.12: Gene expression levels in the BLA of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P56	BLA			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.584 +/-0.143	0.340	0.741	n.s.
Gabra2	0.262 +/-0.221	-1.020	0.332	n.s.
GAD65	2.966 +/-0.720	-2.657	0.026*	sign.
GAD67	-0.481 +/-0.277	1.615	0.137	n.s.

Table A.13: Gene expression levels in the DG of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P56	DG			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.256 +/-0.124	0.174	0.865	n.s.
Gabra2	0.340 +/-0.114	-1.554	0.151	n.s.
GAD65	0.307 +/-0.205	-0.403	0.701	n.s.
GAD67	0.073 +/-0.178	-0.346	0.736	n.s.

TABLE A.14: Gene expression levels in the hilus of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P56	hilus			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.254 +/-0.369	0.702	0.514	n.s.
Gabra2	0.280 +/-0.281	-0.889	0.415	n.s.
GAD65	1.223 +/-0.548	-2.419	0.060	n.s.
GAD67	-0.595 +/-0.235	2.452	0.036*	sign.

TABLE A.15: Gene expression levels in the CA3 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P56	CA3			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.275 +/-0.203	-0.759	0.465	n.s.
Gabra2	0.260 +/-0.130	-0.840	0.421	n.s.
GAD65	0.233 +/-0.395	-0,325	0.752	n.s.
GAD67	-0.167 +/-0.117	1.005	0.339	n.s.

TABLE A.16: Gene expression levels in the CA1 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P56	CA1			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.312 +/-0.126	-1.808	0.101	n.s.
Gabra2	0.093 +/-0.154	-0.429	0.677	n.s.
GAD65	-0.215 +/-0.493	0.187	0.856	n.s.
GAD67	-0.203 +/-0.240	0.722	0.487	n.s.

TABLE A.17: Gene expression levels in the BLA of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P112	BLA			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.346 +/-0.116	-2.223	0.043*	sign.
Gabra2	0.008 +/-0.090	-0.054	0.958	n.s.
GAD65	0.128 +/-0.264	-0.307	0.764	n.s.
GAD67	-0.180 +/-0.075	1.299	0.215	n.s.

TABLE A.18: Gene expression levels in the DG of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P112	DG			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.502 +/-0.151	-2.473	0.027*	sign.
Gabra2	0.823 +/-0.287	-2.058	0.059	n.s.
GAD65	-0.007 +/-0.310	0.017	0.987	n.s.
GAD67	0.106 +/-0.124	-0.746	0.473	n.s.

TABLE A.19: Gene expression levels in the hilus of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P112	hilus			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.063 +/-0.157	-0.278	0.785	n.s.
Gabra2	0.060 +/-0.128	-0.212	0.836	n.s.
GAD65	1.147 +/-0.197	-4.291	0.001**	n.s.
GAD67	0.298 +/-0.097	0.388	0.124	n.s.

TABLE A.20: Gene expression levels in the CA3 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P112	CA3			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	0.154 +/-0.217	-0.489	0.632	n.s.
Gabra2	0.612 +/-0.205	-1.806	0.092	n.s.
GAD65	0.879 +/-0.161	-3.363	0.005**	sign.
GAD67	0.143 +/-0.155	-0.624	0.544	n.s.

TABLE A.21: Gene expression levels in the CA1 of GAD65(+/–) mice relative to GAD65(+/+) mice of the same age. n.s.: not significant

P112	CA1			
	ddCT mean +/-SEM	T-value	P-value	Sign.
Gabra1	-0.276 +/-0.232	0.810	0.433	n.s.
Gabra2	0.669 +/-0.494	-1.063	0.313	n.s.
GAD65	0.603 +/-0.101	-3.980	0.002**	sign.
GAD67	-0.228 +/-0.088	1.260	0.232	n.s.

A.6 Study 4 – supplement: within–session extinction

Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice maintained high freezing levels throughout extinction, when the first 2 minutes of each session were assessed. To gain a more detailed picture of how the fear response changes within a single session, I analyzed fear development in five 2–minute intervals (i1–i5) for each extinction session. The fear response in Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice declined in the course of an extinction session, but mice were unable to consolidate this new information and retain it until the next session.

A.6.1 Freezing duration

In the retrieval and first extinction session R/E1 a significant main effect for the interval was observed ($F(4,92) = 21.388$, $p < 0.001$), but no effect of the genotype ($F(2, 23) = 0.739$, $p > 0.05$) or a genotype x interval interaction ($F(8,92) = 1.649$, $p > 0.05$) emerged. *Post hoc* comparison revealed a reduction of freezing compared to the first interval i1 in all three genotypes ($Hprt^{3L\alpha185L+/0}$: i1 vs. i3–i5: $p < 0.05$; $Hprt^{3L\alpha185L+/0}$; $Camk2a^{Cre+/-}$: i1 vs. i3, i4: $p < 0.05$; $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$: i1 vs. i4, i5: $p < 0.01$) (Fig. A.3 A).

In session E2 a significant effect was evident for the interval ($F(4,92) = 7.964$, $p < 0.001$) as well as for genotype x interval interaction ($F(8,92) = 3.043$, $p < 0.01$), but no significant effect for the genotype alone ($F(2, 23) = 3.168$, $p > 0.05$). $Hprt^{3L\alpha185L+/0}$ mice reduced the time spent freezing from the first to the fourth interval ($p < 0.05$). $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice displayed high freezing levels in the beginning, which declined during the session (i1 vs. i3: $p < 0.05$, i1 vs. i4: $p < 0.001$, i1 vs. i5: $p < 0.01$; i2 vs. i3: $p < 0.05$, i2 vs. i4: $p < 0.001$, i2 vs. i5: $p < 0.01$). $Hprt^{3L\alpha185L+/0}$; $Camk2a^{Cre+/-}$ showed not significant changes between intervals (Fig. A.3 B).

A similar pattern emerged in E3 with $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice showing high freezing levels in i1 and i2 and alignment with the other 2 genotypes during i3–i5. Thus a significant interval effect ($F(4,92) = 9.278$, $p <0.001$) and a significant genotype x interval interaction ($F(8,92) = 3.857$, $p < 0.01$), but no main genotype effect emerged ($F(2, 23) = 1.509$, $p > 0.05$). Pair wise comparisons confirmed a significant reduction from the first 2 intervals to the last 3 in $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice only (i1 vs. i3–5: $p < 0.01$, i2 vs. i3–5: $p < 0.01$) (Fig. A.3 C).

The same pattern was observed in session E4 with a significant interval effect ($F(4,92) = 4.487$, $p < 0.01$) and a significant interaction ($F(8,92) = 3.49$, $p < 0.01$), but no genotype effect ($F(2, 23) = 0.59$, $p > 0.05$). Again, freezing diminished only in $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice during the session (i1 vs. i4, i5: $p < 0.01$; i2 vs. i4, i5: $p < 0.05$; i3 vs. i5: $p < 0.05$) (Fig. A.3 D).

Finally, for E5 a significant interval effect ($F(4,92) = 5.972$, $p < 0.001$) and a significant interaction ($F(8,92) = 2.409$, $p < 0.05$) arose. The genotype alone did not yield a significant effect ($F(2, 23) = 0.016$, $p > 0.05$). In the $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice the first interval i1 differed significantly from each i3, i4 and i5

($p < 0.05$), and the i2 differed from i4 ($p < 0.05$). Again, no significant habituation was found in the other groups (Fig. A.3 E).

A.6.2 Freezing bouts

Overall, al similar picture arose for the number of freezing bouts. In the first retrieval/extinction session repeated measures ANOVA revealed a significant effect for the interval ($F(4, 92) = 17.593$, $p < 0.001$), but not for the interaction ($F(8, 92) = 1.265$, $p > 0.05$) or the genotype ($F(2, 23) = 2.398$, $p > 0.05$). Freezing levels in all three groups declined in the course of the first extinction session ($Hprt^{3L\alpha185L+/0}$: i1 vs. i3, i4: $p < 0.01$, i1 vs. i5: $p < 0.05$; $Hprt^{3L\alpha185L+/0}$; $Camk2a^{Cre+/-}$: i1 vs. i3–i5: $p < 0.01$; $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$: i1 vs. i4, i5: $p < 0.01$) (Fig. A.3 F).

In the second extinction session a significant interval effect ($F(4, 92) = 7.915$; $p < 0.001$) and a significant interval x genotype effect ($F(8, 92) = 4.873$, $p < 0.001$) appeared. A significant genotype effect was not observed ($F(2, 23) = 3.028$, $p > 0.05$). While $Hprt^{3L\alpha185L+/0}$– and $Hprt^{3L\alpha185L+/0}$; $Camk2a^{Cre+/-}$ mice display a low number of freezing bouts throughout the session, $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ show initially a high number of freezing bouts that declines until the end of the session (i1 vs. i3: $p < 0.01$, i1 vs. i4–i5: $p < 0.001$) (Fig. A.3 G).

On the third day of extinction training a significant interval effect ($F(4, 92) = 10.023$, $p < 0.001$) and a significant interaction effect ($F(8, 92) = 3.829$, $p < 0.05$), but no genotype effect ($F(2, 23) = 2.014$, $p > 0.05$) arose. Again freezing bouts at i1 were more frequent than at i3–5 (i3:$p < 0.001$, i4:$p < 0.01$, i5:$p < 0.001$) in $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice (Fig. A.3 H).

Similarly, at E4 significances were observed for the interval ($F(4, 92) = 3.034$, $p < 0.05$) and the interaction ($F(8, 92) = 2.507$, $p < 0.05$), but not for the genotype alone ($F(2, 23) = 0.225$, $p > 0.05$). In the $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ group i1 differed significantly from i4 ($p < 0.01$) and i5 ($p < 0.01$) (Fig. A.3 I).

In the last extinction session interval ($F(4, 92) = 6.004$, $p < 0.001$) and interval x genotype ($F(8, 92) = 2.816$, $p < 0.01$) reached statistical significance. The genotype effect alone was not significant ($F(2, 23) = 0.05$, $p > 0.05$). Finally, i1 of $Hprt^{3L\alpha185L+/0}$; $Pvalb^{Cre+/-}$ mice differed from i3, i4, and i5 ($p < 0.01$) (Fig. A.3 J).

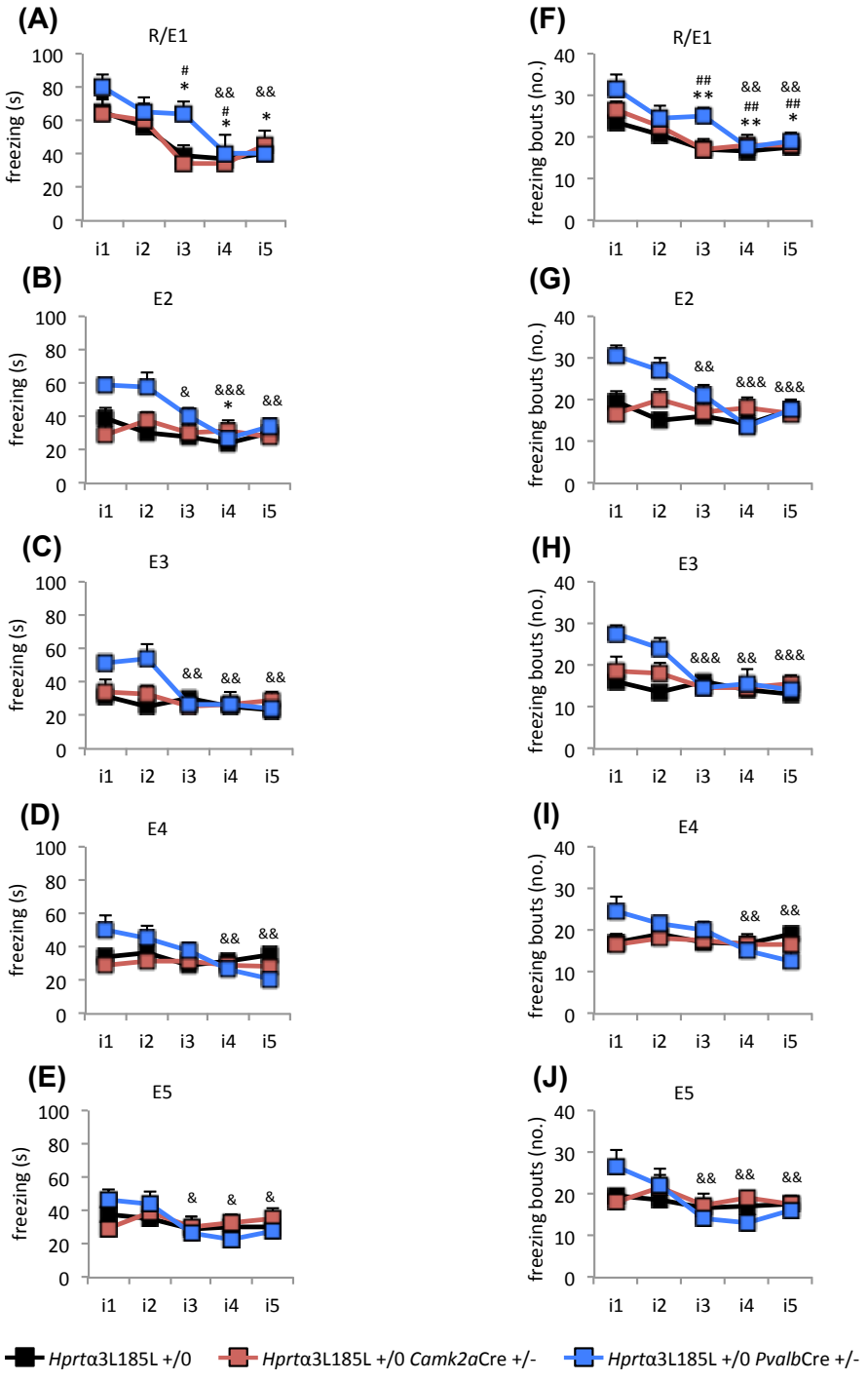

FIGURE A.3: : 2–minute interval analysis within each extinction session R/E1–E5 for (A–E) freezing duration and (F–J) number of freezing bouts. In the first extinction, fear declines in all genotypes, but only Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice were unable to maintain reduced fear responses until the next session. i1–5: interval 1–5; *p < 0.05, **p < 0.01 of Hprt$^{3L\alpha185L+/0}$ mice compared to i1; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01 of Hprt$^{3L\alpha185L+/0}$; Camk2a$^{Cre+/-}$ mice compared to i1; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01, $^{\&\&\&}$p < 0.001 of Hprt$^{3L\alpha185L+/0}$; Pvalb$^{Cre+/-}$ mice compared to i1. For a comprehensive view only significances compared to i1 are displayed, for a more detailed analysis see the description in the text.

A.7 Chemicals

Agaraose	Carl Roth, Karlsruhe, Germany
Cresyl violet acetate	Sigma-Aldrich, Seelze, Germany
Dimethyl dicarbonat (DMDC)	Sigma-Aldrich, Seelze, Germany
DirectPCR-Tail lysis reagent	Peqlab, Erlangen, Germany
di-Nucleotide-Tri-Phosphate (dNTPs)	Thermo Scientific , St. Leon-Roth, Germany
Ethanol 96%	Carl Roth, Karlsruhe, Germany
Ethidium bromid	Carl Roth, Karlsruhe, Germany
Jung Tissue Freezing Medium	Leica, Nussloch, Germany
Methylbutane	Carl Roth, Karlsruhe, Germany
Oligonucleotide (dT)18 primer	Ambion/Life Technologies, Darmstadt, Germany
Poly-L-Lysine 1%	Sigma-Aldrich, Seelze, Germany
Primer for genotyping PCRs	Life Technologies, Darmstadt, Germany
Proteinase K	Carl Roth, Karlsruhe, Germany
Random defamer primer	Ambion/Life Technologies, Darmstadt, Germany
RNAse Zap	Life Technologies, Darmstadt, Germany
β-Mercaptoethanol	Serra, Heidelberg, Germany
SuperaseIN	Ambion/Life Technologies, Darmstadt, Germany

A.8 Solutions

1% Cresyl violet solution	1 g Cresyl violet acetate 50 ml 96% ethanol fill with aqua dd. to 100 ml stir for 7 h at RT, protect from light filter store protected from light
DMDC-treated water	0.1% dimethyldicarbonate in aqua dd. stir for 3 h autoclave
Poly-L-Lysine	1:2 dilution of Poly-L-Lysine 0.1% in aqua dd.

A.9 DNA length standard

GeneRuler^{TM} 100 bp DNA ladder	Thermo Scientific , St. Leon-Roth, Germany

A.10 Kits and enzymes

Dream Taq polymerase	Thermo Scientific , St. Leon-Roth, Germany
RNeasy Micro Plus kit	Qiagen, Hilden, Germany
Sensiscript Reverse Transcription kit	Qiagen, Hilden, Germany
Taq Polymerase	Qiagen, Hilden, Germany

A.11 Consumables and instruments

Animal care

Lignocel BK 8/15	J. Rettenmaier & Söhne, Rosenberg, Germany
Macrolon standard cages (type II long)	Techniplast, Hohenpeissenberg, Germany
	Bioscape, Castrop-Rauxel, Germany
Ssniff R/M-H V-1534	Ssniff Spezialitäten, Soest, germany
Kellogg's Choco Krispies	Kellogg Company, USA

Plastic ware

Adhesive cap 500 clear	Carl Zeiss, Jena, Germany
Micro-Amp Fast Reaction Tubes	Life Technologies, Darmstadt, Germany
Micro-Amp 8-cap strips	Life Technologies, Darmstadt, Germany
Micor-Amp Fast Optical 96-well plate	Life Technologies, Darmstadt, Germany
Micro-Amp Optical Adhesive Film	Life Technologies, Darmstadt, Germany
Plastic syringe for restraint stress	Braun, Melsungen, Germany
Safe lock tubes (1.5 ml)	Eppendorf, Hamburg, Germany
Tissue culture dish	Greiner Bio-One, Frickenhausen,Germany

Glass ware

Glass bottles	Carl Roth, Karlsruhe, Germany
Beaker	Carl Roth, Karlsruhe, Germany
Graduated cylinder	Carl Roth, Karlsruhe, Germany
Staining cuvettes	Carl Roth, Karlsruhe, Germany
Slide holder	Carl Roth, Karlsruhe, Germany
Object slide box	Carl Roth, Karlsruhe, Germany
Membrane slides 1.0 PEN	Carl Zeiss, Jena, Germany

Pipettes

Pipettes	Brand, Wertheim, Germany
Pipette tips	Brand, Wertheim, Germany
Pipette tips with filter	Brand, Wertheim, Germany

Freezers and Fridges

Liebherr KU 2407	Liebherr Hausgeräte, Ochsenhausen, Germany
Liebherr GU 4506	Liebherr Hausgeräte, Ochsenhausen, Germany
Sanyo Ultra Low	Ewald Innovationstechnik, Bad Nenndorf, Germany

Scale

Sartorius TE 2101	Sartorius AG, Göttingen, Germany

Centrifuges

Heraeus Pico 17	Thermo Scientific, Germany

Magnet stirrer

IKA RET basic	IKA-Werke, Staufen, Germany
magnetic stir bar	Brand, Wertheim, Germany

Vortexer

VWR Lab dancer S40 VWR International, Darmstadt, Germany

Rotor incubator

Hybrid 2000 H. Saur Laborbedarf, Reutlingen, Germany

Water bath

LAUDA A103 Lauda Dr. R. Wobser, Lauda-Königshof, Germany

Autoclave

Systec DB-23 Systec Labortechnik, Wettenberg, Germany
Systec VA120 Systec Labortechnik, Wettenberg, Germany

Oven

Binder FP53 Binder, Tuttlingen, Germany

PCR hood

Captair bio Erlab, Köln, Germany

Thermocycler

Veriti Thermal Cycler Applied Biosystems, Darmstadt, Germany

Real-time PCR

StepOne Plus Real-Time PCR system Applied Biosystems, Darmstadt, Germany

Microwave

Clatronic MWG 746 H Clatronic International, Kempen, Germany

Gel electrophoresis system

AGT3 & Maxi-VG VWR International, Darmstadt, Germany

Gel documentation system

InGenius LHR Syngene, Cambridge, UK

Cryostat

CM 1950 Leica, Nussloch, Germany

Hot plate

Medite OTS 40.2530 Medite, Burgdorf, Germany

Laser capture micro dissection system

PALM MicroBeam Carl Zeiss, Jena, Germany

Behavioral test systems

TSE Fear Conditioning System	TSE, Bad Homburg, Germany
Open field	Stoelting Co., Wood Dale, IL, USA
Elevated plus maze	Stoelting Co., Wood Dale, IL, USA
3-compartment social interaction box	Stoelting Co., Wood Dale, IL, USA

Software

Anymaze Video tracking system	Stoelting Co., Wood Dale, IL, USA
SPSS Statistics	IBM, Ehningen, Germany
Micorsoft Office	Mircosoft, Redmond, WA, USA
Palm Robosoftware	Carl Zeiss, Jena, Germany
Step One v2	Life Technologies, Darmstadt, Germany
Mendeley	Mendeley Ltd, London, UK

Mouse lines

GAD65 knock out mouse	Laboratory of Prof. Kunihiko Obata, NIPS, Okazaki, Japan
C57BL/6Tac	M & B Taconic, Berlin, Germany
Hprt$^{3La185L+/0}$ mouse	Prof. Jochen C. Meier, Max Delbrück Center for Molecular Medicine, Berlin, Germany
Hprt$^{3La185L+/0}$; Camk2a$^{Cre+/-}$ mouse	Prof. Jochen C. Meier, Max Delbrück Center for Molecular Medicine, Berlin, Germany
Hprt$^{3La185L+/0}$; Pvalb$^{Cre+/-}$ mouse	Prof. Jochen C. Meier, Max Delbrück Center for Molecular Medicine, Berlin, Germany

Others

Lab clock	Carl Roth, Karlsruhe, germany
Aluminuim foil	Carl Roth, Karlsruhe, germany

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