The Startle Response as a Measure in Mouse Models of Mood Disorders

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Cho
vợ tôi
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Es gibt keinen Neuschnee

Wenn du aufwärts gehst und dich hochaufatmend umsiehst, was du doch für ein Kerl bist, der solche Höhen erklimmen kann, du, ganz allein –: dann entdeckst du immer Spuren im Schnee. Es ist schon einer vor dir dagewesen.

Glaube an Gott. Verzweifle an ihm. Verwirf alle Philosophie. Laß dir vom Arzt einen Magenkrebs ansagen und wisse: es sind nur noch vier Jahre, und dann ist es aus. Glaub an eine Frau. Verzweifle an ihr. Führe ein Leben mit zwei Frauen. Stürze dich in die Welt. Zieh dich von ihr zurück...

Und alle diese Lebensgefühle hat schon einer vor dir gehabt; so hat schon einer geglaubt, gezweifelt, gelacht, geweint und sich nachdenklich in der Nase gebohrt, genau so. Es ist immer schon einer dagewesen.

Das ändert nichts, ich weiß. Du erlebst es ja zum ersten Mal. Für dich ist es Neuschnee, der da liegt. Es ist aber keiner, und diese Entdeckung ist zuerst sehr schmerzlich. In Polen lebte einmal ein armer Jude, der hatte kein Geld, zu studieren, aber die Mathematik brannte ihm im Gehirn. Er las, was er bekommen konnte, die paar spärlichen Bücher, und er studierte und dachte, dachte für sich weiter. Und erfand eines Tages etwas, er entdeckte es, ein ganz neues System, und er fühlte: ich habe etwas gefunden. Und als er seine kleine Stadt verließ und in die Welt hinauskam, da sah er neue Bücher, und das, was er für sich entdeckt hatte, das gab es bereits: es war die Differentialrechnung. Und da starb er. Die Leute sagen: an der Schwindsucht. Aber er ist nicht an der Schwindsucht gestorben.

Am merkwürdigsten ist das in der Einsamkeit. Daß die Leute im Getümmel ihre Standard-Erlebnisse haben, das willst du ja gern glauben. Aber wenn man so allein ist wie du, wenn man so meditiert, so den Tod einkalkuliert, sich so zurückzieht und so versucht, nach vorn zu sehen –: dann, sollte man meinen, wäre man auf Höhen, die noch keines Menschen Fuß je betreten hat. Und immer sind da Spuren, und immer ist einer dagewesen, und immer ist einer noch höher geklettert als du es je gekonnt hast, noch viel höher.

Das darf dich nicht entmutigen. Klettere, steige, steige. Aber es gibt keine Spitze. Und es gibt keinen Neuschnee.

Kaspar Hauser, alias Kurt Tucholsky, in „Die Weltbühne“ vom 7. April 1931

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Nomenclature

αCRH ..... α corticotropin releasing hormone
ADHD ..... attention deficit/hyperactivity disorder
AMY ..... amygdala
ANOVA ..... analysis of variance
ASR ..... acoustic startle response
BLA ..... basolateral amygdala
BNST ..... bed nucleus of the stria terminalis
CA3 ..... cornu ammonis region 3
CCD ..... charged coupled device
CeA ..... central amygdala
ChR2 ..... channelrhodopsin-2
CRH ..... corticotropin releasing hormone
CS ..... conditioned stimulus
CSF ..... cerebro spinal fluid
DA ..... dopamine
DAT-ko ..... dopamine transporter deficient
DR1/2 ..... dopamine receptor 1/2
(E)GFP ..... (enhanced) green fluorescent protein
EPM ..... elevated plus maze
EPSP ..... excitatory postsynaptic potential
EtOH ..... ethanol
ExFC ..... extinction of conditioned fear
FC ..... fear conditioning
FPS ..... fear potentiated startle
GABA ..... γ aminobutyric acid
HAB ..... high anxiety related behaviour
HAB-R ..... high anxiety related behaviour rat
HPA ..... hypothalamus pituitary adrenal

HPC ..... hippocampus
i.c.v. ..... intracerebroventricular
IC ..... inferior colliculus
IPI ..... interpulse interval
ISI ..... interstimulus interval
LAB ..... low anxiety related behaviour
LAB-R ..... low anxiety related behaviour rat
LDTg ..... lateral dorsal tegmental nucleus
LES ..... light enhanced startle
MEMRI ..... manganese enhanced magnetic resonance imaging
MPI-P ..... Max-Planck-Institute of Psychiatry
MRI ..... magnetic resonance imaging
NAB ..... normal anxiety related behaviour
NAC ..... nucleus accumbens
NMDA ..... N-methyl-D-aspartate
NpHR ..... *Natronomonas pharaonis* halorhodopsin
P ..... startle eliciting acoustic pulse
p.o. ..... per os
PAG ..... periaqueductal grey
PCR ..... polymerase chain reaction
PFC ..... prefrontal cortex
PnC ..... caudal pontine reticular nucleus
PP ..... prepulse
PPA ..... prepulse augmentation
PPF ..... prepulse facilitation
PPI ..... prepulse inhibition
PPTg ..... pedunculopontine tegmental nucleus
PS ..... pre-stimulus
PTSD ..... posttraumatic stress disorder
rmANOVA ..... repeated measures analysis of variance
SC ..... superior colliculus
SEM ..... standard error of the mean
SNR ..... substantia nigra
SPL ..... sound pressure level
SR ..... startle response

sw	sine wave
TES	tone enhanced startle
Thy1	thymus cell antigene 1
TRPV1-ko	transient receptor potential cation channel subfamily vanilloid type 1 deficient
UR	unconditioned reaction
US	unconditioned stimulus
vi	various interval
VTA	ventral tegmental area
wn	white noise
YFP	yellow fluorescent protein

Zusammenfassung

Ein großer Teil der Fragestellungen in den Neurowissenschaften beschäftigt sich mit dem Thema, wie das Säugerhirn Verhalten auslöst und steuert. Die Schreckreaktion ist ein relativ einfaches Verhalten, welches bei Säugetieren ohne großen Aufwand ausgelöst werden kann und variabel auf eine Vielfalt von experimentellen Behandlungen reagiert.

Das Ziel der vorliegenden Arbeit war es, Schreckreaktions-Messungen am Max-Planck-Institut für Psychiatrie in München (MPI-P) zu etablieren. Vor dem Hintergrund aktueller Fragestellungen sollten die Experimente zu einsatzbereiten Messmethoden und Verhaltensparadigmen führen.

In der vorliegenden Arbeit gelang es nicht, das Paradigma der furchtpotenzierten Schreckreaktion (FPS) zuverlässig in einem häufig am MPI-P eingesetzten Mausstamm anzuwenden. Das FPS maskierende Phänomen, daß die Präsentation eines unkonditionierten Tons bereits zu einer deutlich verstärkten Schreckreaktion in diesen Mäusen führt („tone enhanced startle“, TES) wurde dann charakterisiert und im Folgenden als ergänzendes Paradigma zur Messung und Abschätzung des Hörvermögens, der Stimulus Adaptation und der Aufmerksamkeit in Mäusen vorgeschlagen.

Eine Literaturrecherche ergab, daß im Paradigma der Furchtkonditionierung („fear conditioning“, FC) und deren aktives Verlernen („extinction of FC“, ExFC) verwendete Stimulus-Parameter eine hohe Varianz zwischen verschiedenen Laboratorien aufweisen. Der im Verhalten ausgelesene Lernerfolg während einer FC wie auch einem ExFC hingen in den vorliegenden Experimenten wesentlich von der verwendeten Stimulusqualität ab (d.h. sinus-Ton oder weißes Rauschen). Im Umkehrschluß empfiehlt die vorliegende Arbeit einen überlegteren Umgang mit den eingetzten Stimulus-Parametern.

Es zeigte sich, daß eine erhöhte Schreckreaktion (Übererregbarkeit) ohne weiteres in einem Tiermodell der Posttraumatischen Belastungsstörung („posttraumatic stress disorder“, PTSD) gemessen werden kann. Im Weiteren konnte gezeigt werden, daß verändertes Hippocampus-Volumen in diesen Tieren, gemessen über ultramikroskopische Aufnahmen und analog zu Hippocampusveränderungen in Patienten, unabhängig von

anderen PTSD-ähnlichen Symptomen dieser Mäuse ist.

In einem weiteren Abschnitt widmet sich die vorliegende Arbeit der laufenden Charakterisierung der Rolle von Dopaminrezeptoren (DR) in der Präpulsinhibition (PPI) und -Faszilitierung (PPF) der Schreckreaktion. Durch lokale injektion von DR-Antagonisten konnte gezeigt werden, daß die Blockade von DR1 wiederholbar PPI verstärkt, während die Rolle von DR2, getestet mit zwei verschiedenen Antagonisten, als ambivalent gedeutet werden muß.

Basierend auf diesen Experimenten wurden optogenetische Methoden in die Schreckreaktionsmessung eingeführt. Transgenen Mäusen, die lichtsensitive Ionenkanäle in ihren neuronalen Zellmembranen bestimmter Zellpopulationen tragen, wurden Lichtblitze ins Gehirn appliziert. Auf diese Weise konnten PPI und PPF unabhängig voneinander manipuliert werden. Daraus folgend, und im Unterschied zur populären Summationshypothese der PPF, schlägt die vorliegende Arbeit einen eigenständigen, von der PPI unabhängigen PPF-Schaltkreis vor, der Pyramidenneuronen der präfrontalen Kortexschicht V beinhaltet.

Die vorliegende Arbeit konnte erfolgreich verschiedene Protokolle und Verhaltensparadigmen der Schreckreaktionsmessung am MPI-P etablieren und zur sofortigen Nutzung zur Verfügung stellen. Es wurden nicht nur neue Techniken wie z.B. optogenetische Methoden in die Schreckreaktionsmessung eingeführt, die vorliegenden Experiemente leisten auch ihren Beitrag zur aktiven Forschung, in dem sie z.B. die große Bedeutung der Stimulus-Parameter für den Lernerfolg von Versuchstieren nachweisen.

Abstract

In neuroscience major efforts are focused on the question of how the mammalian brain generates and controls behaviour. The startle response is a relatively simple behaviour that can be easily elicited in mammals and is sensitive to a variety of experimental treatments.

The aim of the present work was to establish startle response measures at the Max-Planck-Institute of Psychiatry (MPI-P), Munich, providing a set of readily applicable methods and paradigms, and contributing to questions in behavioural neuroscience.

While the present thesis failed to robustly elicit fear potentiated startle (FPS) in a commonly used mouse strain at the MPI-P, strong unconditioned startle enhancement by acoustic stimulus presentation in that mouse strain was capitalised to propose tone enhanced startle (TES) as an additional paradigm to assess hearing capability, stimulus adaptation and attention in mice.

A literature survey revealed considerably varying parameters used in fear conditioning (FC) and extinction of conditioned fear (ExFC). In the present work FC, ExFC as well as FPS/TES highly depended on stimulus quality (i.e. sine wave or white noise), demanding a more careful handling of stimulus parameters.

Hyper-arousal was readily tested in a mouse model of posttraumatic stress disorder (PTSD). Additionally it was shown that altered hippocampal volume in these animals, assessed by ultramicroscopic measures and mimicking patient data, was independent of other symptoms present in this model.

The present thesis contributes to the ongoing characterisation of the role of dopamine receptors (DR) in prepulse inhibition (PPI) and prepulse facilitation (PPF) of startle, manipulating PPI/F by injections of DR-antagonists into the prefrontal cortex of mice. It was found that blockade of DR1 reliably increases PPI, while the effect of DR2 was inconsistent, using two different DR2-antagonists. Based on this work, optogenetic methods were established. Applying intracerebral light flashes to transgenic mice carrying light sensitive ion channels on their neuronal cell membrane, PPI and PPF were ma-

nipulated independently, proposing the existence of a discrete PPF mediating pathway including prefrontal layer V pyramidal neurons, contrasting the popular summation hypothesis of PPF.

The present work established and developed successfully different startle paradigms that are ready to use for animal characterisation and testing. Apart from combining startle measures with new techniques such as optogenetic methods, the present thesis points out the stimulus parameter dependence of animal learning, suggesting a fundamental discussion about fear conditioning and extinction learning protocols.

Part I.

Introduction

Huminea natura curiosi sunt - humans are curious by nature; this phrase alone could explain why humans study animal behaviour and underlying physiology. The first written evidence of animal testing goes back to the ancient Greek, who used animals for anatomical studies (cf. Maehle and Trohler, 1987 in Close, 2007). It was then in the 12th century AD that Avenzoar introduced animal testing explicitly as an experimental method to test surgical techniques before applying them to patients (Abdel-Halim, 2005). About 300 years before, al-Jahiz published several studies dealing with animal communication and psychology (Haque, 2004) marking the beginning of the specialism of comparative psychology. With the upcoming theory of Behaviourism in the late 19th century (cf. McKenna, 1995), first animal models of psychiatric disorders were developed (cf. Graeff and Jr, 2002), not only to satisfy curiosity, but to investigate underlying mechanisms and possible treatments of these diseases (cf. Flint and Shifman, 2008).

1. The startle response - neurobiology and animal testing

The startle response as an animal model

According to Geyer and Markou (1995), animal models must satisfy the criteria of reliability and predictive validity to establish its value in basic neurobiological research. One paradigm fulfilling these criteria almost entirely and intimately connected with the concept of Behaviourism is classical conditioning, introduced by Pavlov (cf. Pavlov, 1927). An even more fundamental behaviour matching criteria of reliability and predictive validity is the startle response. Already subject to studies as early as 1900 (e.g. Partridge, 1900), it can be measured across species as stereotypic muscle contraction (cf. Landis et al., 1939 in Grillon, 2008), that can be, on the other hand, modified by a variety of internal and external factors (Koch, 1999).

The development of animal models in fields such as emotion (cf. Brown et al., 1951; Davis, 1998), perception (Cohen et al., 1933), and psychiatric disorders (cf. e.g. Braff et al., 1978) was supported by the comparability of startle reflex behaviour found in rats and humans (Ison, 2001). During the past 15 years, advances in molecular biology have

enabled the creation of diverse inbred, transgenic, and knock-out mice, bringing this species also into the focus of startle research (cf. e.g. Geyer et al., 2002).

The study of the startle response and its modifications has expanded our knowledge about biological mechanisms underlying signal processing and behavioural outcome. Reliability and predictive validity of the startle response have enabled detailed study of neuronal functionality on behaviour, brain and cellular level. For the development of a specific treatment for a disease it is indispensable to know aetiology leading to this disease, and biological processes underlying the disorder. Since startle is comparable in a variety of aspects in a number of species, including humans (cf. Baird et al., 1993; Briffa et al., 2008; Cho et al., 2004; Howard and Ford, 1992; Stehouwer, 1992; Stitt et al., 1976; Hoffman and Fleshler, 1963; Parham and Willott, 1988), the knowledge about biological processes underlying startle mediation and modulation may have implications for clinical applications. The startle response is found to be altered, and modifications such as prepulse inhibition are disturbed, in diverse psychiatric diseases (cf. Grillon and Baas, 2002; Swerdlow and Geyer, 1998). Thus, startle response studies in humans, in comparison with animal models of a respective disorder, led to new theoretical framework, but also suggestions for treatment applications (cf. Feifel and Shilling, 2010; Grillon, 2008).

Measuring startle

The acoustic startle response (ASR) is elicited by acoustic stimuli with a steep rise time and intensities $> 75\,\mathrm{dB}$ (Pilz et al., 1987). It can be measured electromyographically in the neck- or limb muscles (Caeser et al., 1989; Cassella et al., 1986; Pilz et al., 1988), but also non-invasively via automated recording systems. Automated recording systems for rodents consist of a cage, fixed to either a mechanical or electronic transducer, with which signals are recorded by an oscillograph or oscilloscope, respectively. Early constructions consisted of a postage stamp scale or similar spring suspension systems, referred to as *rat stabilimeter*, deviced by Mowrer (described by Brown, 1939 cited in Wilson and Groves, 1972). Induced currents in a coil by magnets attached to the cage were the first electronic sensors (e.g. Hoffman and Fleshler, 1964 cited in Wilson and Groves, 1972), subsequently replaced by accelerometer transducers based on the piezoelectric effect (cf. White and Horlington, 1969). However, also more sensitive mechanical systems based e.g. on strain-gauges are still used (Wilson and Groves, 1972). In subsequent years, methods have become more sophisticated and further optimised (cf. Cassella and Davis, 1986). In the late 1980s and early 90s, computer based systems were established, which

enabled fully automated stimulus control and measurement of startle (cf. Blumenthal and Cooper, 1990; Flaten et al., 1989). Today's systems are completely software controlled, triggering stimuli of various modalities and intensities, and measuring startle as voltage output of piezoelectric accelerometer transducers, amplified and digitised by personal computer systems.

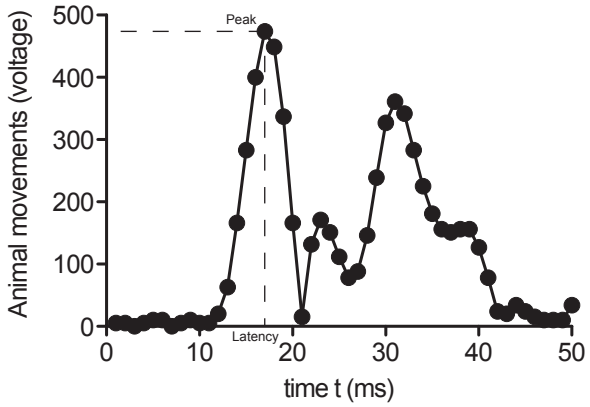


Figure 1.1.: Exemplary startle response trace of a C57BL/6N mouse to a 115 dB(A) noise burst of 20 ms duration, recorded with a piezoelectric accelerometer equipped system (SR-Lab™). After stimulus onset (t = 0), 50 data points were recorded (sampling rate 1 kHz). Peak latency is ca. 15 ms, peak voltage ca. 470 mV (= reported startle response) (Mauch, unpublished).

It should be noted however, that there are various other systems, especially made for measures of startle and its modifications for other species (cf. e.g. Hoffman and Ruppen, 1996). In humans for instance, electromyograms (EMG), preferably of the orbicularis oculi muscle, but also other muscles (cf. e.g. Siegmund et al., 2001), are still the method of choice, although recently infrared based measures have been introduced (Lovelace et al., 2006).

The neuronal basis of startle

Although the basic characteristics of startle are preserved throughout taxa, differences are reported for stimulus parameters eliciting and modifying startle already at the level of rodents (i.e. rats and mice, cf. Ison, 2001) as well as brain areas involved in modulation of the response (for review cf. Swerdlow et al., 2001).

However, the acoustic startle response (ASR) itself is thought to be mediated by phylogenetically old brain areas, involving the auditory nerve, the ventral cochlear nuc-

leus, the dorsal nucleus of the lateral lemniscus, the caudal pontine reticular nucleus (PnC), spinal interneurons and spinal motor neurons (Davis et al., 1982a). The PnC plays a central role in mediation of ASR, receiving direct input from different nuclei of the auditory pathway (cf. fig. 1.2). In particular the giant reticulospinal neurons of the contralateral PnC have been shown to receive neuronal input from cochlear nucleus, lateral superior olive and the cochlear root nucleus (Kandler and Herbert, 1991; Lee et al., 1996; Lingenhöhl and Friauf, 1992, 1994). Projections of the PnC neurons are found onto cranial, facial and spinal motor neurons (Lingenhöhl and Friauf, 1992, 1994), therefore acting as sensorimotor interfaces for the ASR. Additionally, in vivo intracellular recordings during acoustic stimulation from reticulospinal PnC giant neurons of the rat revealed an excitation threshold of about 75 dB, fitting well with the behavioural ASR threshold found in theses animals (Ebert and Koch, 1992; Lingenhöhl and Friauf, 1992, 1994). Furthermore, an averaged latency of ca. 2.6 ms for excitatory postsynaptic potentials (EPSP) and mean spike latency of 4.4 ms of these neurons (Lingenhöhl and Friauf, 1992, 1994) are consistent with the latency of 10 - 15 ms of the ASR, suggesting the giant reticulospinal neurons of the PnC to play the central role in acoustic startle mediation.

Startle modifications and mediating circuits

Enhancement of startle. According to the assumption of the startle reflex being a protective response, ASR is observed enhanced in diverse situations related to fear and anxiety. Thus, cues prediciting an aversive event (e.g. fear conditioning, cf. Pavlov, 1927; Davis and Astrachan, 1978) enhance startle (i.e. fear potentiated startle, cf. Brown et al., 1951; Davis, 1993). Startle is also found enhanced after sensitisation (e.g. application of electric shocks, Plappert et al., 1999) or in the presence of bright light (i.e. light enhanced startle, Walker and Davis, 1997a). In line with enhanced startle in animal models of fear and anxiety, startle is increased in humans anticipating an electric shock (Grillon et al., 1991), or being exposed to unpleasant odour or aversive pictures (Ehrlichman et al., 1995; Lang et al., 1990). Exaggerated startle is additionally found in patients suffering from anxiety disorders (for review see Grillon, 2008). Moreover, human startle responses are potentiated in darkness (Grillon et al., 1997), mimicking the finding of increased startle in rats in a bright lit environment (i.e. LES, cf. p. 13 and Walker and Davis, 1997a).

Startle enhancement is also apparent after stimulation of brain areas associated to anxiety and fear behaviour, namely the amygdala (Koch, 1993; Koch and Ebert, 1993;

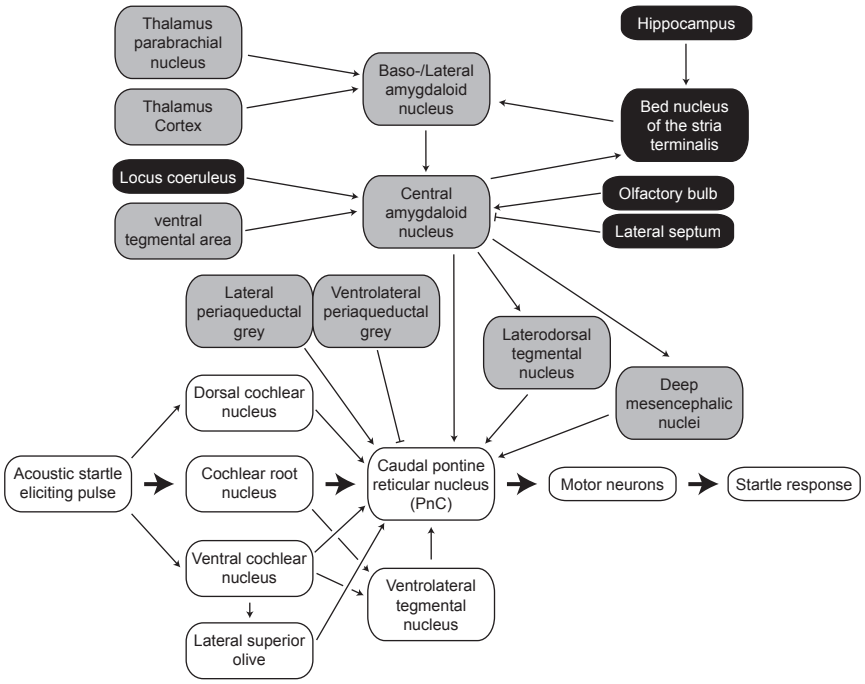


Figure 1.2.: A hypothetical circuit of brain regions mediating acoustic startle responses (white boxes) and its modifications by sensitisation (black boxes), and fear conditioning and sensitisation (grey boxes). Bold arrows indicate the probably fastest route of transmission. ⊣: inhibitory input, →: excitatory input. Adapted from Koch (1999).

Rosen and Davis, 1988; Yeomans and Pollard, 1993), the ventral tegmental area (VTA) and the periaqueductal grey (PAG) (Borowski and Kokkinidis, 1996). Beside stimulation, results from lesion as well as microinjection studies allowed the creation of a basal neuronal circuit that mediates startle enhancement by fear potentiation (cf. section 6.1), and stress and sensitisation (cf. fig. 1.2). Regarding sensitisation, data suggest the enhancement of startle via the medial central amygdala at the level of the PnC (Boulis and Davis, 1989; Davis et al., 1982b; Hitchcock et al., 1989), receiving amygdaloid input directly (Rosen et al., 1991) or via several relay nuclei, such as the periaqueductal grey (Fendt et al., 1994a), the laterodorsal tegmental nucleus (Hitchcock and Davis, 1991; Krase et al., 1994), or the deep mesencephalic nuclei (Frankland and Yeomans, 1995).

The role of corticotropin releasing hormone (CRH) in increase of startle is complex. Startle is enhanced after infusion of CRH into the lateral ventricle (Risbrough et al., 2003; Swerdlow et al., 1989), into the PnC (Birnbaum and Davis, 1998), and into the bed nucleus of the stria terminalis (BNST, Lee and Davis, 1997). Lee and Davis (1997)

also showed that lesions of, or infusion of the specific CRH receptor blocker αCRH into the BNST, but not the central amygdala (CeA), block CRH enhanced startle. The CeA has been shown to be crucial for mediating conditioned fear (cf. Davis et al., 1993). Consistently, Liang et al. (1992) reported no effect on startle after CRH infusion into the CeA. Additionally, fear potentiated startle and other paradigms of condtioned fear have been shown to be insensitive to infusion of CRH receptor blockers (de Jongh et al., 2003; Walker et al., 2009) as well as to lesions of the BNST (Gewirtz et al., 1998; Lee and Davis, 1997; Sullivan et al., 2004), while being susceptible to lesions of the CeA (Lee and Davis, 1997; Sullivan et al., 2004; Walker and Davis, 1997b). Contrary, light enhanced startle (LES) is not altered after CeA lesion, but disrupted after lesion of the BNST (Walker et al., 2009) and it is also found to be affected by CRH treatment (de Jongh et al., 2003; Walker et al., 2009). These observations led to the theory of phasic fear-like and sustained anxiety-like responses, where fear potentiated startle would belong to the first and light enhanced or CRH induced increase of startle to the latter phenomenon (Walker et al., 2009).

The ASR is also increased in an environment of loud noise (Hoffman and Fleshler, 1963). However, parametric analysis revealed different efficacy depending on startle eliciting pulse intensity and noise frequency band, and non-monotonic function of noise intensity (Davis, 1974; Gerrard and Ison, 1990). Additionally, Schanbacher et al. (1996) found this phenomenon to be independent of the amygdala, overall questioning a connection of this phenomenon to fear and anxiety (cf. section 6.2).

Attenuation of startle. Beside enhancement, there are several phenomenona causing attenuation of the ASR. Among habituation and pleasure attenuated startle, prepulse inhibition (PPI) has been most extensively studied. PPI is mediated by brainstem structures (cf. fig. 1.3). After perception of an auditory prepulse by peripheral auditory systems (ear, cochlea nuclei), the information is probably passed on to the inferior colliculus (IC). Complete lesion of IC totally abolishes the inhibitory effect of prepulse (Leitner and Cohen, 1985), while small lesions only decrease the amount of inhibition (Li et al., 1998). Furthermore, stimulation of the IC mimics the effect of prepulse on startle with an optimal interstimulus interval of 15 - 30 ms (Li et al., 1998), consistent with maximal prepulse inhibition at intervals of about 50 - 100 ms, considering response latencies of IC neurons of 7 - 40 ms (Li and Kelly, 1992).

The superior colliculus (SC) might serve as a integration centre, receiving inputs from auditory, somatosensory and visual areas (Meredith et al., 1992), and passing informa-

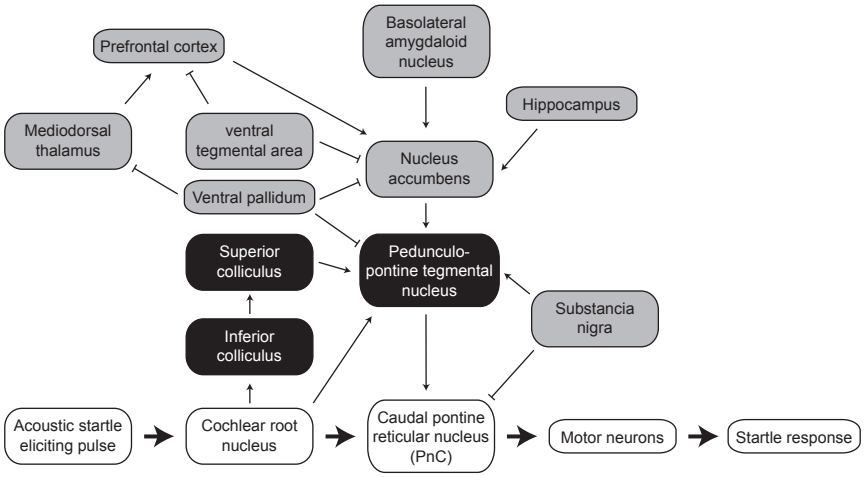


Figure 1.3.: A hypothetical circuit of brain regions mediating prepulse inhibition (black boxes) of the acoustic startle response (white boxes), and modifications of prepulse inhibition (grey boxes). ⊣: inhibitory input, →: excitatory input. Adapted from Fendt and Yeomans (2001) and Swerdlow et al. (2001).

tion about prepulses of these modalities to the PPI mediating structures. Fendt et al. (1994b) demonstrated that lesions of the SC attenuate and pharmacological stimulation facilitates PPI (Fendt, 1999). Furthermore, and analogue to manipulation of the IC, electrical stimulation of the SC mimics the effect of acoustic prepulses on startle (Li and Yeomans, 2000).

Among other structures, the SC projects to the pedunculopontine tegmental nucleus (PPTg, Redgrave et al., 1987; Semba and Fibiger, 1992), which in turn is crucial in mediating PPI. Lesions as well as pharmacological inactivation of the PPTg strongly disrupt PPI of startle (Kodsi and Swerdlow, 1997; Swerdlow and Geyer, 1993), and stimulation of the PPTg are shown to mimic prepulse inhibition by acoustic prepulses (Li and Yeomans, 2000). Additionally, the laterodorsal tegmental nucleus (LDTg) is similarly critical involved in PPI mediation (cf. Jones and Shannon, 2004), while both structures send cholinergic fibres to the PnC (cf. p. 6).

Prepulse inhibition itself is under influence of other brain structures. Koch et al. (2000) found prepulse inhibition to be reduced after lesions of the substantia nigra (SNR). SNR lesion protects against PPI disruption caused by treatment with PPI modulating drugs (Bakshi and Geyer, 1998; Swerdlow et al., 1990 and cf. section 7), and the SNR has direct connections to the PPTg as well as γ-aminobutyric acid (GABA) -ergic projections to the PnC (Beninato and Spencer, 1986; Yasui et al., 1992). Thus, SNR is thought to be

a part of the PPI mediating pathway, but also to be a relay mediating PPI modulation by higher brain areas (cf. p. 25).

2. The startle response in paradigms of anxiety and fear

The repertoire of behavioural and physiological phenotypes of fear and anxiety is well preserved within the vertebrates, including increase of heart rate and blood pressure, freezing, startle, and flight behaviour (cf. Belzung and Philippot, 2007; Misslin, 2003; Stiedl and Spiess, 1997). Anxiety disorders are among the most frequently diagnosed psychiatric disorders and maladaptive fear and anxiety behaviour is found as a symptom accompanying almost all psychiatric diseases (cf. Kessler et al., 2005; Lang and McTeague, 2009). Commonly fear is stated to be object related and anxiety object un-related. Work by Mobbs et al. (2007) additionally demonstrates that the intensity of fear reactions is associated with threat distance, and that brain activity shifts from higher cortical areas (prefrontal) to lower reflex related areas (periaqueductal grey) as the threat distance decreases.

The question, whether fear is an emotional state or merely a reflexive response is important, since laboratory animals can only be measured for physiologic markers (e.g. heart rate) and behaviour, but not emotion. On the other hand, emotion and the ability to interfere with emotional states are of high interest in terms of human psychiatric disorders, and implications for the treatment of these diseases drawn from animal experiments. A shift in emotion is always accompanied by a shift in physiological markers and the behavioural repertoire (e.g. smiling and crying). From a human point of view, a specific sensation is intrinsically tied to these measurable biological expressions of emotion, and there is no reason why one should doubt that at least higher vertebrates share this property with humans. In fact there is evidence that some animals are able to show empathic behaviour, while empathy implicitly requires emotional sensation (Fraser and Bugnyar, 2010; Langford et al., 2006, cf.). Together, the author states that fear is an emotional state, but according to work by Mobbs et al. (2007) one has to dissociate fear to distal (avoidable) and proximal (unavoidable) threats, that can be differentiated by their respective behavioural response.

These different behaviours associated with fear or anxiety are excited reliably under laboratory conditions, and can usually be easily observed and quantified. Hence, fear and anxiety are the most studied emotional states in animal experiments.

A frequently used paradigm in fear research is classical (or Pavlovian) fear conditioning (Pavlov, 1927). The experimental subject is presented a neutral stimulus before receiving a stimulus that unconditionally leads to a physiological response (unconditioned stimulus, US). The subject learns to associate the neutral stimulus (now termed conditioned stimulus, CS) with the US, the CS itself now leading to the same physiological response. The CS usually consists of visual or acoustic stimuli (cf. Goldstein, 1975), the latter playing the most important role in mouse experiments (cf. fig. 2.1A). Unfortunately, acoustic CS are applied in a huge variety of duration and quality combinations (cf. fig. 2.1A,B), while it remains unclear how these different stimulus parameters interfere with behaviour and learning of the animal. According to fear conditioning, extinction of conditioned fear is conducted with the same variety of stimulus parameters. Extinction is a paradigm of memory inhibition. During repeated presentation of the unreinforced CS the experimental subject learns that the CS does not predict the US any more (cf. Ji and Maren, 2007 and section 6.4).

Like elemental cues (light, tones, etc.), animals can be conditioned to whole contexts (cf. Rudy and O'Reilly, 2001). There is an ongoing debate in the literature, as to whether and under which circumstances the hippocampus (HPC), generally thought to be indispensable in spatial, configural and contextual learning tasks, plays a role in contextual conditioning (cf. Anagnostaras et al., 2001; Ji and Maren, 2007; Maren, 2008). HPC lesions do not necessarily lead to disturbed contextual conditioning, but may lead to impaired cue learning as well (for review see Maren, 2008). This may indicate that the function of the HPC is to form a unitary representation of what is called a context (Rudy et al., 2004) via a process of pattern completion within the hippocampus (HPC) put forward by Marr (1971) and developed by McNaughton and Morris (1987), as well as Wickelgren (1979), theorising the HPC to form a unitary representation from independent experiences, and Rudy and Sutherland (1995), describing the HPC as a configural association system. By now, theoretical network models based on empirical findings support the idea of pattern completion and, reversely, separation as a function of the CA3- and DG-region of the hippocampus, respectively (cf. Myers and Scharfman, 2010).

In most studies of fear and anxiety, these emotional states are quantified by means of the amount of freezing behaviour during a defined period of time. Usually, freezing is defined as total immobility of the observed animal except for respiratory movements (cf. e.g. Blanchard et al., 1975). Apart from freezing, the acoustic startle response (ASR) is frequently used as a measure of the emotional state of an animal in fear and anxiety related experiments. Not earlier than 1951, Brown et al. introduced the startle response as a measure of fear in animal studies. Based on the anecdotal evidence that humans startle more when they are afraid, Brown and colleagues showed that the ASR is increased by a preceding acoustic stimulus that has been previously conditioned to an averse stimulus (US).

Including drug withdrawal states, Schizophrenia or the post-traumatic stress disorder (PTSD), the startle response is found to be altered in the context of a diverse range of psychiatric disorders (cf. Howard and Ford, 1992). Today, the ASR has evolved to a standard measure not only in human psychiatric research, but also in animal models of respective diseases (cf. Braff et al., 2001; Geyer et al., 1990; Grillon, 2002). The paradigms include light enhanced startle (LES) in rats, where ASR is augmented when stimuli are presented in a lit environment (Walker and Davis, 1997a); fear potentiated startle (FPS), where a previously conditioned stimulus presented before startle stimulus presentation leads to increased reactivity (Brown et al., 1951); prepulse inhibition (cf. section 3); sensitisation, where the animal is subjected to aversive situations or stress (e.g. electric footshocks or forced swimming) before ASR is measured (e.g. Davis, 1972); and baseline startle measurements elicited by stimuli of suitable parameters (e.g. Mansbach et al., 1992).

LES is commonly interpreted as a measure of anxiety. In contrast to FPS, where fear to a harm-predicting stimulus is tested, the light stimulus in LES does not predict any harmful experience, but creates a potentially harmful environment for rats, which are usually active during dawn or night and avoid bright light (Walker and Davis, 1997a). As indicated above (p. 12), mice seem to be more susceptible to acoustic stimuli than to light. Hence, LES is of minor interest in mouse studies, although there is some work reporting successful application (cf. Hironaka et al., 2002; Salam et al., 2009).

As freezing behaviour, FPS is thought to be a measure of fear (e.g. Hitchcock and Davis, 1991). The presentation of the CS predicts a concrete threat to the animal. In this state of fear, the experimental subject is more susceptible to startling stimuli, resulting in a higher ASR (Brown et al., 1951). As LES, FPS is susceptible to anxiolytic drugs such as benzodiazepines (Davis, 1979; Smith et al., 2010; Walker and Davis, 2002a). Applied

to mice only about a decade ago (Falls et al., 1997), FPS compared to analysis of freezing behaviour has some advantages. For instance the ASR can be measured easily by means of automated data recording, thus ensuring objective data acquisition. On the other hand, the startle reflex can be modulated by a variety of internal and external factors (cf. Koch, 1999), demanding careful experimental design and measuring. Additionally, the variability of the ASR necessitates repeated measuring to achieve reliable data, including repeated presentation of the CS, that could interfere with the experimental design in e.g. extinction experiments.

Sensitisation of the ASR is simply achieved by putting the experimental subject through aversive or stressful situations. Thus, sensitisation is present in almost all paradigms of ASR measurement. Even repeated presentation of startle eliciting stimuli may put animals to a sensitised state (Davis and Sheard, 1974; Groves and Thompson, 1970; Plappert et al., 1999), leading to increased ASR or impaired habituation of the ASR. Sensitisation may serve as a tool to characterise animal strains in terms of stress coping abilities (e.g. Gonzales et al., 2008), but can also be used to study the effect of genes on the neural mechanism of behaviour (cf. Plappert and Pilz, 2001). Additionally, the ASR is found to be increased in animal models of PTSD (e.g. Servatius et al., 1995; Khan and Liberzon, 2004 and cf. section 6.5) like it is found in patients (e.g. Butler et al., 1990; Grillon et al., 1996; Ornitz and Pynoos, 1989), showing the sensitising effect of trauma-like events.

Baseline startle usually is measured presenting startle eliciting stimuli of three or more intensities. It is a common measure of baseline emotional states, such as anxiety (cf. Grillon, 2008) or arousal (cf. Samuels et al., 2007), but is also used to assess hearing capabilities in animals (Willott et al., 1984 and cf. section 6.5.1).

After a short excursion characterising FPS in a mouse-strain used as background strain of genetic mutants (e.g. Marsicano et al., 2002) and model of PTSD (e.g. Golub et al., 2009) at the Max-Planck-Institute of Psychiatry (MPI-P), Munich (section 6.1), this chapter introduces a paradigm which may be applicable in measures of hearing capability, acoustic stimulus adaptation or attention in mice. Based on the work by Hoffman and Fleshler (1963) and educed from FPS measurements, the paradigm of tone enhanced startle (TES) is characterised and applied in three experiments which shall demonstrate the use of TES as the proposed methods (section 6.2).

Being aware of the importance of the chosen parameters in startle paradigms, section 6.3 tries to elucidate the pitfalls of stimulus parameters in fear conditioning. Pure

tones (sine wave) and noise stimuli have been similarly often and uncritically applied as conditioned stimuli in fear conditioning and extinction of conditioned fear (cf. fig. 2.1). The present data unequivocally demonstrate that white noise and sine wave stimuli differ markedly in their impact on animal learning and behaviour.

Fear conditioning is not only dependent of the parameters of the used conditioned stimuli (CS), but can also depend on the context where learning takes place (cf. Effting and Kindt, 2007). The proposed process of pattern completion might take place also during memory retrieval upon a single reminder, such as the CS. In section 6.4 the hypothesis is tested that during fear extinction training the presentation of the CS also weakens other associations to the US acquired during conditioning, such as the conditioned context. This would lead to alleviated fear response not only to the CS, but to the conditioned context as well. The hypothesis is refused while demonstrating that the fear response (i.e. freezing) to the CS tone can be readily measured by means of animal movements recorded by a piezoelectric device usually used to record the startle response.

In section 6.5 the usability of measuring startle is demonstrated applying this measure to two mouse models established at the MPI-P. It is shown that animals related to high anxiety of the HAB/LAB mouse-model of trait anxiety (cf. Krömer et al., 2005) acquire conditioned fear better than animals related to low anxiety. Subjecting these mice to measures of baseline startle and TES (cf. section 6.2), and response to electric stimuli, it is proposed that this difference cannot be attributed to differences in hearing capability or shock sensitivity.

Measuring baseline startle in a mouse-model of PTSD (cf. Siegmund and Wotjak, 2007) it is demonstrated that the ASR can be readily used to measure hyper-arousal in these animals (Golub et al., 2009 and section 6.5). Having shown that this measure is an independent factor of the symptoms of this PTSD-model (Pamplona et al., 2010), the present work also demonstrates the independence of hippocampal shrinkage following a traumatic event (i.e. intense footshock) and hyper-arousal.

2.1. Fear potentiated startle in C57BL/6N mice

Fear potentiated startle (FPS) is a paradigm to measure and quantify fear in animals. Introduced in 1951 by Brown et al., it is frequently used in animal studies of fear and anxiety (for review see Davis, 1990). It involves a conditioning session, where the experimental subject learns to associate a neutral stimulus, such as a tone, with a stimulus (US) leading to an unconditioned response (UR), the now conditioned stimulus (CS) leading

then to the same response (cf. p. 12). In the test session, the experimental subject is then presented with a sequence of startle eliciting stimuli alone (pulse P) and preceded by the CS (CS + P). The potentiation of the startle response (SR) is then expressed as either difference or percental change. Since prior conditioning or drug treatment or else may affect baseline startle of the animals compared, Walker and Davis (2002b) recommended the use of percental rather than absolute (difference) values, thereby controlling for baseline startle changes. Grillon and Baas (2002) argue that since the nature of startle changing effects might be unknown, difference and percental change should be analysed. The data presented here will be reported in both ways.

FPS can be elicited across species, also in humans (Grillon and Davis, 1997). This has the rare advantage to corroborate the idea that physiological signs of fear measured in animals and humans reflect indeed a state of emotional fear, since these physiological measures are accompanied by verbal reports of a state of fear in humans.

Since FPS is a paradigm based on fear conditioning, brain areas involved in mediating the enhancement of startle via CS - US association are the same (cf. fig. 1.2). The association is built in the lateral and basolateral amygdala (Campeau and Davis, 1995; Miserendino et al., 1990), integrating input from sensory and nociceptive brain structures (Doron and Ledoux, 1999; Linke et al., 1999; Shi and Davis, 1999). The amygdala mediates the potentiation of the startle response via direct efferent pathways (Davis et al., 1993), probably by corticotropin releasing hormone (CRH) or glutamate (Fendt et al., 1996b, 1997). Additionally, other structures such as the periaqueductal grey or the laterodorsal tegmental nucleus were shown to affect fear potentiated startle (Fendt et al., 1996a; Fendt and Fanselow, 1999; Hitchcock and Davis, 1991) while sending projections to the startle mediating caudal pontine reticular nucleus (PnC) (Borowski and Kokkinidis, 1996; Koch et al., 1993), suggesting these nuclei as relays between amygdala and PnC (Koch, 1999).

As other behaviour associated with conditioned fear, FPS is attenuated by several inhibitory manipulations such as extinction (cf. section 6.4) or latent inhibition (Schauz and Koch, 1998, 1999, 2000). Among others, compared to freezing (cf. p. 13) (fear potentiated) startle has the advantage not only to detect response changes by means of increase, but also decrease. Hence, FPS could be a useful measure in experiments of fear-, but also security learning, introducing a conditioned inhibitor that predicts the absence of the US (cf. Falls and Davis, 1995, 1997).

Additionally there might be animal models where animals exhibit a high locomotory

drive, thereby not being able to freeze even in their highest state of fear. Since the ASR is a basic reflex, it is very reliable and thus could be used as an alternative measure of fear in such animal models.

In the following, FPS is characterised in the C57BL/6N mouse strain in terms of CS (i.e. light and tone), pulse intensity and context dependency. For future application of the FPS paradigm it was of particular interest to strictly differentiate between the conditioning and the test context. Thus, conditioning was conducted in the FC-apparatus (cf. p. 35) in a room separate from the room where startle measures took place. Finding strong non-associative startle enhancing tone effects (i.e. TES, cf. section 6.2) that masked conditioned effects, FPS was not followed up further.

2.2. Tone enhanced startle as a measure of hearing capability, stimulus adaptation and attention

Unconditioned alterations of the acoustic startle response (ASR) may occur in the presence of increased acoustic environments. Hoffman and Fleshler (1963) first described increased ASR in an environment of steady background noise in rats. In subsequent studies, they analysed temporal characteristics of this phenomenon, showing that seconds-long, continuously presented noise facilitates ASR, while discrete noise pulses inhibits ASR (later termed PPI, cf. section 7, Hoffman and Wible, 1969). In subsequent years the phenomenon of startle enhancement by background sound was further characterised by Hoffman, Ison and colleagues (Gerrard and Ison, 1990; Hoffman and Searle, 1965; Ison and Hammond, 1971; Ison et al., 1973; Ison and Russo, 1990), evaluating dependence of background sound intensity and spectral composition, and the intensity of the eliciting stimulus (Davis, 1974).

The paradigm of prepulse facilitation of startle (PPF) by preceding long stimuli ($\geqslant 2\,\mathrm{s}$, cf. PPF by short prepulses, p. 27) has been descibed for humans and rats (Hsieh et al., 2006; Reijmers and Peeters, 1994; Reilly and Hammond, 2001), and sometimes applied in measures of attention (cf. Conzelmann et al., 2010; Wynn et al., 2004). The phenomenon is also apparent in mice, although simply described as unconditioned effects of pre-stimuli by Falls and colleagues (Falls et al., 1997; Falls, 2002; Heldt et al., 2000) applying the paradigm of fear potentiated startle (cf. section 6.1). While Carlson and Willott (2001) demonstrated that the phenomenon of background sound startle alteration is apparent and equally complex in mice as in rats, work by Hoffman and Wible (1969) already

suggests that enhancement of startle by background sound presentation and facilitation by startle pulse preceding stimuli is equivalent.

Having found strong and reliable enhancement of the ASR by preceding sine wave tones in the C57BL/6NCrl mouse strain (cf. section 6.1), the phenomenon of *tone enhanced startle* (TES) is characterised and the usefulness as a tool in mouse behavioural experiments is tested.

There is usually the need of invasive techniques or manipulation of the emotional state (e.g. fear conditioning) to test for experimentally relevant properties of an animal, such as hearing capability or stimulus adaptation. Startle can be reliable elicited in a variety of species and data acquisition is today easily achieved by automated response recording. Modifications of the startle response can be achieved by divers parameter- or environmental changes which enables to draw conclusions on stimulus neuronal processing. Therefore the startle reaction is ideally suited as a basal measure for characterisation of a naive animal. Since in the paradigm of TES the startle response is affected by a preceding acoustic stimulus, TES offers the possibility to measure properties related to stimulus perception. It is therefore proposed that TES might be applicable in terms of measuring hearing capability, stimulus adaptation and attention.

2.3. Fear conditioning parameters - the matter of fact

Our knowledge about communicational and functional processes in the brain is based to a large extend on studies of fear conditioning (FC) and extinction of conditioned fear (ExFC) in rodents. During the last decade, fear conditioning as well as extinction in the mouse gained more and more importance. Its ability to reliably acquire memory in a FC- as well as ExFC-task together with the possibilities of genetic manipulation make the mouse the most important animal model to study gene-memory interactions in mammals.

Stimulus parameters of startle and startle modulation have been well defined and characterised (cf. Blaszczyk and Tajchert, 1997; Hoffman and Searle, 1965; Ison et al., 1997; Plappert et al., 2004; Stoddart et al., 2008), demonstrating that they may have considerable impact on animal behaviour. Nevertheless, the characteristics of applied stimuli in FC and ExFC vary considerably between the different laboratories. This is shown not only by the stimulus frequency-spectrum of more than three octaves, whereby the frequencies used often do not match the perceptibility of the mouse ear (Ehret, 1976; Marsch et al., 2007; fig. 2.1A,C). It is also shown by the use of stimulus intensities, which

cover a range of about 50 dB (50 - 100dB, fig. 2.1B).

While the latter may be due to the mouse hearing abilities at different frequencies or results from constructional aspects of the FC-system, the indifferent use of stimulus length and stimulus quality (i.e. sine wave vs. noise) in FC (fig. 2.1A) and ExFC (fig. 2.1C) could be more critical. Kamprath and Wotjak (2004) have shown that non associative processes like sensitisation and habituation may determine expression of conditioned fear. Thereby the duration of the conditioned stimulus (CS) may affect acquisition and/or extinction of conditioned fear. Stimulus quality has been shown to affect animal behaviour in measures of acoustic startle response (ASR) and prepulse inhibition (PPI) as well as prepulse facilitation (i.e. TES, see p. 27 and section 6.2, and cf. Hsieh et al. (2006); Stoddart et al. (2008); Wynn et al. (2000)). Additionally, the natural acoustic environment of mice mainly consists of broadband noises and multiple-frequent sounds. Therefore, pure sine wave tones may differ in their ecological significance from sounds and noises. Thus, sine wave and noise stimuli could alter animal behaviour in a FC or ExFC experiment.

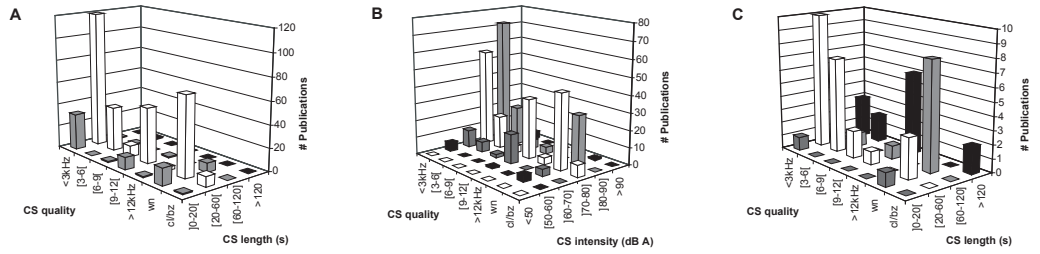


Figure 2.1.: Number of publications dealing with auditory cue fear conditioning in mice during the past ten years (2000 - 2010). Combinations of stimulus parameters length and quality (A,C) as well as stimulus quality and intensity (B) vary considerably in fear conditioning studies (A,B) and in extinction of conditioned fear (C). wn: white noise, cl/bz: clicking/buzzing.

In a literature survey an indifferent use of different qualities (i.e. noise and sine wave stimuli) and length, as well as intensity of acoustic stimuli in FC and ExFC in mice became apparent (fig. 2.1). Apparatus differences, mostly regarding orientation of speaker and FC-chamber material and structure of chamber walls, etc., could necessitate application of different stimulus intensities. The experiments described below focused therefore on stimulus quality and length. The following experiments shall clarify, whether stimulus quality and length in terms of FC and ExFC are leading to differences in animal behaviour and thereby could lead to different interpretations of measured data.

To assess the importance of length and quality of conditioned stimulus (CS) in fear con-

ditioning (FC) and extinction of conditioned fear (ExFC) experiments, acoustic startle response (ASR) and acquisition as well as extinction of conditioned fear to either sine wave (sw) or white noise (wn) stimuli of different duration is measured. It is shown that freezing response as well as ASR differ remarkably between sw and wn in mice during the course of conditioning and extinction retrieval, and basal as well as fear potentiated (FPS) and tone enhanced startle (TES, cf. section 6.2), respectively.

2.4. Extinction of conditioned fear to context by cue extinction training

By repeated unreinforced presentation of an previously conditioned stimulus, the fear response to this stimulus is alleviated. This process of fear extinction neither represents forgetting of the initially learned pairing of CS and US, nor does it overwrite the initial fear memory, but rather suggests a new memory built up (i.e. CS + ~~US~~, Bouton (2004); Delamater (2004); Myers and Davis (2002)). This is indicated by several recovery effects (renewal, spontaneous recovery, reinstatement), not only demonstrating an intact initial memory trace (for review see Ji and Maren, 2007), but also that extinction is highly context specific and context dependent (e.g. Bouton and King, 1983).

As in other spatial, configural and contextual learning tasks, context association in conditioning was long thought to be a function exclusively of the hippocampus (HPC), indicated by impaired context conditioning after HPC lesion. However, findings of intact context conditioning with impaired or lesioned HPC question this concept (for review see Maren, 2008).

Preliminary data in the group of Dr. Carsten T. Wotjak at the Max-Planck-Institute of Psychiatry, Munich (MPI-P), show that two days after conditioning an olfactory part of the conditioned context mimics the fear response to a contiguously conditioned stimulus (asymptotic increase vs. exponential decrease of freezing, respectively) when an animal is exposed to this context after the HPC was inactivated by injection of muscimol (Dr. Carsten T. Wotjak, personal communication). This observation makes it plausible to postulate a direct encoding of cues of all sensual modalities parallel to the encoding of the summary of these cues (i.e. context) by the HPC. Otherwise, if contextual information would be only processed by the HPC, a context feature would not be able to elicit a fear reaction in retrieval when the HPC is inactivated. Interestingly, Sacco and Sacchetti (2010) found that excitotoxic lesions of auditory, visual, or olfactory secondary sensory

cortices modality-specific impaired remote, but not recent, fear memories, arguing for direct modality-specific memory encoding.

Conversely, Rudy et al. (2004) proposed the theory of dual representation of the context,

> [...] the features view, where context is represented as a set of independent features or elements that each can enter into association with an event [...]

and

> [...] the conjunctive view, where the separate features are bound into a new unitary representation that encodes their conjunction or co-occurrence [...].

Thus, context conditioning per se can also be achieved without HPC function by association of single features and US by neocortical systems (Nadel and Willner, 1980), but interaction with the HPC is necessary to elaborate a unitary conjunctive representation out of all contextual features (Rudy and O'Reilly, 2001).

If contiguously paired cues (i.e. CS) and the so called contextual features are processed directly and through the HPC in parallel, extinction training of the CS should lead in turn not only to a decreased fear reaction to the CS, but also to the context. The present work therefore predicts that the presentation of the CS during extinction training leads to a recall of the configural representation (pattern completion), and thereby also to extinction of context fear. This of course only as long as extinction training is performed in a short time period after conditioning, when the HPC is still active in pattern completion (i.e. memory consolidation, cf. Rudy and O'Reilly, 1999). In turn, context extinction through CS extinction should not occur when extinction training is done a long time after conditioning.

This hypothesis is tested by conducting fear extinction in the startle apparatus. Thus conditioning and extinction training takes place in two very different contexts in different rooms, ensuring complete independence of fear and extinction learning. Demonstrating that the fear response to the CS can be readily measured by means of movement scores in the startle apparatus, it is shown that extinction to a high degree depends on the context while the hypothesis of context fear extinction via CS presentation induced recall of the configural representation has to be rejected.

2.5. ASR measures in mouse-models of trait anxiety and PTSD

The reliability of anxiety related behaviour makes it possible to draw conclusions from animal studies which also account for other vertebrates, including humans (cf. Belzung and Philippot, 2007). This allows the creation of animal models to study the physiological background of fear and anxiety, as well as circumstances which lead to pathological changes in anxiety and fear behaviour.

One model of maladaptive behaviour established at the Max-Planck-Institute of Psychiatry, Munich (MPI-P), is the high/low anxiety related behaviour mouse line (Krömer et al., 2005).

Based on the performance in the elevated plus-maze (EPM) paradigm, a bidirectional breeding approach led to rats which are characterised as high (HAB-R) and low (LAB-R) anxiety related behaviour rats (Liebsch et al., 1998a,b). This animal model is found to mimic symptoms of trait anxiety like it is found in patients (Neumann et al., 2005). As proteomic analysis already revealed differences in protein-expression patterns (Salomé et al., 2004) and hormone activity (Landgraf et al., 1999; Murgatroyd et al., 2004) between these animals of high and low anxiety related behaviour, the same breeding approach was applied to mice. This enables the use of powerful genetic methods available in these animals to study the genetic contribution to the different behavioural phenotypes and to look for putative biomarkers of trait anxiety (Ditzen et al., 2006, 2010).

In this section it is shown that HAB mice express higher fear to conditioned stimuli than LAB or NAB (normal anxiety related behaviour) mice. Measuring startle response to acoustic and electric stimuli, and tone enhanced startle (TES, cf. section 6.2), it is proposed that differences in hearing capability or electric footshock susceptibility do not account for these differences. Interestingly, HAB mice differ tremendously from LAB and NAB mice, showing very low ASR, but high TES.

Another model recently established at the MPI-P is a model of the post-traumatic stress disorder (PTSD) in mice (Siegmund and Wotjak, 2007) based on a single intense electric footshock.

PTSD was first defined in Diagnostic and Statistical Manual of Mental Disorders, 3rd edition (Spitzer, 1980). Early following studies already effectively identified significant

risk factors and established the disorders symptoms (Peleg and Shalev, 2006). Although in subsequent years animal models were established to study the pathophysiology of PTSD, the processes of sensitisation and conditioning, named as critical psychobiological processes underlying PTSD (Charney et al., 1993), were studied in independent branches of research (Siegmund and Wotjak, 2006). Emphasising the involvement of associative (i.e. conditioning) and nonassociative (i.e. sensitisation) processes in the development of PTSD, Siegmund and Wotjak (2006) defined criteria to establish animal models of PTSD that meet not only face, but also predictive as well as construct validity.

The model subsequently presented by these authors (Siegmund and Wotjak, 2007) was then shown to fulfil criteria for face and predictive validity and was further studied in terms of risk and prediction factors (Siegmund et al., 2009a,b), treatment strategies (Golub et al., 2009) and interdependency of the observed symptoms (Pamplona et al., 2010; Siegmund and Wotjak, 2007).

Among others, known symptoms of PTSD are abnormal levels of corticotropin-releasing hormone (CRH) and increased startle responsiveness, as well as decreased hippocampal (HPC) volume. PTSD patients have been shown to exhibit high CRH concentrations in the corticospinal fluid (CSF) compared to healthy people (Baker et al., 1999; Bremner et al., 1997; Sautter et al., 2003) and dysfunction of hypothalamus-pituitary-adrenal (HPA) axis (Yehuda et al., 1991; Handwerger, 2009). Pharmacological enhancement of cortical CRH elevate the startle response at least in animal experiments (Risbrough et al., 2003; Swerdlow et al., 1986; Walker et al., 2009) which suggests a linkage between CRH hyperfunction and exaggerated startle response found in these patients (e.g. Holstein et al., 2010; Kinzie et al., 1984).

Among other structural changes found in the brain of PTSD patients compared to healthy people, the decrease in hippocampal (HPC) volume is very prominent. It is still controversially discussed whether the observed HPC volume and functional alterations are related to the symptomatology of PTSD or merely to trauma experience per se. Brohawn et al. (2010) stated that the HPC, in interplay with the amygdala, is closely related in processes mediating the enhancement of emotional memory and the integrity of this interplay may be compromised in PTSD. On the other hand, Winter and Irle (2004) failed to find differences in HPC volume between healthy trauma-exposed individuals and trauma-exposed PTSD patients. Furthermore, Gilbertson et al. (2002) found an association between HPC volume and PTSD prevalence in monozygotic twins which indicates HPC volume to be a risk factor for rather than a symptom of PTSD. These different observations demand further studies, presumably under controlled conditions

that can only be provided by animal models.

The PTSD mouse model of Siegmund and Wotjak (2007) has been shown to be a useful tool to study PTSD in various aspects of the disease (Golub et al., 2009; Pamplona et al., 2010; Siegmund and Wotjak, 2007; Siegmund et al., 2009a). This is further illustrated by the present work, applying measures of acoustic startle response (ASR) and hippocampal volume on mice that experienced the PTSD-protocol (i.e. single electrical footshock, cf. Siegmund and Wotjak, 2007). It is shown that also in terms of startle reactivity and HPC volume this mouse model of PTSD resembles patient data. Demonstrating that intracerebroverntricular CRH injections lead to increased ASR, which is prevented by co-treatment with alphahelical CRH (αCRH), a CRH-receptor blocker, and that mice which underwent the PTSD-protocol show increased ASR, too, further investigations are suggested, addressing a linkage between increased cerebral CRH levels and PTSD symptoms in this mouse model. Additionally, while hyper-arousal (i.e. increased startle response) has been recently shown to be an independent emotional dimension in this model (Pamplona et al., 2010), the present data demonstrate that hyper-arousal is also independent of HPC function and, by analysing HPC volume with the imaging technique ultramicroscopy (cf. Dodt et al., 2007), these mice have decreased HPC volume which is prevented by enriched housing conditions.

3. Pharmacological and optogenetical manipulation of prepulse inhibition

Prepulse inhibition (PPI) of the acoustic startle response (ASR) is a reliable behavioural tool to measure sensorimotor gating in vertebrates (cf. e.g. Burgess and Granato, 2007; Sasaki et al., 1998; Schall et al., 1999; Swerdlow et al., 2001). Most excessively studied in rats, this paradigm is also well characterised in the mouse (cf. Plappert et al., 2004) and frequently used preferably in animal models of schizophrenia, resembling the finding of disturbed sensorimotor gating in these patients (for review see van den Buuse, 2010).

Inhibitory reflex modification was first studied and described in 1862 by Sechenov (quoted in Ison and Hoffman, 1983). He found the cutaneous flexor reflex in the frog inhibited after presentation of midbrain stimulations preceding the tactile stimulus. In the auditory system, Peak (1939) reported an inhibition of the perceived intensity of an acoustic stimulus when it followed the same stimulus by 177 ms. The paradigm of PPI is based on the work by Hoffman and Fleshler (1963), who studied the effects of different background sounds on the ASR in rats. They found the ASR to be inhibited when startle eliciting pulses (P) were presented in a pulsed background noise of 1 Hz. Eventually it was found that a single prepulse (PP) presented in a certain time interval before the pulse is sufficient to decrease the ASR up to 80 - 90 %, depending on the chosen parameters (Hoffman and Searle, 1965). Findings by Buckland et al. (1969) and Pinckney (1976) that the ASR is also inhibited by visual and tactile prepulses, respectively, supported the hypothesis of PPI as a general mechanism of reflex inhibition.

Although some authors describe PPI simply as an attentional deficit caused by the distracting prepulse (cf. Filion et al., 1998; Schell et al., 2000), most authors follow the theory of Graham (1975). He proposed low-intensity changes in the sensory environment to automatically trigger a gating mechanism attenuating irrelevant responses, thereby protecting the perceptional processing of the leading stimulus.

This process of *sensorimotor gating* is modulated under a variety of experimental

conditions. Alterations of PPI have been found among others to depend on ovarian hormones (Koch, 1998, but cf. Plappert et al., 2005), breeding conditions (Geyer et al., 1993) and genetic background (Swerdlow et al., 2007), and is also found to be disrupted in some psychiatric disorders, such as Huntington's disease (for review see Abbruzzese and Berardelli, 2003), Tourette Syndrome (Castellanos et al., 1996), obsessive compulsive disorder (Swerdlow et al., 1993), or, most prominent, Schizophrenia (for review see Powell et al., 2009).

Brain structures associated with modulation of PPI are the hippocampus (HPC), amygdala (AMY), nucleus accumbens (NAC), and the prefrontal cortex (PFC, cf. p. 29) (fig. 1.3). Prepulse inhibition is modulated by cholinergic as well as glutamatergic activity within the hippocampus (Caine et al., 1991; Koch, 1996; Wan et al., 1996). The HPC has been shown to have direct projections to the prefrontal cortex (Ferino et al., 1987; Swanson, 1981), which in turn affects PPI mainly in a manner of dopamine, but also glutamate activity (Bubser and Koch, 1994; Koch and Bubser, 1994; Schwabe and Koch, 2004; Swerdlow et al., 2006 and cf. section 7.1.2). The nucleus accumbens has a pivotal role in mediating modulation of PPI. HPC as well as PFC directly innervate the NAC (cf. Carr et al., 1999; French and Totterdell, 2002). As within the PFC, dopaminergic (Swerdlow et al., 1986, 1990; Wan et al., 1994; Zhang et al., 2000) as well as glutamatergic transmission in the NAC is crucially involved in PPI modulation (Grauer and Marquis, 1999; Reijmers et al., 1995; Wan and Swerdlow, 1996).

In addition, Decker et al. (1995) and in more detail Wan and Swerdlow (1997) found that also the amygdala, in particular the basolateral part (BLA) is involved in modulation of prepulse inhibition. Other manipulations, such as electrical kindling (Koch and Ebert, 1998), or microinfusions of GABA(A)- or NMDA-receptor antagonists (Fendt et al., 2000) also potently disrupt PPI. Fendt et al. (2000) also demonstrated that these effects are reversed by haloperidol, suggesting a dopamine dependency of BLA mediated PPI disruption. As described above, also alteration of PPI via the PFC is dopamine dependent, and like the PFC, the BLA innervates the core region of NAC (cf. Groenewegen and Trimble, 2007), which suggests a mechanism of direct subcortical dopamine transmission for PPI effects of PFC and BLA. Hippocampal effects on the other hand are not dopamine dependent, and may be mediated by glutamatergic mechanisms within the NAC shell (Wan and Swerdlow, 1996), which is innervated by parts of the hippocampal system (cf. Groenewegen et al., 1987).

PPI can be measured across species (cf. Burgess and Granato, 2007; Sasaki et al.,

1998; Schall et al., 1999; Swerdlow et al., 2001), and findings suggesting similarities also in the neurochemical regulation such as the dopaminergic system and disturbed dopaminergic neurotransmission in some of the before mentioned disorders (cf. e.g. Swerdlow et al., 2001) lit the development of animal models of these diseases. On the other hand, even mice of different strains seem to differ in their response to dopamine interference (DR-antagonists, agonists, transporter blocker, etc.) (Varty et al., 2001), and pharmacological models such as apomorphine treatment cannot readily be transferred from one species to another (for review see Geyer, 2006). Hence, there is still a need for detailed characterisation of gating processes and associated neuronal substrates in animals subjected to disorder models.

Prepulses may not only induce inhibition, but also enhancement of ASR. This only rarely studied phenomenon is termed prepulse facilitation (PPF) (cf. Ison et al., 1997), sometimes prepulse augmentation (PPA) (cf. Willott and Carlson, 1995). There are apparently two types of PPF: facilitation caused by short prepulses at short interpulse intervals (IPI < 15 ms) (cf. Plappert et al., 2004) and facilitation caused by long stimuli and long IPI (> 1 s) (cf. Reijmers and Peeters, 1994). The latter is a phenomenon of increased startle response in an environment of high background sound (cf. Hoffman and Fleshler, 1963) and related to measures of *tone enhanced startle* (cf. section 6.2). With respect to short IPIs, some authors propose PPF as a functional mechanism like PPI (e.g. Plappert et al., 2004), others see PPF as kind of an artefact of PP + P summation in the startle mediating circuit (Hoffman and Ison, 1980). PPF can be observed only at short IPIs, supporting the summation hypothesis; on the other hand, PP-intensity in PPF does not follow a linear but an "inverted U-shaped" function (Plappert et al., 2004) and is found to be more pronounced at lower intensities (Ison et al., 1997; Plappert et al., 2004). Yet, there are only hints that dopamine in the nucleus accumbens might play a role in net PPF (Mohr et al., 2007), but no studies that examined the neuronal basis of PPF.

Like the startle response itself, PPI/F can be elicited and measured almost infinitely with the experimental subject serving as internal control. This fact and the possibility to boost or to constrain PPI/F via various parametric adjustments makes it a valuable paradigm to study inter brain-region functionality and communication. Applying methods such as systemic or intra-cerebral administration of drugs (e.g. Swerdlow et al., 2005), local electrolytic or excitotoxic lesion (e.g. Pouzet et al., 1999) or local elec-

tric stimulation (e.g. Yeomans et al., 2006), there are multiple ways to interfere with and study neuronal processes by means of PPI/F. An only recently introduced way of intra-cerebral interference is the optogenetic approach (Arenkiel et al., 2007). Employing transgenic mice that carry light sensitive ion-channels in their neuronal cell membranes, it is possible to put these cells to a depolarised (channelrhodopsin-2, ChR2, cf. Nagel et al., 2003) or hyperpolarised (halorhodopsin, NpHR, cf. Hegemann et al., 1982) state. Using genetic approaches which enable the expression of these light-sensitive proteins in specific populations of neurons, these neurons can be depolarised or hyperpolarised by short, area specific light-flashes to study their contribution in for example behavioural tasks or to oscillation patterns.

Although parameters for eliciting PPI/F are well known, most of the studies published examine the effects of treatment on PPI only in a very small range of parameters. The present work presents a protocol which covers a wider range of parameters, showing that also other areas of the parametric spectrum than those usually applied can provide useful information.

Interfering with the dopaminergic system in mice of the BALB/c and the C57BL/6J strain, it is shown that systemic blockage of dopamine (DA) receptor type 1 (DR1), but not DR2 (cf. section 7.1.1), and prefrontal blockage of DR1 or DR2 (cf. section 7.1.2), both result in increased PPI and decreased PPF. Showing that the prefrontal cortex (PFC) is involved in mediating PPI in the mouse, PPI and PPF are also successfully manipulated by applying light stimuli to the PFC of transgenic ChR2-positive mice (section 7.2).

3.1. Prefrontal DR1 and DR2 mediate modulation of prepulse inhibition

The dopamine (DA) receptor (DR) 1 and 2 play an essential role in mediating prepulse inhibition (PPI) of startle. Various studies show that direct or indirect DA-agonists, such as apomorphine or d-amphetamine (Mansbach et al., 1988; Swerdlow et al., 1991) result in disruption of PPI. In rats, this effect is reliably prevented by DR2-antagonists (Mansbach et al., 1988; Swerdlow et al., 1991; Wan et al., 1996) and it has been shown that the disrupting effects largely depend on DR2, since direct stimulation of DR2 decreases PPI (Peng et al., 1990; Wan et al., 1996). Contrary, DR1 seems to have a more limited role in mediating modulation of PPI. Studies by Peng et al. (1990) and Wan et

al. (1996) suggest an auxiliary function of DR1 over DR2 in rats, since sub threshold DR2 agonists potently disrupt PPI in the presence of DR1 agonists, while each alone does not yield any PPI change.

In mice, DR1 has a more prominent role in PPI mediation. Like in rats, amphetamine effects have been shown to be a function of DR2 (Ralph et al., 1999; Ralph-Williams et al., 2002). Similarly, PPI deficits shown by DA transporter deficient mice are ameliorated by DR2-antagonists, but not by DR1 blockage. On the other hand, DR1 agonists are found to be much more effective than DR2 agonists in disrupting PPI in mice (Ralph-Williams et al., 2002, 2003; Ralph and Caine, 2005).

Animals were treated with DR-agonists (direct or indirect) in most studies published so far reporting successful PPI alteration by DR-antagonists. Baseline PPI alterations by DR blockage were reported by Schwarzkopf et al. (1993), who found PPI to be enhanced when rats were treated with a DR1- or DR2-antagonist (SCH23390 and haloperidol, respectively). Contrary, Ellenbroek et al. (1996) reported PPI disruption after systemic or prefrontal infusion of DR1- or DR2-antagonist. Also Swerdlow et al. (2005) found PPI in rats decreased after systemic or intra-prefrontal injections of the DR1-antagonist SCH23390, although shown only for prepulse intensities of $\leqslant 5$ dB above background. Here, SCH23390 mimicked PPI decrease after treatment with amphetamine, but SCH23390 mediated decrease in PPI was not completely reversed by pretreatment with DR2-antagonist haloperidol, while amphetamine effects were successfully rescued by DR2 blockage, indicating that this effect was not entirely mediated by increased DA transmission at DR2.

BALB/c mice have been shown to have higher cerebral DA levels compared to C57BL/6J (B6J) mice (George et al., 1995). To further examine the role of DR1 and DR2 in mice and clarify the effect of baseline DR1 and DR2 blockage also in an environment of high DA concentrations, BALB/c and B6J mice are treated with DR2-antagonist haloperidol or sulpiride, and DR1-blocker SCH23390 and are subsequently measured for PPI/F of startle. While haloperidol potently encreased baseline PPI (and decreased PPF), sulpiride treatment did not yield any PPI change. On the other hand, SCH23390 reliably increased PPI, but effects were less pronounced than with haloperidol.

The prefrontal cortex (PFC) has been shown to play a key role in regulation of PPI (cf. p. 25) and being susceptible to dopaminergic interference in terms of PPI. In rats, under some experimental conditions PPI is disrupted by systemic administration (Swerdlow et al., 1991, 2005; Wan et al., 1996) or prefrontal infusion (Ellenbroek et al., 1996; Shoemaker et al., 2005; Swerdlow et al., 2005; Zavitsanou et al., 1999) of DR1-antagonists.

Since blockage of systemic and prefrontal DR1 leads to the same phenotype in rats, PFC is considered a reasonable target to investigate the side of action of DR1 blockage enhancing PPI in mice. Asking whether prefrontal infusion of specific inhibition of DR1 or DR2 by receptor-antagonists would mimic the findings in systemic treated animals, BALB/c and B6J mice are subjected to intra-cerebral drug infusion. It is demonstrated that the PFC indeed is involved in mediating PPI in the subjected mouse lines and that DR1 blockage in the PFC successfully increased PPI in BALB/c, and in B6J mice.

3.2. Mimicking pharmacological interference by optogenetic stimulation

A recently introduced method to interfere with neuronal circuits in vitro (Boyden et al., 2005) and in vivo (Arenkiel et al., 2007) is the optogenetic approach. Here, light sensitive ion-channels/-pumps are expressed in neuronal cells which can then be triggered by illumination with the appropriate wavelength. To date, the cation-channel channelrhodopsin-2 (ChR2), originating from the alga *Chlamydomonas reinhardtii*, and the chloride-pump halorhodopsin (NpHR), found in the archaea Halobacterium *Natronomonas pharaonis*, are applied for neuron excitation or inhibtion, respectively (e.g. Grossman et al., 2010; Tønnesen et al., 2009). Using transgenic mice that express the gene for these channels on specific cell types or transfecting cells in vivo via viral vectors (Kravitz et al., 2010) or electroporation (de Vry et al., 2010), the contribution to e.g. behaviours, memory encoding, or neuronal oscillation patterns of these cells can be studied. Although electrical stimulation has been successfully used for brain stimulation as well as inhibition (cf. Deniau et al., 2010), it is unspecific for cell types. Genetic tools such as the cre/lox-system (Sauer and Henderson, 1988) and the transgenic channel type with its corresponding excitation wavelength define for cell-type specificity and excitation or inhibition, respectively.

The ChR2-positive mouse line Thy1-YFP-18 has been shown to have strong expression of ChR2 on cortical layer V pyramidal neurons (Wang et al., 2007). As has been shown by Bubser and Koch (1994) and others, as well as above (cf. section 7.1.2), function of prefrontal cortex is crucial for modulation of PPI and anatomical changes of layer V pyramidal neurons are found in parallel with altered modulation of PPI (Grant et al., 2007). Subjecting Thy1-YFP-18-mice to measures of PPI/F while applying light flashes to the PFC, the present work demonstrates the usability of optogenetic manipulation in startle experiments. Showing that PPI and PPF are affected by light stimulation, a neuronal

basis of prepulse facilitation different than the suggested prepulse/pulse summation (cf. Hoffman and Ison, 1980; Stoddart et al., 2008) is proposed.

Aims

The rise of new technologies, such as genetic manipulation two decades ago, or more recently the possibility of specific excitation and inhibition of defined cell populations via optogenetics (cf. Arenkiel et al., 2007), has opened the doors to new aspects and deeper insights into the neurobiology, also of startle; hence, startle is still not only a tool to study aspects of mood disorders, but also an object of research itself. The aim of the present work was to establish behavioural paradigms of the startle response and its modifications at the Max-Planck-Institute of Psychiatry:

- Fear potentiated startle as a tool for security learning: possible implications for treatment of specific phobia.
- Parameters governing fear conditioning, fear potentiation of startle, and extinction of conditioned fear: stimulus parameters are crucial for animal learning.
- Applicability of startle measuring systems in control of animal movements: compound via cue extinction.
- Startle response as a tool for animal characterisation: hearing capability and electric susceptibility assessed by startle measures in an animal model of trait anxiety.
- Symptomatology of an animal model of post-traumatic stress disorder: hyperarousal and its independence of hippocampal volume changes.
- Prepulse inhibition of startle: contribution to understand the complex interactions of dopamine receptor subtypes in the prefrontal cortex.
- Elucidate the startle paradigm: feasibility study of optogenetical manipulation of startle response.

Part II.

Materials and Methods

4. General materials and methods

Animals

In the present work, a total of 1187 animals were used. Mice were kept under standard laboratory housing conditions in the animal facility of the Max-Planck-Institute of Psychiatry (inverse 12:12 h light-dark schedule with lights off at 09:00 am, at $22 \pm 2\,^{\circ}$C room temperature and $55 \pm 5\,\%$ humidity). Mice were single housed in Macrolon type II cages with sawdust bedding and food and water ad libitum for at least ten days before starting the experiments. All experiments were performed during the activity phase of the animals between 09:30 am and 08:00 pm.

All experiments were approved by the Committee on Animal Health and Care of the State of Bavaria (Regierung von Oberbayern, Germany) and performed in strict compliance with the European Economic Community recommendations for the care and use of laboratory animals.

Fear conditioning and sensitisation

Procedures were performed in conditioning chambers (ENV-307A, MED Associates Inc., Georgia, VT, USA) with house light (0.6 Lux, ENV-215M, MED Associates), and a floor of stainless steel rods for electrical footshock application (grid harness package: ENV-407; Shocker/Scrambler: ENV-414, MED Associates). The chamber has a cubic shape with two walls made of aluminium and two of acrylic glass. This context was cleaned with 70 % ethanol after each session. The same chambers were also used to test contextual fear memory. For extinction training and test of conditioned stimulus (CS) memory, chambers of cylindric form were used. The acrylic glass cylinder with sawdust as bedding was illuminated with a light (0.3 Lux, ENV-215M, MED Associates) and cleaned with 1 % acetic acid. It has been shown that mice perceive sine wave tones best in a frequency-range of 9 - 14 kHz (Ehret, 1976; Marsch et al., 2007). Since many investigators have problems perceiving frequencies above 10 kHz at least at lower intensities, it was decided to utilise stimuli of the lower border of the animals optimal

perception window. Therefore, 9 kHz sine wave tones (sw) and white noise (wn) were used as acoustic stimuli in the experiments described below.

Note that fear conditioning and extinction training was also conducted partly in the startle apparatus (cf. p. 37).

All chambers were located in soundproof isolation cubicles (ENV-018M, MED Associates) that were additionally isolated with acoustic foam (Conrad Electronic SE, Hirschau, Germany). Tones were generated by audio stimulus generators (ANL-926, MED Associates) and applied by speakers (DTW 110 NG, Visaton GmbH & Co. KG, Haan, Germany) mounted to the ceiling of the isolation cubicle above each chamber. Sound pressure levels (SPL) were checked by means of the SPL Measurement Package (ANL-929A-PC, MED Associates) at floor level. Animal behaviour was observed and videotaped using charged coupled device (CCD) cameras (Conrad Electronic), mounted to the back plane of the isolation cubicles. Offline analysis of freezing behaviour (immobility except for respiration movements) was performed using counter based analysis software (EVENTLOG, Robert Henderson, 1986) and the amount of freezing is displayed as percentage in a defined time window. Experiments were controlled by commercial software (MED-PC for Windows v1.17) via interfaces (DIG 715) and the respective control panels (SG 215, all MED Associates). Two conditioning or four testing setups were used simultaneously.

Acoustic startle response

The startle reflex was measured in the dark in up to eight identical mouse cages, consisting of non-restrictive acrylic glass cylinders (inner diameter 4 cm, length 8 cm) mounted on an acrylic glass platform. Each cage was placed in a sound attenuated chamber (SR-LAB™, San Diego Instruments, San Diego, CA, USA) which was located in cubicles isolated with acoustic foam (Conrad Electronic). Cages were cleaned with soap water after each session. The cylinder movement was detected by a piezoelectric element mounted under each platform and the voltage output of the piezoelectric device was amplified and then digitised (sampling rate 1 kHz) by a computer interface (all San Diego Instruments, SDI). The startle amplitude was defined as the peak voltage output within the first 50 ms after stimulus onset (cf. fig. 1.1). To assure identical output levels for each chamber, response sensitivity was calibrated before each startle experiment.

Startle stimuli and background noise were delivered through a high-frequency speaker placed 20 cm above each cage. Sine wave stimuli were generated by a SDI-Software controlled function generator (BK Precision 4011A, B & K Precision Corporation, Yorba

Linda, CA, USA). The signal was amplified (STR-DE197 FM Receiver, Sony Corporation, New York, NY, USA) and the stimuli were delivered through a high frequency speaker mounted 10 cm above the cage (SDI, pure tone kit). Stimulus intensities were measured in decibel with filter A (dB(A)) sound pressure level (SPL, re. 20 µPa) using an audiometer (33-2055, RadioShack Corporation, Fort Worth, TX, USA). All stimuli were presented in background noise of 50 dB(A). On control trials only background noise was present.

For measures of baseline ASR, white noise pulses of 75, 90, 105, and 115 dB(A) (30 times each) were presented to the animal. On 18 no-stimulus trials, only background noise was present. Pulses were presented after an acclimation period of 5 min in a pseudo-randomised order, where each stimulus was repeated only once before another stimulus-type was presented.

Fear conditioning and extinction training took place in the same cages where the startle response was measured. For fear conditioning and extinction training, sine wave tones were generated using SDI pure tone kit (cf. p. 36). For fear conditioning, grids of seven stainless steel rods were added to the floor of the cages for electric footshock application. Scrambled shocks were produced and parameters adjusted using SDI fear potentiated startle kit.

Surgery

Surgery was performed by fixing the animal to a stereotactic frame (TSE Systems GmbH, Bad Homburg, Germany). Animals received an injection of analgesic before surgery started (0.5 mg/kg Metacam®, Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany). During surgery, mice were deeply anaesthetised by inhalation of isoflurane (Forene®, Abbott GmbH & Co. KG, Ludwigshafen, Germany) using a self-made inhalation apparatus. Body temperature was kept at constant 36 °C by a heating pad (Harvard Apparatus, Holliston, MA, USA). The skull of the animal was exposed and a cannula to guide the injection cannula during injections (cf. below) was implanted and a screw was inserted into the skull. This guide cannula was fixed to the skull and the screw by dental cement (Paladur®, Heraeus Kulzer GmbH, Hanau, Germany). The wound was disinfected and closed with sutures. Analgesic treatment was continued at 0.5 mg/kg for another 3 - 4 days via drinking water and mice were allowed to recover for 10 - 12 days before starting an experiment.

Drug administration

Drug administration was carried out 40 min intra peritoneal (i.p.), 60 min subcutaneously (s.c.) or 30 min intracerebral or intracerebroventricular (i.c.v.) before testing after a short isoflurane anaesthesia. Intracerebral and i.c.v. injections were performed with a 30 G cannula connected to a microlitre syringe via a calibrated tubing. The infusion device was filled with distilled water. A small air bubble was sucked into the injection cannula in front of the injection solution. The air bubble provided protection against contamination of the syringe with the injection solution and provided a visual mean of volume control by calibration marks on the tubing. The injection cannula topped the guide cannula by 1 mm and by this was able to reach the target area. After insertion the solutions were infused slowly over the course of 30 - 60 s. After injection, the cannula remained in place for another 30 s to allow complete diffusion. Animals were then taken back to their home cages. The injection cannula was cleaned carefully with 70 % ethanol and Ringer solution (Fresenius Kabi AG, Bad Homburg v.d.H., Germany) between injections. Different injection devices were used for vehicle and drugs.

For detail experimental material and methods please refer to the respective section.

5. Detailed materials and methods

5.1. The startle response in paradigms of anxiety and fear

5.1.1. Fear potentiated startle in C57BL/6N mice

Animals

A total of 98 male single housed C57BL/6NCrl mice purchased from Charles River (Charles River Laboratories, Research Models and Services, Germany GmbH, Sulzfeld, Germany) or bred at the Max-Planck-Institute of Psychiatry, Munich (MPI-P) were subjected to fear conditioning and measures of freezing behaviour and startle at the age of 8 - 12 weeks.

Procedures

Mice were subsequently subjected to baseline acoustic startle response (ASR) measurements, fear conditioning, measures of freezing behaviour to CS and fear potentiated startle (FPS). In Experiment 2, 3, and 4 animals were also measured for startle following unconditioned tone presentation, before being subjected to fear conditioning. According to baseline ASR, animals were assigned to the experimental groups in a counterbalanced manner. Fear conditioning was conducted in the fear conditioning apparatus or in the startle apparatus (cf. p. 37 and Experiment 4).

The conditioned stimulus (CS) consisted of a sine wave tone of 9 kHz and 70 dB(A) (80 dB(A) in Experiment 4) intensity with a duration of either 4 s (Experiment 1 - 3) or 20 s (Experiment 3 and 4) or light (4 s, 12 Lux, Experiment 1). Unconditioned stimulus (US) was an electric footshock of 0.4 (experiment 4), 0.5 (Experiment 1 and 2) or 0.7 mA (Experiment 3) of 500 ms duration which co-terminated with the CS. After an acclimation period of 180 s, six (five, Experiment 4) CS - US pairings were presented to the animals at a various interstimulus intervall (ISI) of 15 to 145 s. After the last CS - US pairing, animals remained in the apparatus for another 60 s before they were carried back to their home cage (Experiments 1 - 3) or were carried back immediately to

A

B

	US intensity (mA)	CS type	CS duration (s)
Exp 1	0.5	70dB tone vs. light	4
Exp 2	0.5	70dB tone	4
Exp 3	0.7	70dB tone	4 vs. 20
Exp 4	0.4	80dB tone	20

Figure 5.1.: Parameters used in fear potentiated startle experiments. (A) Scheme of general stimulus presentation. Black bar: CS, white bar: US. (B) Table of parameters used in experiments 1 - 4. Note that tone frequency was 9 kHz for all experiments and that for US-control groups US intensity was 0 mA.

minimise context conditioning (Experiment 4). For parameters used see also fig. 5.1.

Baseline ASR was measured as described above (cf. p. 36). For measures of FPS in Experiment 1 - 3, 60 startle eliciting pulses (P) were presented, half of them preceded by the particular CS (i.e. light or tone of the assigned duration). Pulses had an intensity of 115 dB(A) (Experiment 1 and 2) or half of the pulses and half of the CS + P had an intensity of 105 or 115 dB(A), respectively (Experiment 3). In Experiment 4, 20 pulses and 20 CS + P were presented to the animal at an intensity of 105 dB(A) and six control readings were taken where no stimulus was present.

All stimuli were presented in a pseudo-randomised order after 5 min acclimation period, where no stimulus was repeated more than once before another stimulus-type was presented.

Freezing behaviour was measured and analysed as described above (cf. p. 35). To control for CS memory, animals were presented a 30 s CS after 180 ms acclimation time in a neutral context. After CS presentation, animals remained in the context for another 60 s.

Statistics

Alteration of the startle response (SR) was calculated as either percental change (%ASR)

$$\frac{SR(CS+P) - SR(P)}{SR(P)} \cdot 100\%$$

or difference between ASR of P and CS + P trials (ΔASR)

$$SR(CS + P) - SR(P)$$

Measured values of a given animal and trial-type were averaged and data were then analysed using Statistica v5.0 (StatSoft Europe GmbH, Hamburg, Germany). Analyses of variance (ANOVA) with factor CS duration (4 s and 20 s) and repeatedly measured factors pulse intensity (105 and 115 dB(A)) and CS presentation (+CS, and -CS for no CS presentation) was calculated with SPSS® v19.0 (SPSS Inc., Chicago, IL, USA). Data of freezing behaviour were analysed as freezing per 30 s interval, calculating t-test with Graphpad Prism v5.0 (GraphPad Software Inc., La Jolla, CA, USA). For startle amplitudes 1-way repeated-measures (rm)ANOVA was conducted with the between-subjects factor group (CS light or tone, or shock and no shock, or shock, no shock and unpaired). Within-subject factors were CS presentation or pulse intensity, and 1-way ANOVA or t-test for difference values and percental change. Newman-Keuls posthoc was calculated if appropriate. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

5.1.2. Tone enhanced startle as a measure of hearing capability, stimulus adaptation and attention

Animals

A total of 276 male single housed C57BL/6NCrl mice purchased from Charles River or bred at the MPI-P, and 24 transient receptor potential vanilloid 1 deficient (TRPV1-ko) mice bred at die Max-Planck-Institute of Biochemistry, Martinsried, were subjected to startle measurements at the age of 8 - 12 weeks. In Experiment 2, animals were measured six times for either TES or PPI/F. All other animals were measured only once for TES.

Procedures

Mice were subsequently subjected to baseline acoustic startle response (ASR) measurements and tone enhanced startle (TES). According to baseline startle, mice were assigned to different groups in a counterbalanced fashion.

The pre-stimulus (PS) consisted of a sine wave tone of 9 kHz, or white noise (Experiment 7), and 70 dB(A) (and 60 and 80 dB(A), Experiment 1) intensity with a duration of 20 s, or PS was light (1445 Lux, 2 s or 20 s, Experiment 4 and 7, respectively).

Baseline ASR was measured and animals of Experiment 3 were sensitised in the apparatus described above (cf. p. 36 and p. 35, respectively). For sensitisation, a single electric footshock of 0, 0.5, 0.7 or 1.5 mA and 2 s duration was given to the animal after 198 s acclimation time. After the footshock, animals remained in the apparatus for another 60 s and TES was measured 30 days after. For measures of TES in Experiment 1 - 6, 40 startle eliciting pulses (P) of 105 dB(A) intensity were presented, half of them preceded by the particular pre-stimulus (PS, i.e. light or tone). On PS + P trials in Experiment 2, pulses were presented at different time points during pre-stimulus presentation (cf. fig. 6.6A).

Experiment 2 aimed to characterise possible prepulse effects of prestimulus offset on startle response changes (i.e. prepulse inhibition, -facilitation, PPI/F, cf. section 3). For measures of PPI/F in Experiment 2, prepulse again was a 9 kHz, 70 dB(A) pre-stimulus. Duration was either 10 ms or 20 s. For 10 ms duration, onset of the pre-stimulus (PS) served as prepulse, while for 20 s PS prepulse was stimulus offset. Two protocols were used, both starting with an acclimation period of 5 min, followed by 20 initial startle eliciting pulses of 105 dB(A) for startle habituation. Then, the first protocol was designed to measure PPI/F at different interpulse-intervals (IPI, cf. p. 52, and fig. 6.6A), presenting 28 pulses and nine conditions where a prepulse (PP) preceded the pulse (IPI = 3000, 1000, 500, 50, 10, 0, -10, -20 ms), 16 times each. If IPI < 0, then prepulse onset was after pulse onset. If startle enhancement observed in experiments of sections 6.1 and 6.2 was due to prepulse like effects of pre-stimulus offset and not pre-stimulus presentation pre se, then 10 ms and 20 s pre-stimuli should lead to comparable startle enhancement, especially at PI < 0. The second protocol was designed to extend the first protocol by IPI = 3 ms. 24 pulses and three PP + P conditions (IPI = 10, 3, 0 ms), 16 times each, were presented.

In Experiment 7, the testing-protocol was designed to enable measurement of TES and habituation of TES. After 5 min acclimation period, ten habituation pulses were followed by another ten pulses, which served to assess habituated baseline ASR as reference value to enable calculation of TES habituation. Then, twelve pulses and 24 PS + P were presented for measures of TES. For calculation of TES per se (see below, p. 43), average of the twelve pulses during pre-stimulus presentation phase were used as reference value. This protocol was also used in Experiment 8.

In Experiment 9, animals were presented 78 startle eliciting pulses. 16 of these pulses were preceded by the tone, 16 by 2 s of light and another 16 by both, tone and light, where the light stimulus was presented during the last 2 s of tone presentation. All other pulses were presented without any pre-stimulus. Additionally, animal movements were

measured during presentation of six light stimuli which were not followed by a pulse, and also six times when only background noise was present. In addition, the animal's reaction was also controlled during onset of tone.

All stimuli were presented in a pseudo-randomised order after 5 min acclimation period, where no stimulus was repeated more than once before another stimulus-type was presented.

Drugs

In Experiment 5, mice were treated with the GABA(A) agonist diazepam (Diazepam-Lipuro®, Braun Melsungen AG, Melsungen, Germany). Diazepam was freshly dissolved in saline (0.9 %) and injected as described on p. 38. Drugs were administered i.p. 40 min before measuring TES in dosages of 0 (vehicle), 0.3, 1 and 2 mg/kg.

In Experiment 6 the effect of the selective serotonin reuptake inhibitor paroxetine (Desitin Arzneimittel GmbH, Hamburg, Germany) on TES was tested. Paroxetine was administered per os (p.o.) on oat flakes. 10 mg saccharin was dissolved in tap water and a suspension was made with 20 mg paroxetine. 15 µl of this suspension were pipet on one oat flake and given to an animal after the suspension on the flake was dry. Thus an oat flake carried a dosage of 10 mg/kg, assuming a body weight of 25 g per mouse. Control animals got oat flakes with saccharin-water only. Animals were habituated to oat flakes by feeding them saccharin carrying oat flakes for four days before the experiment. On the day of the experiment, one flake per mouse was dropped into the animal's cage, and the mice ate the flakes within a latency of about 30 s. Feeding was conducted 1 h before TES measures started.

Statistics

Alteration of the startle response (SR) to an intense noise burst (pulse P) caused by a acoustic pre-stimulus (PS) was calculated as either percental change

$$\frac{SR(PS+P) - SR(P)}{SR(P)} \cdot 100\%$$

or difference between ASR of pulse and PS + P trials

$$SR(PS+P) - SR(P)$$

It was observed that ASR is affected by pre-stimuli, even in measures where pre-stimuli were not presented contiguously to the pulses, suggesting dehabituation of ASR resulting

from arousing effects of the pre-stimulus after habituation phase. To analyse stimulus adaptation, %ASR was therefore calculated relative to the last ten pulses averaged during habituation phase, where ASR is habituated to the presented pulse. ASR to pulse alone trials during the phase of pre-stimulus presentation (i.e. after habituation phase) were used for analysis of TES (%ASR or ΔASR) per se. Analysis of TES to assess pre-stimulus adaptation was calculated using SPSS® with within-subject factors day and trial (i.e. repeated presentation) and between-subject factors pre-stimulus quality and -duration. Linear regression to assess the amount of habituation was calculated using Graphpad Prism.

For analysis of TES, measured values of a given animal and trial-type were averaged and data were then analysed using Statistica. 1-way repeated-measures analyses of variance (rmANOVA) were conducted with the between-subjects factor group (PS intensity, shock intensity, PS type, PS duration, treatment, or genotype) and the within-subject factors PS presentation (+PS, or -PS for no PS presentation) for startle amplitudes and 1-way ANOVA or t-test for percental change and difference scores. For measured of attention or distraction, respectively, 1-way rmANOVA was calculated with the within subject factor PS + P condition. Newman-Keuls posthoc was calculated if appropriate. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values $\pm$SEM.

5.1.3. Fear conditioning parameters - the matter of fact

Animals

A total of 96 male single housed C57BL/6NCrl mice bred at the MPI of Biochemistry, Martinsried, were subjected to fear conditioning and extinction of conditioned fear, and startle measurements at the age of 8 - 12 weeks.

Procedures

Mice were subjected to either baseline acoustic startle response (ASR) measurements, fear conditioning and fear extinction, or fear potentiated (FPS) and tone enhanced startle (TES), respectively.

Baseline ASR was measured presenting startle eliciting pulses (P) of 75, 90, 105 or 115 dB(A) intensity. Pulses consisted of 9 kHz sine wave (sw) tones or white noise (wn). Each intensity in combination with each quality was presented 20 times together with eight control readings in a pseudo-randomised order after 5 min acclimation period.

For fear conditioning (FC), a single electric footshock of 0.7 mA of 2 s duration co-terminated with the conditioned stimulus (CS, 9 kHz sine wave tone (sw) of 80 dB or white noise (wn) of 80 dB, 20 s or 120 s duration), which was presented to the animal after 180 s acclimation time. After the footshock, animals remained in the apparatus for another 60 s. For extinction training, animals received ten CS-presentations (all 20 s in duration presented at various interstimulus intervals of 20 - 170 s) of the respective quality (i.e. sw or wn) after 180 s acclimation time, and remained in the apparatus for another 60 s after the last CS. Extinction of conditioned fear (ExFC) following FC was conducted on three consecutive days (day 1 - 3) and test of ExFC memory was performed on day 9 post shock. While FC and test of context memory was conducted in the conditioning context, ExFC test of CS memory took place in the extinction context (cf. p. 35).

TES and FPS were measured as described above (cf. p. 42). However, in the current experiment all 20 initial startle eliciting pulses were discarded as habituation phase, and TES and FPS were calculated using average response of twelve pulses that were presented after habituation phase as reference value (see below).

Statistics

Alteration of the startle response (SR) was calculated as either percental change

$$\frac{SR(CS+P) - SR(P)}{SR(P)} \cdot 100\%$$

or difference between ASR of pulse alone (P) and conditioned stimulus (CS) + P trials

$$SR(CS+P) - SR(P)$$

where SR-data were analysed after averaging measured values of a given animal and trial-type. Data of freezing behaviour were analysed by freezing in 20 s intervals. 2-factor repeated-measures analyses of variance (rmANOVA) was conducted with the within-subject factors pulse quality and pulse intensity using SPSS®. 2-way rmANOVA was calculated using Statistica with the between-subjects factor CS duration (20 s and 120 s) and CS quality (sw and wn), or CS quality and conditioning (shock and no shock). The within-subject factor were CS presentation number (i.e. 1 - 10), day of measurement, and +CS (CS presentation) or -CS (no CS presentation) for SR amplitude and differences. For ASR percental change, 2-way ANOVA with between subject factor CS quality and conditioning was calculated. Newman-Keuls posthoc was calculated if appropriate. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

Between session extinction was measured comparing the freezing response to the very first CS of each day of testing (cf. Plendl and Wotjak, 2010).

5.1.4. Extinction of conditioned fear to context by cue extinction training

Animals

A total of 24 male singlely housed C57BL/6NCrl mice bred at the MPI-P were subjected to fear conditioning and extinction of conditioned fear at the age of 8 - 12 weeks.

Procedures

Mice were subjected to trace fear conditioning (FC), extinction of conditioned fear (ExFC), and test of CS- and context memory.

Fear conditioning (FC) was conducted as described above (p. 44), except that a 15 s interval (*"trace"*) was inserted between CS offset and shock onset to favour importance of the hippocampus (HPC) in this experiment. HPC function has been shown to be critical for trace-conditioning (cf. McEchron et al., 1998; Moyer et al., 1990; Wanisch et al., 2005). After footshock, animals remained in the apparatus for another 60 s.

For ExFC, animals were transported to another room where extinction training took place in the startle apparatus (cf. p. 36). After 3 min acclimation period, half of the animals received ten CS presentations of 20 s duration at various interstimulus-intervals (ISI, 20 - 170 s) on three consecutive days (days 1 - 3 post shock). To allow quantification of the animal's fear response while not being able to monitor freezing behaviour, voltage output of the peizo element of the startle apparatus was recorded. Instead of analysing peak values of a given time interval (cf. p. 36), all recorded values were averaged during a 10 s interval during the whole procedure. CS presentation was omitted for the other animals (extinction control).

After the last extinction session, animals were transported back to the FC facilities and were measured for CS- and context-memory on day 7 and 9 post shock, respectively. Memory for CS was tested in a new, neutral context, while test for context memory took place in the context of shock application during conditioning. During memory tests, mice were presented four CS spaced by various ISI (40 - 120 s) after 180 s acclimation time, and remained in the apparatus for another 60 s after the last CS presentation.

Statistics

Freezing-data were averaged to 20 s intervals. 1-way repeated-measures analyses of variance (rmANOVA) was calculated using Statistica with the between-subjects factor extinction (ex or no ex) and the within-subject factors CS presentation or interval (1 - 4 for CS, 1 - 9 for context-memory, respectively). For statistical analysis of animal movement during extinction training, acquired data before and during CS presentation were averaged, respectively, and compared day by day calculating independent t-test with Graphpad Prism. Newman-Keuls posthoc was calculated if appropriate. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

5.1.5. ASR measures in mouse-models of trait anxiety and PTSD

Animals

To achieve mice of high, low, and normal anxiety related behaviour (HAB, LAB, NAB), male CD1 mice had been selectively inbred in the animal facilities of the MPI-P as described by Krömer et al. (2005). Briefly, inbreeding started with >250 animals from 25 litters of outbred Swiss CD1 mice purchased from Charles River. With at least six families routinely maintained within each selected line, males and females that spent either the least, intermediate or most time on the open arms of the elevated plus-maze (EPM) were mated to establish the HAB, NAB, and LAB mouse lines, respectively. 49 HAB, 37 NAB and 48 LAB (all male) were subjected to either fear conditioning (FC) and subsequent CS memory test, startle response (SR, acoustic or electric) or tone enhanced startle (TES) measures. Mice were single housed for at least two weeks before the experiment started.

Measuring CRH enhanced startle and enhanced ASR in the mouse model of post-traumatic stress disorder (PTSD), 21 male single housed C57BL/6NCrl mice, bred at the MPI of Biochemistry, Martinsried, and 29 male singlely housed C57BL/6NCrl purchased from Charles River, both at the age of 8 - 12 weeks, were subjected to either cerebroventricular injection-cannula implantation, and ASR measures after CRH or αCRH infusion, or to PTSD-protocol and ASR measures 30 days after, respectively.

To evaluate the applicability of ultramicroscopy in terms of measures of HPC volume and arborisation of hippocampal (HPC) pyramidal neuron dendrites, HPC of 24 male mice expressing green fluorescing protein (GFP, thy1-GFP mouse line M, (cf. Feng et al., 2000)) and bred at the MPI-P were dissected and cleared for imaging (cf. p. 49).

Animals were housed in groups of four and either kept under standard (cf. p. 35) or enriched housing conditions (enriched environment, EE), the latter providing a bigger cage (Makrolon type IV), a running wheel and weekly changed toys.

For measures of ASR and hippocampal (HPC) volume in the PTSD model, 64 male C57BL/6NCrl mice were purchased from Charles River and assigned to four groups of 16 animals, each. Animals were housed in groups of four animals per cage, either under standard conditions or in an enriched environment.

Surgery and drug treatment

For intracerebroventricular (i.c.v.), CRH-injection surgery was performed as described above (p. 37). Coordinates for injection based on the stereotaxic mouse brain atlas (Franklin and Paxinos, 1997) were 0.3 mm posterior, 1 mm lateral and 1.2 mm deep from the level of the skull surface with respect to bregma. I.c.v. injection of vehicle (0.9 % saline), 0.1 µg CRH, or 0.1 µg CRH and 10 µg αCRH was performed as described on p. 38.

Procedures

Fear conditioning (FC) in HAB/NAB/LAB mice was conducted, and TES and SR were measured as described above (cf. p. 44, p. 42, and p. 36, respectively), except that CS - US pairing during FC was repeated twice with an interstimulus interval (ISI) of 20 and 30 s. CS memory was tested analysing freezing behaviour during presentation of a 180 s long CS in a neutral context (cylinder, cf. p. 35) on the following day. To assess footshock sensitivity in HAB/LAB/NAB mice, ten CS - US (footshock, 0.7 mA, 1 s duration) pairings were presented with an interstimulus interval of 30 - 160 s, and animal movements (cf. p. 46) were measured after 5 min adaptation to the startle chamber.

For test of CRH-enhanced startle response, baseline ASR was measured as described on p. 37.

To achieve trauma-related behaviour, half of the animals were subjected to a single electric footshock (1.5 mA, 2 s) after acclimation time of 198 s, and then remained in the apparatus for another 60 s. For the other half of the animals, footshock was omitted (exposure control). One month later mice were consecutively analysed for contextual freezing in the shock context, a context containing a shock-context reminder (grid), and a neutral context (data not shown, cf. Golub et al., 2009) and ASR.

To study HPC volume changes during development of PTSD symptoms, animals were

housed six weeks under either enriched or standard conditions. Then, half of the animals of each housing condition were subjected to a single electric footshock (PTSD-protocol). For the other half of the animals, footshock was omitted (exposure control). Mice were then returned to their homecage and kept under the respective conditions for another month. Mice were then tested for hyper-arousal (i.e. ASR) as described above (p. 37). Behavioural measurements were followed by manganese enhanced magnetic resonance imaging (MEMRI, cf. Kay et al., 1987; Golub et al., 2010) and ultramicroscopy (Dodt et al., 2007 and see below).

Ultramicroscopy

For measures of hippocampal volume, tissue clearing and ultramicroscopic imaging were performed and the setup used as described by Dodt et al. (2007). Briefly, brains were fixed in 4 % and then 0.4 % paraformaldehyde (PFA) at 4 °C for ten and four days, respectively, followed by HPC dissection blind to the history of the animals. Hippocampi were then dehydrated using a series of graded ethanol (EtOH, 50 %, 80 % and 96 %, for 1 h each). After 100 % EtOH over night and a final step of 100 % EtOH for 1 h, HPC were transferred to a mixture of benzylalcohol and benzylbenzoat (BABB, Sigma-Aldrich Chemie GmbH, Munich, Germany) at a ratio of 1:2.

Specimens were placed on a black platform in a small chamber with glass-walls filled with BABB. The argon-ion laser beam (Innova 90, Coherent) with a wavelength of 488 nm was channelled to the specimens via two mirrors. A cylinder lens (80 mm focal distance) and a slit aperture (4 mm) were used to form the light sheet. Images were recorded by a charge-coupled device (CCD) camera (CoolSnap Cf, 1392 x 1040 pixels, Roper Scientific, Ottobrunn, Germany) using a 2.5 x objective (NA = 0.12, Zeiss Fluar, Carl Zeiss AG, Oberkochen, Germany). Above the objective a band pass filter was positioned (Brightline HC536/40, AHF analysentechnik AG, Tübingen, Germany). In this configuration, 1 pixel accounted for 13.32 μm^2. The camera was mounted on a modified microscope (Zeiss) which was oriented perpendicular to the light beam. About 700 images were then taken by moving the specimen chamber in increments of 3.65 µm vertically through the light sheet. Images were processed using Amira (Visage Imaging GmbH, Berlin, Germany).

For analysing HPC volume and dendritic arborisation, images were loaded into a self-written IGOR routine (WaveMetrics Inc., Portland, OR, USA). The area containing the HPC image on each recorded image was calculated by counting the number of pixels containing a grey value above a given threshold (i.e. fluorescence resulting from GPF

excitation for assessing volume occupied by cells and dendrites, or scattered light from HPC tissue assessing total HPC volume). This number was multiplied with the image thickness (i.e. 3.65 µm, the step size the specimen was moved through the laser beam), giving the number of voxels per image. The sum of all recorded voxels was then reported as the neuronal or HPC volume in mm^3, respectively.

Note that the calculated thickness of the light sheet illuminating the image area during recording was higher than the chosen step size (cf. Addendum); since the equation of thickness calculation also takes into account areas of low light beam intensity, which are not sufficient for GFP excitation, the step size was chosen based on test series and imaging experience (cf. Dodt et al., 2007).

Genotyping

To verify green fluorescent protein (GFP) expression, a tissue biopsy from the animal's tail was taken and digested over night at 56 °C adding 100 µl EDTA, 480 µl nuclein lysis solution and 20 µl proteinase K (both Qiagen GmbH, Hilden, Germany). DNA purification was achieved by subsequently adding 200 µl protein precipitate (Qiagen) and 600 µl isopropyl alcohol (Sigma), and 2 min centrifugation. After discarding supernatant, 600 µl ethanol (70 %) were added, centrifuged, and the supernatant discarded again. After drying for 20 min at 37 °C, DNA was resolved with 200 µl rehydration solution (Qiagen) and 2 h incubation on a shaker at 65 °C.

Polymerase chain reaction (PCR) was conducted adding 3 µl $MgCl_2$, 5 µl Buffer, 1 µl dNTPs (all Qiagen), 39.3 µl H_2O, 0.1 µl primer (100 pM, Sigma, sense 5´- CCT-ACG-GCG-TGC-AGT-GCT-TCA-GC -3´ and anti-sense 5´- CGG-CGA-GCT-GCA-CGC-TGC-GTC-CTC -3´, respectively) and 0.5 µl Taq polymerase (Sigma) to 1 µl DNA solution. PCR parameters were:

5 min 94 °C
  start cycle (32 x)
    30 s 94 °C — 30 s 60 °C — 1 min 72 °C
  end cycle
5 min 72 °C
∞ 4 °C

The PCR product (EGFP sense/antisense = 345 bp, respectively) was then analysed in an agarose gel (1.5 %) containing ethidium bromide (both Sigma).

Statistics

Freezing-data were averaged to 20 s intervals and ASR values were averaged for a given animal and a given pulse intensity. 1-way repeated-measures analyses of variance (rmANOVA) was calculated using Statistica with the between-subjects factor mouse line (HAB, LAB, NAB), treatment, or footshock, and the within-subject factor pulse intensity (0, 75, 90, 105 or 115 dB(A)). ANOVA was calculated on tone enhanced startle change and difference scores (cf. p. 43) and electric footshock susceptibility was assessed by subtracting animal movement scores before tone and shock pairing from animal movement scores during tone and shock pairing. Then, ANOVA was calculated to compare difference values. 2-way rmANOVA was calculated with the between-subject factors enrichment and shock (EE, nEE and S, nS, respectively) and the within-subject factor pulse intensity for ASR analysis, or 2-way ANOVA for analysis of HPC volume. Newman-Keuls posthoc was calculated if appropriate. t-tests to evaluate differences in HPC volume and GFP volume in the HPC were calculated using Graphpad Prism. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

5.2. Pharmacological and optogenetical manipulation of prepulse inhibition

5.2.1. Prefrontal DR1 and DR2 mediate modulation of prepulse inhibition

Animals

A total number of 208 singlely housed BALB/cAnNCrl (BALB/c) and 175 singlely housed C57BL/6JAX (B6J) mice purchased from Charles River or bred at the MPI-P, were subjected to startle measurements at the age of 8 - 12 weeks. For all data reported here, animals were treated only once with the respective compound, except for data displayed in fig. 7.4. Here, animals treated with 0.3 mg/kg SCH23390 were injected the same again two days later, and animals treated with 0.1 mg/kg SCH23390 were used as vehicle control injecting 0.9 % saline.

Surgery

Surgery was performed as described above (cf. p. 37). After exposure of the skull, a hole was drilled and a guide cannula (23 Gauge, stainless steel) was implanted. Coordinates

based on the stereotaxic mouse brain atlas (Franklin and Paxinos, 1997) were anterior + 1.9 mm, lateral + 0.8 mm and ventral + 2.0 mm with an angle of 20° for PFC infusions as referred from the animal's bregma.

Drugs

For systemic drug administration mice were treated subcutaneously (s.c.) with 0.1 or 0.3 mg/kg SCH23390 (BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany), 5 or 20 mg/kg sulpiride (Dogmatil®, Sanofi-Aventis GmbH, Frankfurt, Germany), or 0.3 or 1.0 mg/kg haloperidol (Haldol®, Janssen-Cilag GmbH, Neuss, Germany). The injection volume was 0.01 ml/g body weight. For PFC injection, 100 or 500 ng SCH23390, 30 or 100 ng sulpiride, 250 ng muscimol (BIOZOL) or 10 ng NBQX (Sigma) were administered in a volume of 0.5 µl. For systemic injections 0.9 % saline, and for PFC injections Ringer solution (Fresenius Kabi AG, Bad Homburg v.d.H., Germany) was applied as vehicle. Drugs were applied as described on p. 38.

Startle-procedure

ASR was elicited using short noise pulses of 115 dB(A) (startle eliciting pulse P) intensity with a duration of 20 ms at a background noise level of 50 dB(A). Each test session consisted of a 5 min acclimation period followed by 20 pulses for habituation to the ASR eliciting stimulus. Another 22 pulses, 210 prepulse (PP) -condition trials and 18 prepulse control trials were arranged in a pseudo-randomised order where no stimulus condition was presented repeatedly more than once before another stimulus type was presented. Intertrial interval (ITI) was 15 s averaged, ranging from 13 to 17 s. 15 different prepulse conditions were presented, each for 14 times. Three different prepulse intensities were used (55, 65 or 75 dB(A)) with an interpulse interval (IPI, prepulse onset to pulse onset) of 5, 10, 25, 50 or 100 ms. The prepulse duration was 10 ms, or 5 ms if IPI was 5 ms. On prepulse control trials only the prepulse was presented. Each of the three prepulse intensities was presented six times without any startle pulse. Each animal was tested for baseline PPI and PPF before any drug-treatment. According to basal PPI, each animal was then assigned to one of the differently treated groups, so as that statistical analysis revealed no significant group differences under basal conditions, respectively.

Statistics

Alteration of the startle response (SR) was calculated as percental change (%ASR):

$$\frac{SR(PP+P) - SR(P)}{SR(P)} \cdot 100\%$$

where on (PP + P)-trials a prepulse (PP) preceded the startle eliciting pulse (P) and on (P)-trials P was presented without prepulse.
For normalisation of %ASR (Δ%ASR) of each mouse (y_i) to vehicle treated animals it was calculated:

$$\bar{x} - y_i$$

with the average value of vehicle treated animals $\bar{x}$ and the %ASR of each pharmacological treated mouse y_i. Measured values of a given animal and trial-type were averaged and data were then analysed using Statistica. 1-way repeated-measures analyses of variance (rmANOVA) were conducted with the between-subjects factor group (verum high dose and low dose, vehicle) and the within-subject factor IPI. Newman-Keuls posthoc was calculated if appropriate. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

5.2.2. Mimicking pharmacological interference by optogenetic stimulation

Animals

14 male singlely housed B6.Cg-Tg(Thy1-COP4/EYFP)18Gfng/J-mice, bred at the MPI-P (parental generation: The Jackson Laboratory, Bar Harbor, ME, USA), were subjected to surgery and startle measurement at the age of 4 - 5 month. These transgenic mice, founder line 18 (cf. Wang et al., 2007) express the light activated ion channel channelrhodopsin-2 derived from the green alga *Chlamydomonas reinhardtii*, and yellow fluorescent protein (YFP) fusion gene (ChR2-YFP) under the control of the mouse thymus cell antigen 1 (Thy1) promoter.

Surgery

Procedure and coordinates were the same as described above (cf. p. 51). Instead of injection cannula, a guide cannula (external guide, PlasticsOne Inc., Roanoke, VA, USA) to hold the glass-fibre was implanted.

Apparatus

Glass fibres of 70 cm length were purchased from Thorlabs (Thorlabs GmbH, Dachau, Germany). A cap (PlasticsOne) with a hole was pulled over the glass fibre, which was later on used to fix the glass fibre to the external guide. The coating was removed and the bare glass fibre was inserted into a guide cannula (internal guide, PlasticsOne) and fixed with superglue. To minimise tissue damage, the length of the internal guide was chosen to round off with the external guide and the excess length of the glass fibre was defined to reach the target area .

The glass fibre was connected to another glass fibre (5 m, Thorlabs) via an optical commutator (custom made, Doric Lenses Inc., Québec, Canada) to prevent fibre twisting during animal movements. The light of a laser (488 nm wavelength, Sapphire 488-75 CDRH, Coherent (Deutschland) GmbH, Dieburg, Germany) was coupled into the glass fibre. Light pulses were generated using a shutter (Uniblitz® LS3ZM2-NL, and driver VCM-D1, Vincent Associates, Rochester, NY, USA), which was triggered by a function generator (Master 8, A.M.P.I., Jerusalem, Israel). This in turn was triggered by a modified SR-Lab™ system (cf. p. 36), where the SDI-software driven tactile-out interface (cf. SR-Lab™ manual) triggered a 5 V voltage source to generate TTL-pulses for function generator control.

Stimulation consisted of light pulses of 10 or 15 ms at 50 or 5 Hz (cf. Tsai et al., 2009), respectively, at 70 % Laser power (max. = 75 mW).

To ease animal plugging and unplugging to the glass fibre and to ensure free animal movement and accurate glass fibre position in the measuring cage during testing, a self-made cage and sensor platform were built. The cage consisted of an acrylic glass cylinder on an acrylic glass platform with a gap in the ceiling ranging from end to end of the cylinder to guide the glass fibre. The cage was removable mounted with two clips to another acrylic glass platform that carried a piezoelectric element (Conrad Electronic SE, Hirschau, Germany) on the rear side, which signals were amplified and digitised by the equipment described above (cf. p. 36).

The glass fibre was plugged onto the animal and the animal was carried to the measurement cage after a short isoflurane anaesthesia. Testing was started after the animal started showing exploring behaviour (cf. e.g. Brennan MJ, 1981).

Startle-procedure

ASR was elicited using short noise pulses of 115 dB(A) (startle eliciting pulse P) intensity with a duration of 20 ms at a background noise level of 50 dB(A). Each test session consisted of a 5 min acclimation period followed by 20 pulses for habituation to the ASR eliciting stimulus. Another 40 pulses, 80 prepulse (PP) -condition trials and eight no-pulse control trials were arranged in a pseudo-randomised order where no stimulus condition was presented repeatedly more than once before another stimulus condition was presented. Intertrial interval (ITI) was 15 s on average, ranging from 13 to 17 s. Two different prepulse conditions were presented, a "PPI condition" (interpulse interval IPI = 100 ms) and a "PPF condition" (IPI = 10 ms), based on PPI/F measures in section 7.1.1. Prepulses were of 10 ms duration and were presented at 65 dB(A) intensity. Half of all stimuli of each type were preceded by a period of light (1 and 5 s for 50 and 5 Hz stimulation, respectively), where prepulse or pulse (on pulse alone trials) was presented after the last light-pulse cycle.

Statistics

Alteration of the startle response (SR) was calculated as percental change (%ASR) for PPI and PPF of ASR:

$$\frac{SR(PP+P) - SR(P)}{SR(P)} \cdot 100\%$$

for effects of light stimulation on PPI/F:

$$\frac{SR(L+PP+P) - SR(P)}{SR(P)} \cdot 100\%$$

for effects of light stimulation on ASR:

$$\frac{SR(L+P) - SR(P)}{SR(P)} \cdot 100\%$$

where on (PP + P)-trials a prepulse (PP) preceded the startle eliciting pulse (P), on (P)-trials pulse was presented without prepulse, and on (L+)-trials light stimulation preceded prepulse or pulse presentation, respectively. Measured values of a given animal and trial-type were averaged and data were then analysed by paired t-test or one-sample t-test (for effects of light stimulation on ASR when %ASR was calculated) using Graphpad Prism. Statistical significance was accepted if $p < 0.05$, and data are presented as mean values ±SEM.

Part III.

Results

6. The startle response in paradigms of anxiety and fear

6.1. Fear potentiated startle in C57BL/6N mice

6.1.1. Fear potentiated startle using CS light or tone

The first experiment was designed to test whether CS light or sine wave tone can be paired with US to measure fear potentiated startle (FPS) when the CS is afterwards presented before startle eliciting noise pulses of 115 dB(A) in a context (startle context, cf. p. 36) different from the fear conditioning context. Mice were conditioned to either a sine wave tone or a light CS. On the following day, 115+ trials (CS presentation) showed augmented startle response compared to 115- trials (no CS presentation, four mice were excluded from analysis due to apparatus malfunction), but not equal for both CS type, indicated by significant interaction of CS type and CS presentation ($F(1,14) = 31.51$ $p < 0.05$; fig. 6.1A). CS tone significantly increased startle (posthoc -CS vs. +CS: $p < 0.05$). Contrary, CS light did not yield potentiated startle responses ($p > 0.05$), while tone and light groups did not differ in baseline startle ($p > 0.05$). Congruent, light conditioned animals showed significant less freezing when confronted with the respective CS compared to tone conditioned mice ($t(12) = 6.04$ $p < 0.05$; fig. 6.1D).

Additionally, tone conditioned animals differed significantly in percental ASR change (%ASR) and difference values (ΔASR) from light conditioned animals (fig. 6.1B,C), the latter showing almost no change from baseline ASR after CS presentation ($t(15) = 5.04$ $p < 0.05$ and $t(15) = 5.61$ $p < 0.05$, respectively).

Therefore, tone appeared to be an adequate CS to train animals for measures of FPS, while light stimuli seemed to be largely ignored by the animals. Following experiments will thus apply acoustic stimuli for conditioning.

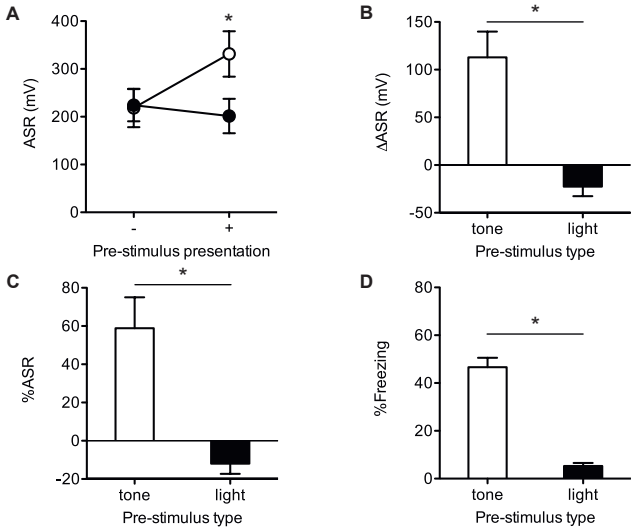


Figure 6.1.: FPS (mean ± SEM) following presentation of conditioned stimulus light (black circles and bars, n = 10) or tone (white circles and bars, n = 6), expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C), and freezing to light or tone (D). *: difference light vs. tone ($p < 0.05$).

6.1.2. Unconditioned tone effect alters startle and masks conditioned FPS

Although Experiment 1 indicated successful FPS by footshock conditioning applying tone CS, the possibility of unconditioned ASR enhancing effects demanded a pre-shock FPS-test to show that observed ASR increase could indeed be attributed to animal conditioning. Unconditioned stimulus enhancing effects on startle have been described for rats in detail by Hoffman and colleagues (Hoffman and Fleshler, 1963; Hoffman and Wible, 1969) and were also mentioned for mice by Falls and co-workers (Falls et al., 1997; Falls, 2002; Heldt et al., 2000), and partly characterised by Carlson and Willott (2001). Thus, in Experiment 2 two groups of mice were first measured for startle alterations after unconditioned tone presentation. Then, one group was conditioned to tone, while for the other group footshock was omitted (no-shock control), and FPS was measured with the same protocol that was used for unconditioned measurements before.

Statistical analysis revealed a significant effect of unconditioned tone presentation ($F(1,18) = 47.85$ $p < 0.05$), while group had no significant effect ($p > 0.05$; fig. 6.2A, left). This was also indicated by %ASR and ΔASR (fig. 6.2B,C), where no significant differences were found between the groups ($p > 0.05$, respectively), but showed a startle

increase after tone presentation of 60 - 70 % (cf. fig. 6.1C, Experiment 1: FPS ca. 60 % !).

FPS after conditioning was apparent in both groups (fig. 6.2A, right), indicated by significant effect of CS ($F(1,18) = 53.92$ $p < 0.05$); effect of group (i.e. conditioning) and interaction of CS and group were not significant (both $p > 0.05$), as it was seen in unconditioned measures of startle enhancement (fig. 6.2A, left). In parallel to unconditioned data, no significant differences occurred in ΔASR ($p > 0.05$, fig. 6.2B). Significant differences with shocked group showing higher potentiation than unshocked control only appeared in %ASR values ($t(18) = 2.39$ $p < 0.05$, fig. 6.2C). Comparing all parameters (i.e. conditioning +/- and measure 1/2), ANOVA detected an interaction of these factors ($F(1,18) = 6.05$ $p < 0.05$) indicating strong FPS in the conditioned group on measure 2 (posthoc $p < 0.05$). However, testing startle behaviour of shocked animals before and after conditioning alone using paired t-test, no significance occurred ($p > 0.05$), indicating that this significant difference was at least partly due to a decrease of FPS from measure 1 (i.e. conditioning -) to measure 2 (i.e. conditioning +) in unconditioned animals (cf. fig. 6.2C).

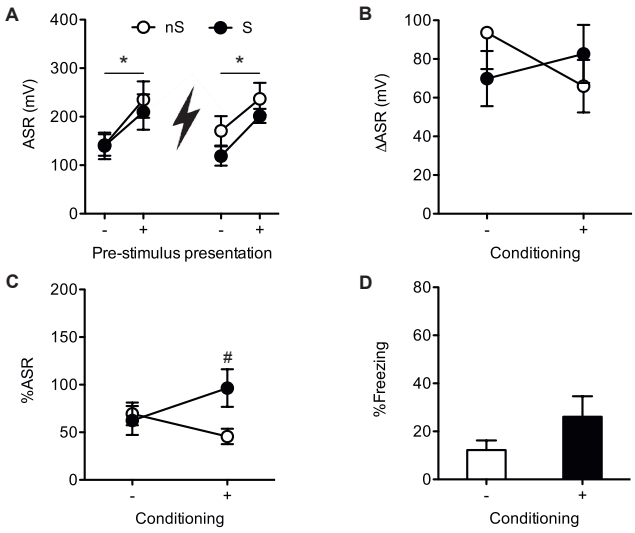


Figure 6.2.: FPS (mean ± SEM) before (-) and after conditioning (↯, +) following tone (CS) presentation in shocked (S, black circles and bars, $n = 10$) and non shocked (nS, white circles and bars, $n = 10$) mice. Data are expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C), and freezing to CS (D). *: ASR changing effect of tone presentation vs. no tone presentation before (left) and after conditioning (↯, right) ($p < 0.05$). #: %ASR increasing effect of shock vs. no shock ($p < 0.05$).

Thus, this experiment suggests that FPS observed in Experiment 1 was rather due to

unconditioned tone effects. However, freezing to CS was insignificantly different between no-shock control and shocked animals (fig. 6.2D, $p > 0.05$), the latter showing only about 20 % freezing to CS (Experiment 1: ca. 40 %), indicating weak conditioning, probably resulting from latent inhibition effects that occur after the CS was frequently presented in the unconditioned test-session before it was eventually conditioned, and which may have resulted in low FPS, too.

6.1.3. Optimising parameters to measure FPS

Experiment 2 suggested that FPS found in Experiment 1 may have been simply due to unconditioned effects of the tone preceding the startle eliciting pulse, but also revealed only weak CS memory in terms of freezing behaviour, which could account for weak FPS. In the next Experiment 3, shock intensity was increased and the influence of pulse intensity and CS duration was examined to find optimal parameters favouring FPS.

Unconditioned tone presentation again revealed strong enhancement of ASR (fig. 6.3A, left). ANOVA with within subject factors tone presentation and pulse intensity and the between subject factor tone duration showed significant effects of within subject factors (tone: $F(1,22) = 66.13$ $p < 0.05$ and P(int): $F(1,22) = 95.12$ $p < 0.05$). Tone duration had no significant effect ($p > 0.05$), but an interaction of tone duration and tone presentation only marginally failed significance ($p = 0.060$). Significant effects of pulse intensity were also apparent when analysing %ASR ($F(1,22) = 16.12$ $p < 0.05$), while ΔASR was insignificantly affected by pulse intensity and rather tone duration seemed to play a role ($p = 0.060$), with 20 s tone resulting in stronger ASR enhancement (fig. 6.3B,C).

FPS post conditioning resembled pre conditioning results (fig. 6.3A, right). ANOVA detected significant effects of CS (i.e. tone) presentation and a significant interaction of pulse intensity and CS duration ($F(1,22) = 82.23$ $p < 0.05$ and $F(1,22) = 5.20$ $p < 0.05$, respectively), indicating that 20 s CS duration were more effective in eliciting FPS with 105 dB(A) pulses than 4 s CS presentation. Also freezing to CS was a little bit stronger in mice conditioned to 20 s tone compared to mice with 4 s tone (fig. 6.3D), although by no means significant ($p > 0.05$). Pulse intensity significantly influenced %ASR as well as ΔASR, where 105 dB(A) led to much higher percental change, but revealed less change of startle amplitude ($F(1,22) = 6.16$ $p < 0.05$ and $F(1,22) = 5.20$ $p < 0.05$, respectively).

Calculating ANOVA on %ASR and ΔASR with within subject factors conditioning and pulse intensity and between subject factor CS duration let one assume, that again unconditioned tone effects masked conditioned FPS, despite pronounced freezing to CS. ANOVA revealed neither significant effects of conditioning nor interactions of condition-

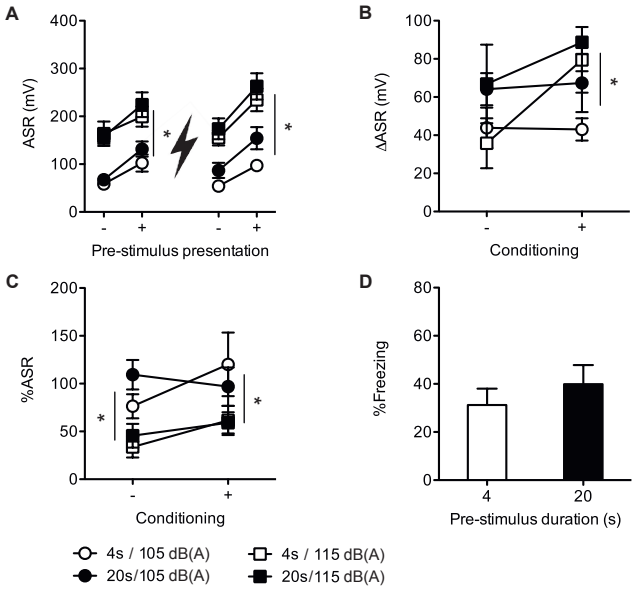


Figure 6.3.: FPS (mean ± SEM) before (-) and after conditioning (↯, +) following tone (CS) presentation of 4 s (white symbols, n = 12) or 20 s (black symbols, n = 12) duration, and startle eliciting pulses of 105 (circles) or 115 dB (squares). Data are expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C), and freezing to CS (D). *: ASR (A), ΔASR (B) or %ASR (C) changing effect of startle pulse intensity (105 vs. 115 dB, $p < 0.05$).

ing and other examined factors (all $p > 0.05$).

6.1.4. Context dependency of FPS

Although the aim was to establish a FPS protocol that strictly differentiates between conditioning and test context, FPS-studies published so far mostly use the same context for conditioning and testing (e.g. Fadok et al., 2010; Gewirtz et al., 2008; Walker et al., 2009). Additionally, Davis and Astrachan (1978) found that higher shock intensities are inversely related to FPS magnitude in rats. As a proof of concept, conditioning as well as FPS testing were conducted in the startle apparatus and shock intensity was decreased to 0.4 mA, after FPS protocols tested so far were not successful (cf. experiments described above). 105 dB(A) pulses seemed to favour potentiation in Experiment 3. Thus, this pulse intensity was applied in Experiment 4. To be congruent with freezing data of other fear related experiments of the current work, 20 s CS duration was chosen for the following experiments. To control for unconditioned tone effects and context conditioning effects, one group of animals received shocks and CS presentation in a not contiguously

manner with varied time intervals between CS and US on each trial of a test session (i.e. temporally unpaired; context control); a second group received no shock (no-shock control), while one group was fear conditioned as usual (cf. p. 12).

As expected, unconditioned tone led to potentiated startle response as indicated by significant effect of tone ($F(1,31) = 30.43$ $p < 0.05$), and unconditioned potentiation did not differ significantly between the groups ($p > 0.05$, %ASR and ΔASR respectively; fig. 6.4A, left). Unlike unconditioned tone effects, conditioned tone (i.e. FPS) on day 1 post conditioning revealed a significant interaction of group (i.e. paired conditioned, unpaired conditioned and not conditioned) and CS presentation (fig. 6.4A, right), indicating stronger potentiation in the shocked groups than in the no-shock control animals ($F(2,31) = 6.71$ $p < 0.05$). However, group effect of %ASR only approached the significance threshold ($p = 0.062$, fig. 6.4C), and while ANOVA on ΔASR showed significant effect of group ($F(2,31) = 6.71$ $p < 0.05$), posthoc comparison revealed only significant differences between paired conditioned animals and no-shock controls as well as unpaired conditioned and no-shock controls ($p < 0.05$, respectively); in contrast, significant differences between paired and unpaired conditioned mice were not observed ($p > 0.05$), indicating that FPS rather resulted from the CS context than contiguously CS tone presentation (fig. 6.4B).

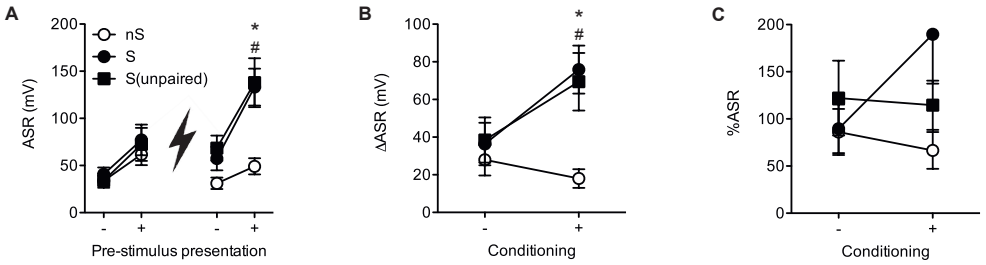


Figure 6.4.: FPS (mean ± SEM) before (-) and after conditioning (↯, +) following tone (CS) presentation in paired shocked (S, n = 11), unpaired shocked (S(unpaired), n = 12) and non shocked (nS, n = 11) mice, expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). White circles: no shock; black squares: unpaired shock; black circles: paired shock. *: ASR (A) and ΔASR (B) changing effect of paired shock vs. no shock. #: ASR (A) and ΔASR (B) changing effect of unpaired shock vs. no shock ($p < 0.05$, respectively).

6.2. Tone enhanced startle as a measure of hearing capability, stimulus adaptation and attention

6.2.1. Tone enhanced startle in mice

Startle is found enhanced during presentation of intense background noise, as well as after presentation of prepulses (i.e. prepulse facilitation, but cf. p. 27) with prepulses of long duration ($\geqslant 2$ s, cf. p. 27) and equally long interpulse intervals (i.e. IPI = duration). These phenomena have been described and characterised in rats and partly in humans, and mice. In the present experiments, detailed information of several aspects of ASR enhancing tone effects are reported and putative applications are presented.

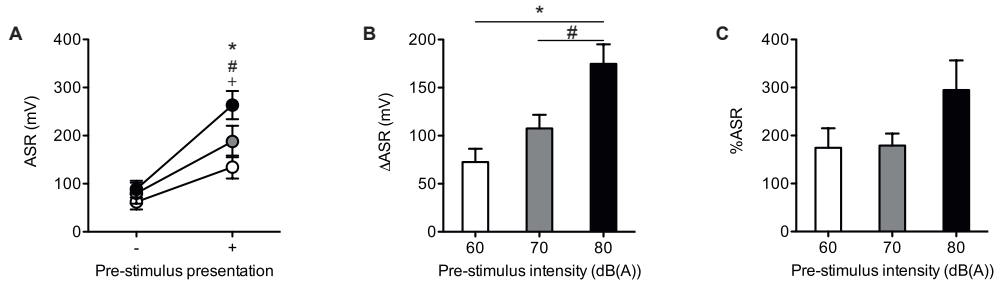


Figure 6.5.: Experiment 1. TES (mean ± SEM) following pre-stimulus (tone) presentation of 60 (white circles and bars), 70 (grey circles and bars) and 80 dB (black circles and bars) intensity (n = 12, respectively), expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). *: ASR (A) and ΔASR (B) difference 60 vs. 80 dB tone. #: ASR (A) and ΔASR (B) difference 70 vs. 80 dB tone. +: ASR (A) difference 60 vs. 70 dB tone ($p < 0.05$, respectively).

Experiment 1

Sine wave (sw, 9 kHz) stimuli of 20 s duration at an intensity of 70 dB(A) were presented preceding 105 dB(A) startle eliciting pulses and a clear-cut ASR increase in tone-compared to no-tone-presentation trials was observed (i.e. $\Delta\text{ASR} \gg 0$).

Fig. 6.5 shows that TES shares the basic properties of sensory tone perception, viz. being susceptible to different tone intensities. Statistical analysis of Experiment 1 revealed significant interaction of tone and tone intensity ($F(2,33) = 10.09$ $p < 0.05$), showing that ASR was significantly increased when pulses were preceded by a tone (pre-stimulus, PS) and that this increase was stronger with higher pre-stimulus intensity (posthoc analysis $p < 0.05$ for 60, 70, and 80 dB(A), respectively). Enhancement differed significantly with increased stimulus intensity (ΔASR: $F(2,33) = 10.09$ $p < 0.05$), although no significant enhancement differences were found in %ASR ($p > 0.05$).

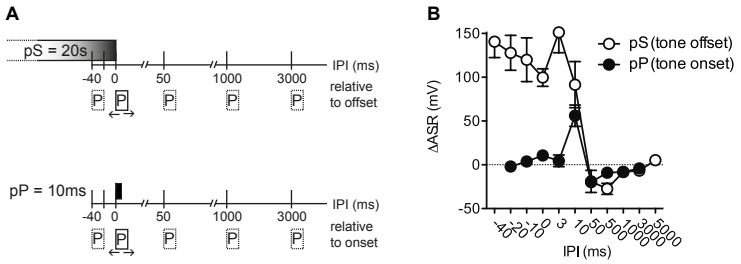


Figure 6.6.: Experiment 2. Absolute startle change (mean ± SEM) following pre-stimulus (tone off) and prepulse (tone on) presentation at various time points before, during or after startle eliciting pulse onset (n = 36). (A) Scheme of pre-stimulus (top) and prepulse (bottom) position relative to pulse onset. If interpulse interval (IPI) < 0, then tone off/tone on happened during pulse presentation. (B) Alteration of startle response expressed as absolute amplitude change.

Experiment 2

To exclude that this increase was simply due to prepulse like effects of pre-stimulus (PS) off-flank (tone off, i.e. prepulse facilitation by short IPI, PPF (cf. p. 27)), ASR increase resulting from tone-off was compared to ASR increase due to prepulse (10 ms duration, tone on) presentation in Experiment 2. Tone off and prepulse (PP, tone on) were presented at various time intervals between tone off/tone on and startle pulse onset (interpulse interval IPI, fig. 6.6A). While at IPI > 10 ms tone off led to prepulse inhibition (PPI, cf. section 3) as did the prepulse, tone off and prepulse led to almost the same amount of ASR increase at IPI = 10 ms (enhancement and facilitation, respectively). At IPI ⩽ 3 ms, prepulse presentation had no pronounced effect on ASR, while tone off led to strong startle enhancement. This indicates that at time intervals t ⩽ 3 ms TES (resulting from tone presentation) rather than PPF (due to tone off) took place (fig. 6.6B).

Experiment 3

To assess the susceptibility of TES to prior sensitisation, four groups of animals were subjected to electrical footshock of four different intensities and tested for TES 30 days later in Experiment 3 (fig. 6.7). Statistical analysis of startle amplitudes again revealed enhancement of ASR, and amplitudes differed between animals that experienced different shock intensities (fig. 6.7A), indicated by significant interaction of PS presentation and shock intensity ($F(3,55) = 5.56$ $p < 0.05$). This was resembled by significant effects of shock intensity when statistics were calculated on ΔASR (fig. 6.7B), but %ASR values (fig. 6.7C) failed to reach significance ($F(3,55) = 5.56$ $p < 0.05$ and $p > 0.05$, respectively).

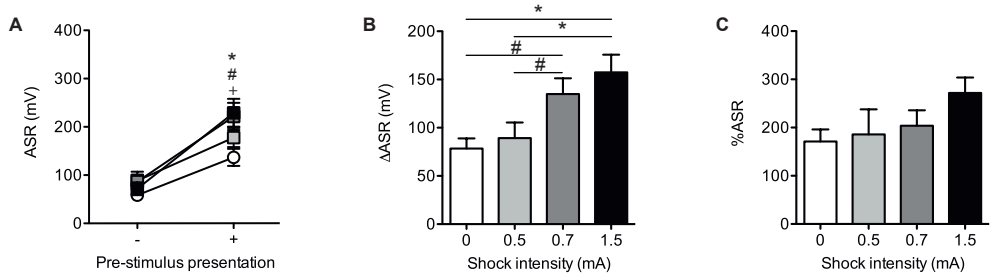


Figure 6.7.: Experiment 3. TES (mean ± SEM) following pre-stimulus (tone) presentation in sensitised mice expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). White circles and bars: no shock (n = 14); light grey circles and bars: 0.5 mA (n = 15); dark grey circles and bars: 0.7 mA (n = 15); black circles and bars: 1.5 mA footshock intensity (n = 15). *: ASR (A) and ΔASR (B) increasing effect of 1.5 mA vs. 0 (A and B) and vs. 0.5 mA (B). #: ASR (A) and ΔASR (B) increasing effect of 0.7 mA vs. 0 (A and B) and vs. 0.5 mA (B). +: ASR (A) increasing effect of 1.5 vs. 0.5 mA ($p < 0.05$, respectively).

Experiment 4

The next Experiment 4 aimed to clarify whether ASR is exclusively enhanced by acoustic stimuli. Mice were presented either light or a sine wave tone (sw) stimulus preceding a startle eliciting pulse. While sw led to expected enhancement of ASR, light did not have any significant effect on ASR amplitude, indicated by significant interaction of pre-stimulus (PS) type and PS presentation ($F(1,14) = 52.79$ $p < 0.05$) as well as significant difference between %ASR light and tone, and ΔASR light and tone ($t(14) = 2.90$ $p < 0.05$ and $t(14) = 4.11$ $p < 0.05$, respectively).

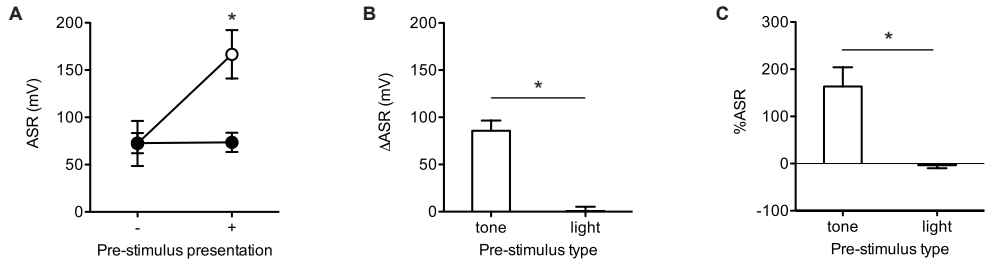


Figure 6.8.: Experiment 4. TES (mean ± SEM) following pre-stimulus presentation tone (white circles and bars, n = 8) or light (black circles and bars, n = 8), expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). *: difference light vs. tone ($p < 0.05$).

This clearly shows that light stimuli are not useful in terms of ASR enhancement at least in the mouse line evaluated in this study. However, acoustic stimuli cannot be chosen freely by means of stimulus quality, either (cf. section 6.2.2 below).

In a last set of experiments, the susceptibility of TES to pharmaceuticals commonly used in anxiety disorders was tested. The enhancing effect of background noise on startle has been demonstrated to be attenuated by treatment with anxiolytic diazepam in rats (Kellogg et al. 1991, but cf. Ison et al. 1997). It was further hypothesised that TES might be an analogous to light enhanced startle (LES) in rats, which measures anxiety in these animals and is also susceptible to benzodiazepine treatment (Walker and Davis, 2002a).

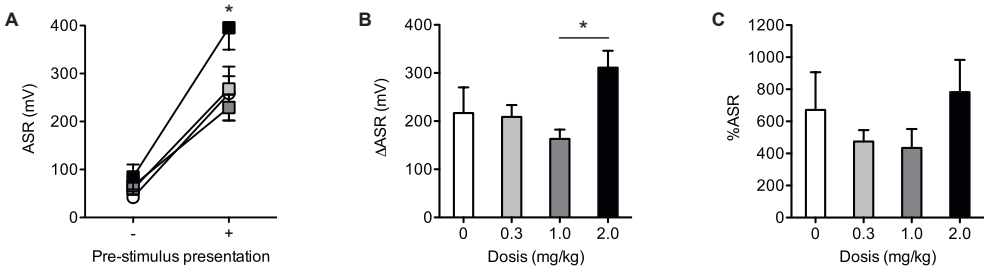


Figure 6.9.: Experiment 5. TES (mean ± SEM) after treatment of mice with 0 (white circles and bars, n = 11), 0.3 (light grey circles and bars, n = 10), 1.0 (dark grey circles and bars, n = 11) or 2.0 mg/kg diazepam i.p. (black circles and bars, n = 11), expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). *: ASR and ΔASR changing effect of 2.0 vs. 1.0 mg/kg diazepam ($p < 0.05$).

Experiment 5

In Experiment 5, again tone enhanced startle response, but this differently in the differently treated animals (five animals had to be excluded from analysis due to apparatus malfunction), indicated by significant interaction of tone presentation and diazepam dosage ($F(3,39) = 2.92$ $p < 0.05$). Only 2 mg/kg diazepam significantly altered the enhancing effect of the pre-stimulus (posthoc analysis, 1 vs. 2 mg/kg, $p < 0.05$, all other comparisons $p > 0.05$, respectively), but surprisingly had a facilitating effect (fig. 6.9A). Baseline startle (i.e. without pre-stimulus) was not affected by diazepam treatment (posthoc $p > 0.05$, respectively). This effect of 2 mg/kg was of course also apparent in ΔASR ($F(3,39) = 2.92$ $p < 0.05$, fig. 6.9B). However, when calculating %ASR, analysing enhancement relative to baseline ASR, no significant effect of diazepam was found

($p > 0.05$, fig. 6.9C).

Experiment 6

Figure 6.10 displays TES after mice were treated with paroxetine. Pre-stimulus presentation significantly enhance startle ($F(1,24) = 15.28$ $p < 0.05$), although not differently in differently treated animals ($p > 0.05$, fig. 6.10A). Paroxetine insignificantly increased both, startle without and startle after pre-stimulus presentation ($p > 0.05$). Additionally, no significant effects were found analysing ΔASR and %ASR (both $p > 0.05$, fig. 6.10B,C).

Thus, it appears that TES is not susceptible to anxiolytic treatment, demonstrating a surprisingly strong immunity to pharmacological intervention.

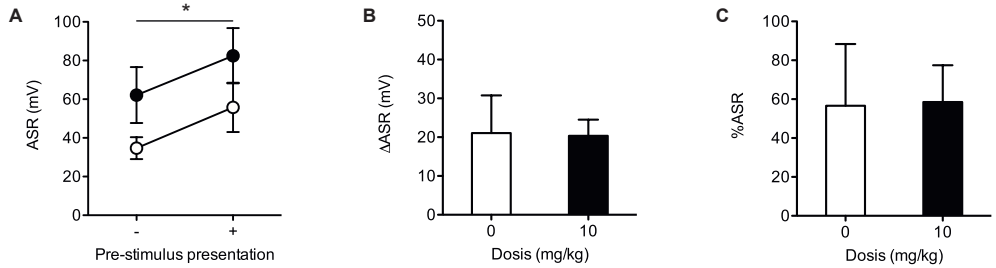


Figure 6.10.: Experiment 6. TES after treatment of mice with 0 (white circles and bars, $n = 13$) or 10 mg/kg p.o. paroxetine (black circles and bars, $n = 13$), expressed as startle response amplitudes (A), absolute amplitude change (B) or percental change (C). *: ASR changing effect of tone presentation vs. no tone presentation ($p < 0.05$).

6.2.2. TES as a measure of acoustic stimulus adaptation

Hoffman and Fleshler (1963) have shown that steady background noise results in enhanced startle response in rats. This increase persists even after hour-long presentation of noise (Hoffman and Wible, 1969). The present data demonstrate enhancing effects of long pre-stimuli or background stimuli since TES is not a tone off-flank but tone presentation effect (cf. p. 66). Are noise stimuli equally effective in the subjected mouse line and does the enhancing effect, in contrast to findings in rats, decrease with ongoing presentation; viz. can the paradigm of tone enhanced startle (TES) be applied to investigate sensory adaptability to acoustic stimuli?

To answer these questions, four groups of animals were presented sine wave tone (sw) or white noise (wn) pre-stimuli (PS) of 20 s or 120 s duration over four days (day 1 - 3 and

day 9, one animal was excluded from analysis due to apparatus malfunction). Again, stimulus presentation significantly altered ASR, but differently with sw and wn stimuli, indicated by significant interaction of stimulus presentation and stimulus quality (i.e. sw or wn, $F(1,160) = 200.44$ $p < 0.05$). While sw stimuli significantly enhanced ASR as expected, wn stimuli led to significant inhibition of ASR (fig. 6.11A) (posthoc analysis -PS vs. +PS, both $p < 0.05$). Stimulus duration had no significant effect ($p > 0.05$), and also day of measurement was not tested significant ($p > 0.05$), indicating that there was no stimulus adaptation from day to day, at least in terms of ASR alteration effects. This was true also for %ASR and ΔASR (fig. 6.11B,C), where statistical analysis revealed significance only for stimulus quality ($F(1,160) = 154.76$ $p < 0.05$ and $F(1,160) = 200.44$ $p < 0.05$, respectively).

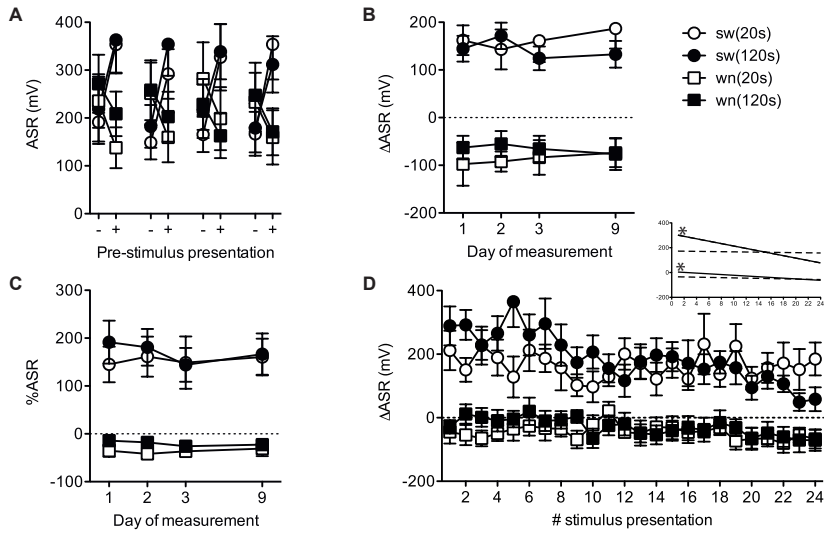


Figure 6.11.: TES (mean ± SEM) following pre-stimulus presentation of different length (20 s and 120 s, white and black symbols, respectively) and quality (sine wave (sw) and white noise (wn), circles and squares, respectively) (all n = 12, except 20 s sw: n = 11). Data are expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). Averaged values (day 1 - 9) of within-day habituation are shown in (D). Inset of (D): linear regression of data depicted in (D) (scattered lines: 20 s, solid lines: 120 s). *: Slope significantly different from zero ($p < 0.05$).

Contrary, within-session TES decreased with repeated presentation of PS + P (i.e. trial number) and this decrease was different between animals presented pre-stimuli of different quality and duration (fig. 6.11D). This was indicated by significant interaction of pre-stimulus quality, pre-stimulus duration and trial number (i.e. repeated presentation), calculating ANOVA on ASR amplitudes ($F(23,989) = 1.55$ $p < 0.05$). When looking at

percental change (i.e. relative to last ten trials of habituation phase, cf. methods pp. 42), ANOVA found significant interaction of trial and pre-stimulus quality ($F(23,989) = 2.20$ $p < 0.05$). Additionally, linear regression calculated on ΔASR as well as on %ASR revealed negative slopes for each stimulus type (positive and close to zero (slope = 0.01) for 20 s wn stimuli in %ASR). Moreover, slopes were tested significantly different from zero only for 120 s stimulus duration (ΔASR: $R^2 = 0.03$, $F = 9.56$ and $R^2 = 0.12$, $F = 40.42$; %ASR: $R^2 = 0.03$, $F = 7.92$ and $R^2 = 0.07$, $F = 21.93$, $p < 0.05$, wn and sw, respectively), viz. the effect of wn (i.e. startle inhibition) got even stronger with prolonged stimulus presentation (fig. 6.11D, inset). These results suggest that stimuli of 20 s duration, but white noise pre-stimuli of any duration in particular are less prone to habituation (which would result in weaker enhancement/inhibition of ASR) than sine wave stimuli of longer duration.

Although ASR to pulse alone trials (-PS) was found to be significantly affected by trial number, too ($F(11,473) = 2.27$ $p < 0.05$), ASR(-PS) was not affected by any factor when related to baseline ASR (%ASR, $p > 0.05$, respectively). Thus, TES indeed measured adaptation to (long) sine wave stimuli.

6.2.3. TES as a measure of hearing capability

TES was shown to be susceptible to different intensities of the pre-stimulus (cf. fig. 6.5). To further illustrate the potency of TES as a primary perception measure paradigm, TES measured in transient receptor potential vanilloid 1 deficient (TRPV1-ko) mice. These mice have been shown to have less pronounced fear response than their wild-type counterparts (Marsch et al., 2007), but do not differ in stimulus perception measured by acoustic brainstem responses. In line with the observation of low fear/anxiety-behaviour by Marsch and colleagues, lower baseline ASR was observed in TRPV1-ko mice (i.e. no pre-stimulus trials, one-tailed t-Test: $t(22) = 1.78$ $p < 0.05$). On the other hand, there was no significant effect of genotype in %ASR or ΔASR (i.e. comparable amount of TES, t-Test: $p > 0.05$, respectively), although tone presentation significantly enhanced ASR in both mouse lines ($F(1,22) = 29.28$ $p < 0.05$), indicating equal tone perceptibility in animals of low anxiety and fear (TRPV1-ko) and wt animals (fig. 6.12), resembling the findings by Marsch et al. (2007).

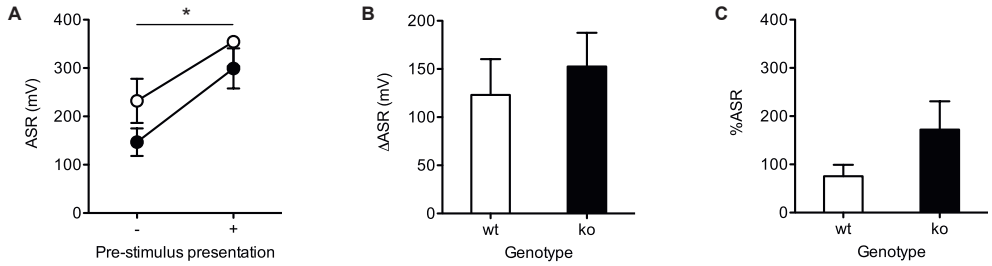


Figure 6.12.: TES in vanilloid receptor deficient mice (ko, black circles and bars, n = 12) and wild type counterparts (wt, white circles and bars, n = 12), expressed as startle response amplitudes (A), absolute amplitude change (B) or percental change (C). *: ASR changing effect of tone presentation vs. no tone presentation ($p < 0.05$).

6.2.4. Attention measured by means of altered TES

Attention to the tone preceding the startle eliciting pulse is probably necessary to achieve strong enhancement of the startle response resulting from tone presentation (i.e. TES). This could be used to measure attention in mice by means of TES. To measure distraction, a second stimulus was introduced to shift the animal's attention to this stimulus. Startle of several mouse lines has been reported to be at most marginally affected by short light prepulses at interpulse intervals of 2 s (Aubert et al., 2006). To make sure that the animal perceives the light stimulus, the last two seconds of the tone preceding the startle eliciting pulse were superimposed with a bright light.

Two animals had to be excluded from analysis due to chamber malfunction and escape from measuring cage. ANOVA detected no influence on animal behaviour of light or tone presentation per se (fig. 6.13A), indicated by insignificant differences between startle measures during light (+L), tone (+T) or background noise presentation only (-) ($p > 0.05$). Contrary, repeated measures ANOVA found significant differences between startle amplitudes of pulses that were either preceded by tone, light, or tone and light stimuli ($F(3,12) = 5.68$ $p < 0.05$). Acoustic startle response (ASR) was significantly higher when preceded by tone alone compared to all other pulse conditions, respectively (posthoc: all $p < 0.05$, respectively). All other conditions did not differ significantly among each other (posthoc $p > 0.05$, respectively). Additionally, %ASR as well as ΔASR differed significantly depending on which pre-stimulus was presented ($F(2,8) = 7.80$ $p < 0.05$ and $F(2,8) = 5.96$ $p < 0.05$, respectively), and in both cases posthoc analysis revealed significant differences between tone and the other both prestimulus conditions ($p < 0.05$, respectively), but only insignificant differences between

changes resulted from light and combined light and tone presentation (fig. 6.13B,C, $p > 0.05$, respectively). This clearly indicates that an additional introduced stimulus attenuates TES resulting from tone presentation, and this attenuation did not simply result from summation of inhibitory and enhancing effect of light and tone presentation, respectively, but was rather due to shift of the animal's attention.

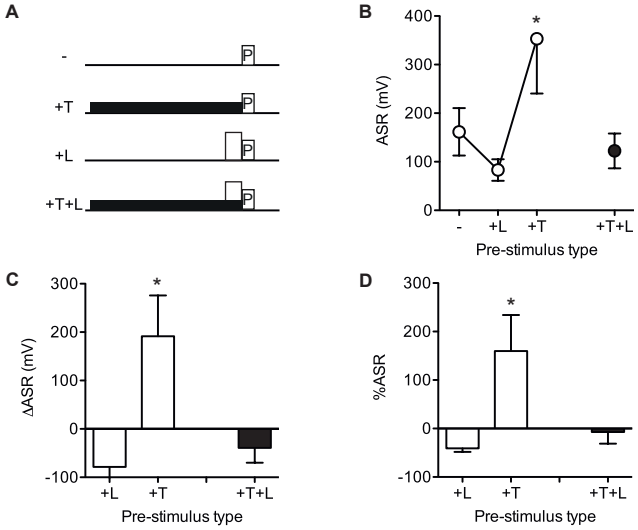


Figure 6.13.: TES (mean ± SEM) following presentation of pre-stimulus tone (+T), light (+L) or tone superimposed by light (+T+L, black circles and bars, $n = 5$). (A) Scheme of stimulus presentation. P: startle eliciting pulse (-), black rectangle: 20 s tone presentation (+T and +T+L), white rectangle: 2 s light presentation (+L and +T+L). TES is expressed as startle amplitude (B), absolute amplitude change (C) or percental change (D). *: ASR (A), ΔASR (B) and %ASR (C) difference [+T] vs. [+T+L] ($p < 0.05$, respectively).

6.3. Fear conditioning parameters - the matter of fact

6.3.1. Mice differ in their behavioural response to white noise and sine wave stimuli

The acoustic startle response (ASR) of mice is an unbiased measure of reflexive behaviour. To test animal behaviour for a priori perception differences to stimuli of different quality (i.e. sw or wn), the ASR to sw and wn startle eliciting pulses (P) was measured (one animal was excluded from analysis due to apparatus malfunction). Statistical analysis revealed a significant interaction of pulse intensity and quality ($F(3,30) = 15.79$

$p < 0.05$). As shown in fig. 6.14, at 105 and 115 dB(A) animals showed differences in their ASR to different stimulus qualities (posthoc analysis: $p < 0.05$, respectively). However, no significant differences occurred at lower intensities, making it convenient to use stimuli of these low intensities in fear conditioning (all $p > 0.05$).

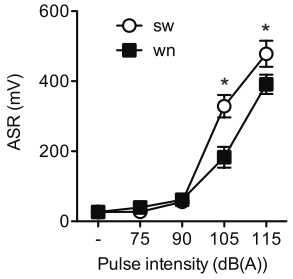


Figure 6.14.: Startle response to acoustic pulses of different intensities and quality (white noise (wn) squares, and sine wave (sw) circles, $n = 11$). *: effect of pulse quality sw vs. wn ($p < 0.05$).

6.3.2. Between-session extinction as a function of quality but not duration of acoustic stimuli

To clarify the role of conditioned stimulus (CS) duration and CS quality in fear conditioning and extinction, on day 0 four groups of mice were conditioned to either sw or wn stimuli of either 20 s or 120 s duration, respectively. Extinction training was performed on day 1 - 3 and extinction retrieval was tested on day 9, presenting each mouse ten times 20 s CS of their respective quality, respectively. CS were presented at various intervals (vi) which has been shown to be most effective in terms of extinction learning (Plendl and Wotjak, 2010).

There was no significant effect of CS duration ($p > 0.05$). In contrast, there was a significant interaction of CS quality and day of measurement ($F(3,126) = 11.74$ $p < 0.05$). CS quality strongly affected fear-memory retrieval (day 1) and extinction retrieval (day 9) (fig. 6.15A, posthoc analysis: $p < 0.05$, respectively). There was also a significant interaction of stimulus quality and stimulus presentation on day 9 in freezing behaviour measured in the extinction- (fig. 6.15B) and conditioning context (fig. 6.15C, $F(1,44) = 27.30$ $p < 0.05$ and $F(1,44) = 13.84$ $p < 0.05$, respectively). During extinction retrieval in the extinction context, freezing levels of wn conditioned mice were significantly higher compared to sw conditioned mice (posthoc analysis sw vs. wn: $p < 0.05$), indicating poor extinction memory acquisition. On the other hand, wn conditioned animals showed de-

creased freezing to CS in the conditioned context (posthoc analysis sw vs. wn: $p < 0.05$), which, contrary to the finding in the extinction context, would indicate good extinction-memory performance. Importantly, mice did not differ significantly in their freezing behaviour to extinction- and conditioned context per se (posthoc analysis sw vs. wn: $p > 0.05$ extinction and conditioned context, respectively), excluding influences of different context-memory between sw and wn group on freezing behaviour, and in both groups freezing to context was much higher in the conditioned context than in the extinction context, showing strong conditioned context memory (cf. fig. 6.15B,C, CS- and CS+, respectively). Additionally, there were significant interactions of CS quality and CS number (i.e. number of CS presentations, 1 - 10) on day 1 and day 2 ($F(9,396) = 6.84$ $p < 0.05$ and $F(9,396) = 3.54$ $p < 0.05$, respectively) as well as significant effects of CS quality and CS number on day 3 of extinction training ($F(1,42) = 8.94$ $p < 0.05$ and $F(9,378) = 2.08$ $p < 0.05$, respectively), indicating impaired within-session extinction in wn conditioned animals (data not shown). This supports the hypothesis of CS quality having strong impact on animal learning, since freezing behaviour to either CS quality did not differ significantly in unconditioned mice (t-Test: $p > 0.05$).

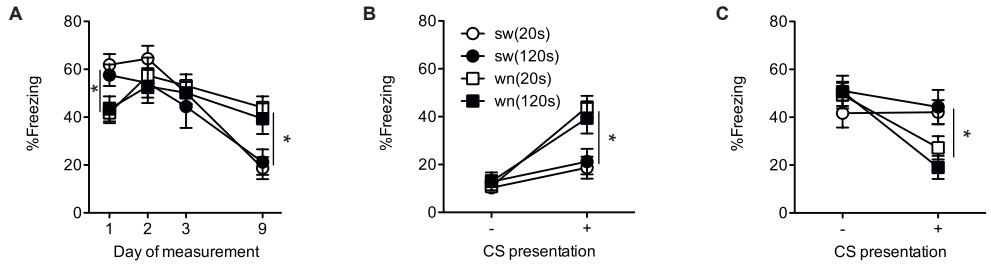


Figure 6.15.: Freezing to stimuli (CS) of white noise (squares) and sine wave (circles) after conditioning to stimuli of either quality and 20 s (white symbols) or 120 s duration (black symbols) (each group $n = 12$). (A) Freezing to first stimulus on each of four days of extinction training. (B) Freezing to extinction context on d9 20 s before (-) and during first stimulus presentation (+). (C) Freezing to conditioned context on d9 20 s before (-) and during first stimulus presentation (+). *: effect of stimulus quality wn vs. sw ($p < 0.05$).

6.3.3. Stimulus quality leads to categorical differences in the FPS/TES paradigm

The previous experiment showed that freezing behaviour is strongly affected by stimulus quality, and white noise pre-stimuli were shown to inhibit rather than enhance ASR in the

TES-paradigm (cf. section 6.2.2). How does stimulus quality affect animal behaviour in a FPS/TES experiment? Four groups of mice were conditioned to either sw or wn stimuli of 20 s duration, respectively. In control animals unconditioned stimulus (footshock) was omitted (no-shock control). The day after conditioning, animals were measured for FPS (conditioned mice) and TES (control mice).

Significant interaction of CS quality and CS presentation, and CS quality and conditioning in ASR amplitudes indicated that again CS quality markedly affected mouse behaviour (fig. 6.16A, cf. section 6.2.2) ($F(1,32) = 69.81$ $p < 0.05$ and $F(1,32) = 5.41$ $p < 0.05$, respectively). In addition to insignificant interaction of conditioning, CS quality and CS presentation ($p > 0.05$), there was only a significant effect of CS quality in ΔASR and %ASR (i.e. FPS/TES, $F(1,32) = 69.81$ $p < 0.05$ and $F(1,32) = 41.38$ $p < 0.05$, respectively), but not conditioning (fig. 6.16B,C; $p > 0.05$). This indicates that pronounced TES effects masked FPS, supporting the previous conclusions (cf. p. 61 and p. 97).

As seen before (cf. section 6.2.2), sw stimuli led to potentiated ASR (posthoc analysis -CS vs. +CS: $p < 0.05$). In contrast, there was no significant effect of CS presentation in wn group (posthoc analysis -CS vs. +CS: $p > 0.05$). CS wn rather decreased ASR when it preceded the pulse in both conditioned and unconditioned mice. Freezing behaviour measured on day 7 after conditioning in a neutral context revealed significant effects only for the factors conditioning and CS presentation ($F(1,31) = 20.92$ $p < 0.05$ and $F(9,279) = 9.74$ $p < 0.05$). No significant effects of CS quality were observed ($p > 0.05$), indicating that the effect of stimulus quality on FPS/TES again was not simply due to differences in perception of the different stimuli, but affected animal behaviour on higher levels of brain function.

6.4. Extinction of conditioned fear to context by cue extinction training

6.4.1. Extinction of conditioned stimulus does not lead to alleviated conditioned context fear

To test the hypothesis of parallel context extinction while cue conditioned stimulus (CS) extinction training, animals were subjected to trace fear conditioning in the conditioning context (cf. p. 35) and extinction training in the startle apparatus (cf. p. 36) to ensure context dependent learning. Trace (i.e. gap between cue CS offset and unconditioned stimulus (US) footshock onset) conditioning has been shown to be highly dependent on

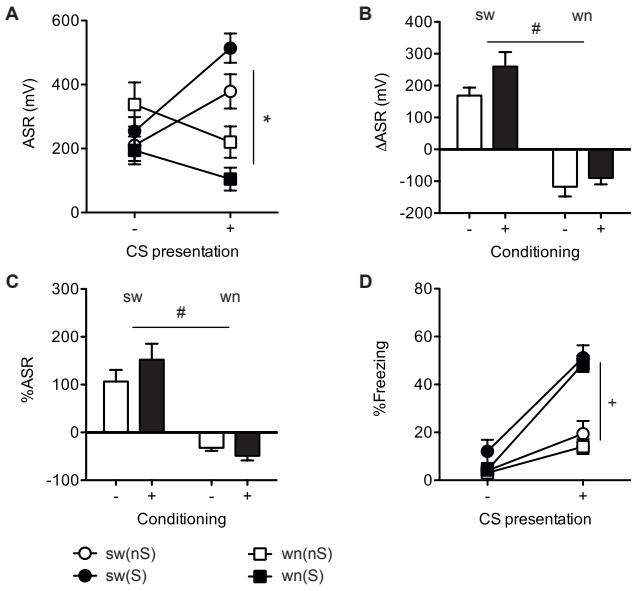


Figure 6.16.: FPS following white noise (wn, squares) or sine wave (sw, circles) stimuli in conditioned (black symbols and bars, S, n = 12 sw and wn respectively) and naive (white symbols and bars, nS, n = 6 sw and wn respectively) mice, expressed as startle amplitude (A), absolute amplitude change (B) or percental change (C). (D) Freezing to neutral context 30 s before (-) and during (+) 30 s stimulus presentation of respective quality. *: ASR (A), ΔASR (B) and %ASR changing effect of stimulus quality wn vs. sw. #: Significant effect of conditioning (shocked vs. non shocked animals, $p < 0.05$). +: effect of conditioning vs. unconditioned ($p < 0.05$, respectively).

hippocampus (HPC) function (cf. McEchron et al., 1998; Moyer et al., 1990), and was performed to favour HPC participation in this paradigm since the working hypothesis argues that context extinction will take place via pattern completion in the HPC during cue CS presentation. Since animal observation was not possible during extinction training, animal movement scores were analysed by means of startle sensor voltage output.

CS presentation resulted in significant movement inhibition (fig. 6.18A,B and fig. 6.17). This behaviour was apparent only on day 1 and (less) on day 2 of extinction training indicated by significant interaction of training (i.e. extinction training (ex) or extinction control (nex)) and CS presentation ($F(1,18) = 22.22$ $p < 0.05$, $F(1,18) = 9.85$ $p < 0.05$ and $p > 0.05$ for day 1, 2 and 3, respectively), suggesting successful extinction learning (fig. 6.18C). To ensure successful extinction of CS memory, freezing behaviour (cf. p. 13) was measured in a neutral context presenting a sequence of four CS tones. CS-extinction was highly context dependent. Freezing to neutral context and during first tone presentation (fig. 6.18C) were not significantly different between ex (i.e. extinction training) and

nex (i.e. extinction control) mice ($p > 0.05$). However, ANOVA revealed a significant interaction of CS number (i.e. 1 - 4) and training ($F(3,66) = 6.52$ $p < 0.05$). While nex animals remained on high freezing levels throughout CS presentation, freezing scores of extinction trained animals decreased from this high to about mottled levels during the course of CS presentations (fig. 6.18B), demonstrating that extinction memory can be contextually generalised after short additional training. Contrary, both groups of mice demonstrated equally intact context memory during exposure to the initial conditioning context, where no significant effect of training was detected ($p < 0.05$, fig. 6.18D).

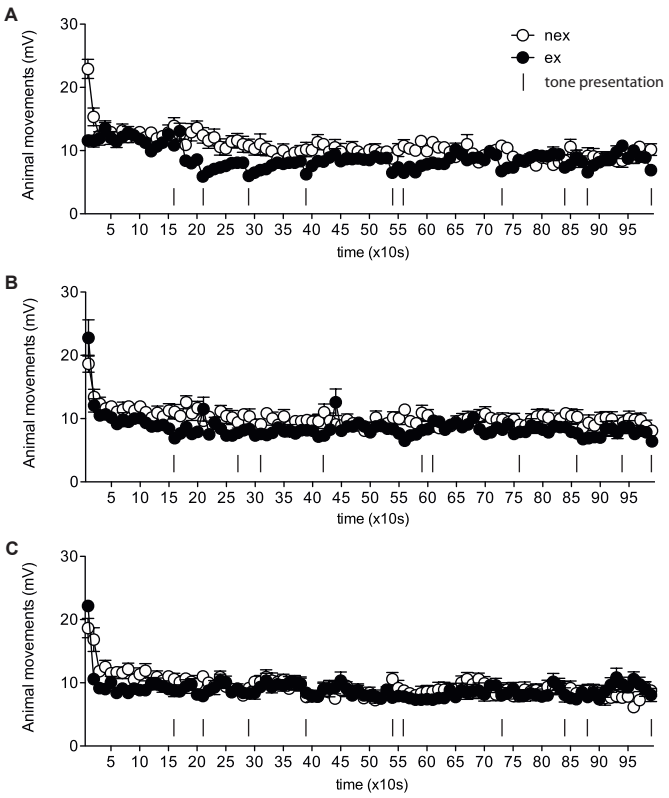


Figure 6.17.: Animal movements during the course of three consecutive days of extinction training. White circles: non extinction (nex, $n = 12$), black circles: extinction training (ex, $n = 12$). Vertical lines indicate conditioned stimulus presentation.

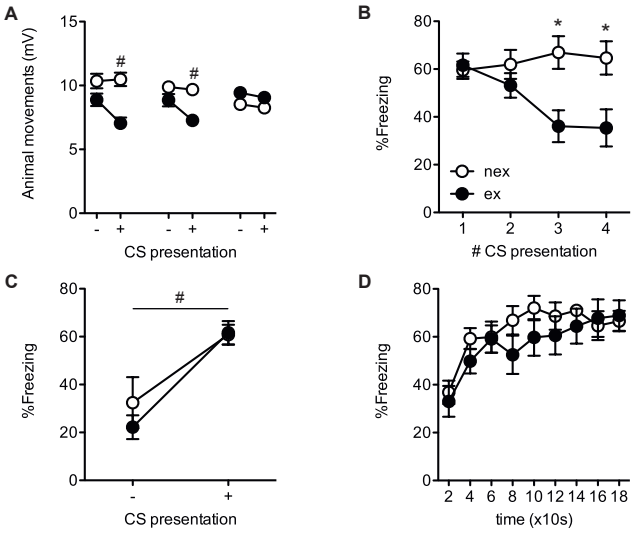


Figure 6.18.: Averaged animal movements on three consecutive extinction training sessions from fig. 6.17A,B,C, respectively, 20 s before (-) and during CS presentation (+, refers to | in fig. 6.17A,B,C) (A). Freezing of extinction trained (ex, black circles, n = 12) and extinction control (nex, white circles, n = 12) mice during extinction training on day 1 - 3 20 s before (-) and during CS presentation (+), on day 7 during memory retrieval of conditioned stimulus (CS) (B) and 20 s before (-) and during first CS presentation (+) (C), and memory retrieval of conditioned context on day 9 (D). *: effect of extinction training ex vs. nex. #: effect of CS presentation vs. no presentation ($p < 0.05$, respectively).

6.5. ASR measures in mouse-models of trait anxiety and PTSD

6.5.1. ASR in mice of high and low anxiety related behaviour

To assess fear learning in mice of the high and low anxiety related behaviour model, animals were conditioned and subsequently tested for conditioned stimulus (CS) memory. Conditioning led to significant differences in freezing behaviour between the different mouse lines ($F(2,23) = 24.36$ $p < 0.05$). HAB and - less - NAB mice showed pronounced freezing during CS presentation, while LAB mice showed almost no freezing (fig. 6.19A). As expected, HAB mice showed significantly higher freezing behaviour to the CS compared to NAB and LAB mice. While this could have arisen either from principal differences in anxiety related behaviour, as the model of HAB/LAB rats suggests (cf. Salomé et al., 2002), it might have resulted also simply from differences in hearing capabilities or shock sensitivity.

To test for hearing capabilities of the three mouse lines, baseline acoustic startle

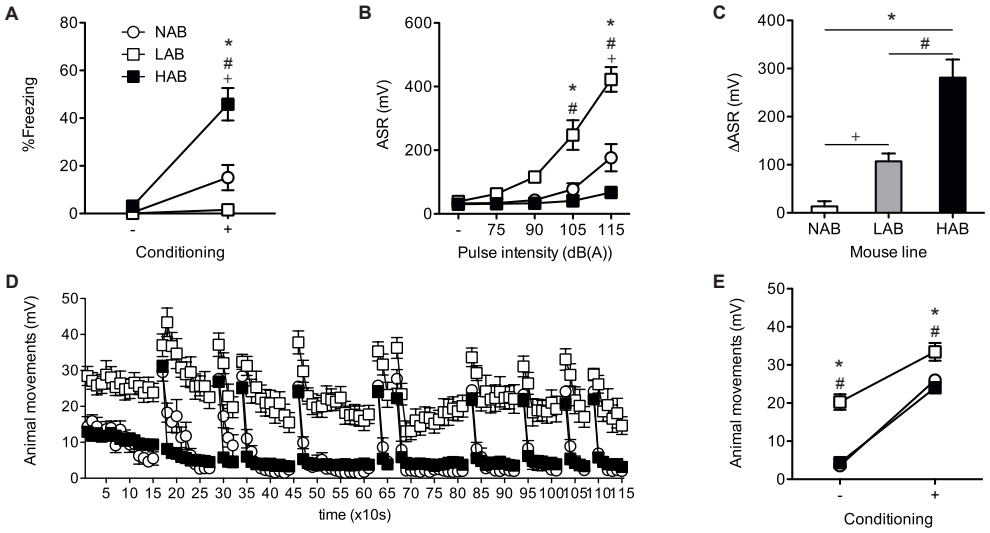


Figure 6.19.: Freezing before conditioning (-) and during conditioned stimulus (CS) memory retrieval (+) (A), startle response to acoustic (B) and electric (D,E) stimuli, and startle enhancement by pre-stimulus presentation (TES, C) in mice of high (black circles and bars), normal (white squares and bars) and low (white squares and grey bars) anxiety related behaviour (HAB/NAB/LAB, n = 9/7/10 (A), n = 8/11/8 (B), n = 11/11/10 (C) and n = 20/8/20 (D,E), respectively). *: effect of mouse line HAB vs. LAB. #: effect of mouse line NAB vs. LAB. +: effect of mouse line NAB vs. HAB ($p < 0.05$, respectively).

response (ASR) was measured. Lines differed tremendously in their startle response (fig. 6.19B), indicated by significant interaction of startle eliciting pulse (P) intensity and line ($F(8,96) = 13.01$ $p < 0.05$). While HAB, showing pronounced freezing to CS, showed almost no SR even to high intense pulses, LAB mice, which show almost no freezing behaviour, reacted potently to each presented pulse intensity. NAB mice showed intermediate responses, thus SR inversely mirroring findings in freezing behaviour of these mouse lines (cf. fig. 6.19A). HAB/NAB/LAB mice were also measured for tone enhanced startle (TES, cf. section 6.2), since it is proposed to be putative applicable as a basal measure of hearing capabilities (cf. section 6.2.3). Tone presentation was differently effective in enhancing ASR, indicated by significant interaction of tone presentation and mouse line ($F(2,29) = 30.97$ $p < 0.05$). Posthoc analysis revealed significant enhancement by tone presentation for HAB and LAB mice, while baseline startle resembled data found before (cf. fig. 6.19B, data not shown). Mouse lines also differed significantly in %ASR ($F(2,29) = 50.83$ $p < 0.05$) as well as in difference scores ($F(2,29) = 30.98$ $p < 0.05$), where HAB mice showed highest potentiation, followed by LAB and NAB mice (fig. 6.19C). Al-

though startle data and TES data are not consistent, which indicates different hearing abilities of the subjected mouse lines, these results render it very unlikely that differences found in fear acquisition can be assigned to differences in hearing capabilities.

To assess susceptibility to electric footshocks, HAB/NAB/LAB mice were subjected to conditioning and their reaction by means of startle amplitude was measured. Figure 6.19D shows animal behaviour during the course of ten condition trials. Statistics calculated on difference values (animal movement before tone and shock pairing (-), subtracted from animal movement during tone and shock pairing (+), fig. 6.19E) revealed significant differences between strains ($F(2,45) = 8.74$ $p < 0.05$), showing that LAB mice increased movements during tone and shock pairing significantly less than HAB and NAB animals (posthoc $p < 0.05$, respectively). This cannot be attributed solely to less shock sensitivity of LAB animals. In fact the absolute movement scores of LAB mice were the highest of all measured mouse lines also during tone and shock pairing, and smaller difference values resulted from very high baseline movement scores in these animals. NAB and HAB mice did not differ significantly in their response to tone and shock pairings (posthoc, $p > 0.05$). Together these data indicate that different freezing scores do not result from differences in shock susceptibility during fear conditioning.

6.5.2. ASR as a measure of hyperarousal in a mouse model of PTSD

CRH and PTSD model, both increase startle responses in mice

Increased corticotropin releasing hormone (CRH) levels and elevated startle responsiveness are found in patients suffering from post-traumatic stress disorder (PTSD). To pave the way for studies of possible interrelations of these symptoms in the mouse-model of PTSD, animals were treated with CRH and mice of the PTSD model were subjected to startle measures to establish the CRH enhanced and PTSD associated enhancement of startle, respectively.

After animals had recovered from surgery, acoustic startle response was measured after intracerebroventricular (i.c.v.) injection of CRH, or CRH in combination with the specific CRH-receptor blocker αCRH. Significant interaction of treatment and startle eliciting pulse intensity showed successful acoustic startle response (ASR) enhancement by CRH treatment ($F(8,72) = 4.71$ $p < 0.05$). This in turn was prevented by co-treatment with αCRH (fig. 6.20). While CRH treated animals showed significantly higher ASR than vehicle or CRH/αCRH treated mice (posthoc, $p < 0.05$, respectively), the latter two did not differ significantly ($p > 0.05$). Thus, ASR is specifically enhanced by increased

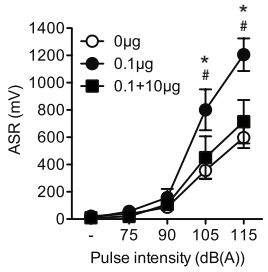


Figure 6.20.: Startle response in mice treated with either 0.1 µg CRH (black circles, n = 7), 0.1 µg CRH and 10 µg αCRH (black squares, n = 7) or vehicle (white circles, n = 7). *: effect of treatment CRH vs. veh. #: effect of treatment CRH vs. CRH/αCRH ($p < 0.05$, respectively).

cerebral CRH.

To examine ASR in mice of the PTSD model, animals received a single intense footshock and were measured for startle response 30 days later. ANOVA observed a significant interaction of footshock and pulse intensity (fig. 6.21), proving that shocked animals had stronger startle reactions than animals which did not experience a footshock ($F(4,108) = 4.22$ $p < 0.05$). Significant differences occurred at 105 and 115 dB(A) (posthoc $p < 0.05$, respectively) as it was observed in CRH treated animals, resembling observations in patients.

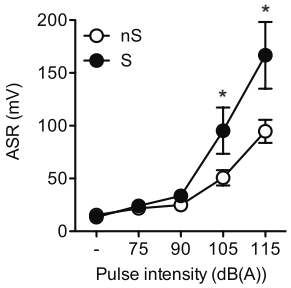
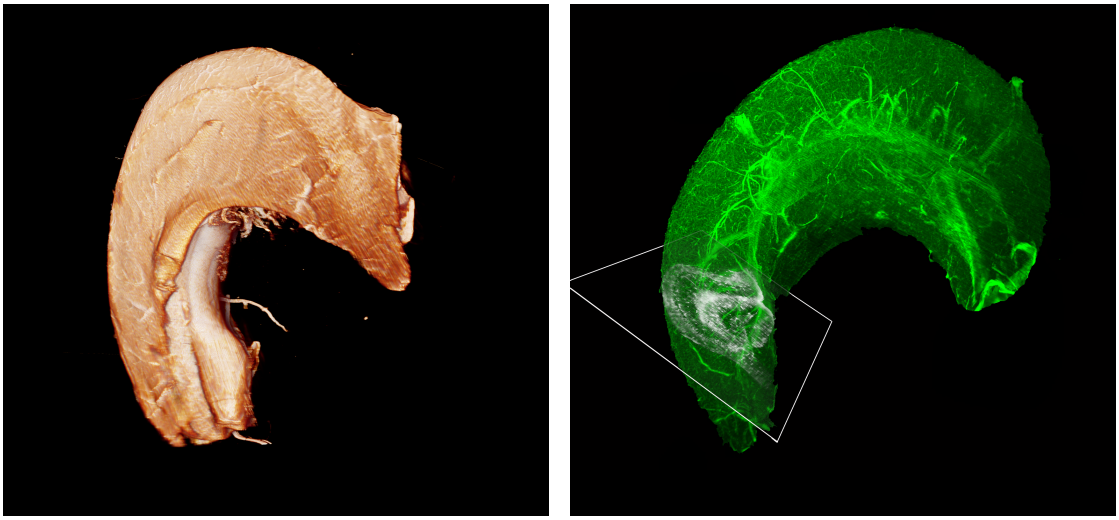


Figure 6.21.: Startle response in mice 30 days after receiving an intense footshock (S, black circles, n = 14) or exposure controls (nS, white circles, n = 15). *: effect of shock vs. no shock ($p < 0.05$).

Enriched housing prevents HPC shrinkage in the PTSD model, but has no influence on startle response

Another prominent symptom found in PTSD patients is decreased HPC volume. While on the one hand it is still discussed whether this finding results from the traumatic ex-

perience or is merely a risk factor for developing PTSD after trauma, or whether it even is a reliable symptom (cf. e.g. Golub et al., 2010), on the other hand it is still unknown, what anatomical changes account for volume loss. To evaluate whether HPC volume changes are a symptom in the PTSD mouse model, too, and - continuative - whether these issues can be studied in the presented model, these mice were subjected to enriched housing, known to favour HPC volume and function (cf. Goshen et al., 2009; van Praag et al., 2000). Subsequently, animals were behaviourally analysed and their HPC volume measured by means of ultramicroscopic imaging.



(a) (b)

Figure 6.22.: Three dimensional reconstruction of a mouse hippocampus (HPC). Imaged sections recorded were loaded into a 3D reconstruction software (Amira), and models rendered using different surface effects. (a) Opaque surface view. HPC is displayed like uncleared tissue. (b) Transparent view. HPC is displayed like it is monitored during imaging process. The white quadrangle indicates the light sheet illuminating the HPC in a discrete plane (white highlighted tissue).

As a proof of concept, GFP-M mice (derived from founder line M, cf. Feng et al., 2000) tested positive for carrying the gene for expression of GFP were housed in standard cages or under enriched conditions. Animals were then measured for HPC volume and neuronal density by means of amount of fluorescence recorded. After four weeks, animals were killed and their HPC dissected and cleared for ultramicroscopic imaging (nine animals had to be excluded from this study due to loss of either left or right HPC during dissection). Analysis of image stacks were compared between the two animal groups (i.e. housing). Statistical analysis revealed indeed significant differences in HPC

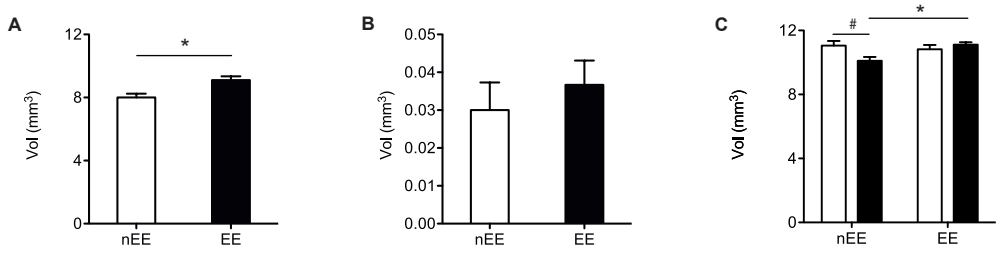


Figure 6.23.: Volume of hippocampus (A,C) or amount of GFP fluorescence (B) of mice after standard housing (nEE, white bar, n = 6) or enriched housing (EE, black bar, n = 9) (A,B), and mice after enriched or standard housing and sensitisation (S, black bars) or no shock (nS, white bars) (nEE-nS n = 10, nEE-S n = 9, EE-nS n = 9, EE-S n = 8) (C). *: effect of EE vs. nEE. #: effect of S vs. nS (nEE) ($p < 0.05$, respectively).

volume (fig. 6.23A), with enriched animals showing larger HPC than animals kept under standard conditions ($t(13) = 3.13$ $p < 0.05$). On the other hand, differences in GFP fluorescence, thought to be a quantitative measure of neuronal tissue in mice expressing this protein in neuronal cells and cellular extensions, did not significantly differ between the animals ($p > 0.05$, fig. 6.23B). Fluorescence did also not differ significantly when analysed for laterality, or differences between dorsal and ventral HPC (data not shown, $p > 0.05$, respectively).

Having shown that HPC volume can be readily quantified by means of ultramicroscopic imaging, the next experiment aimed to analyse HPC volume of animals of the PTSD mouse model. Four groups of mice, either kept under enriched or standard housing conditions, underwent the PTSD-protocol or were exposed to the shock context only (exposure control), respectively. After incubation time, animals were subjected to measures of ASR (one animal had to be excluded due to apparatus malfunction) and HPC volume (28 mice had to be excluded due to loss of either left or right HPC during dissection).

ANOVA detected significant interactions of shock and startle eliciting pulse intensity as well as enrichment and shock intensity ($F(4,236) = 4.27$ $p < 0.05$ and $F(4,236) = 2.81$ $p < 0.05$, respectively). As seen before (cf. section 6.5.2), posthoc analysis revealed significant differences between shocked animals and exposure controls at 105 and 115 dB(A) pulse intensity ($p < 0.05$, respectively), indicating that enrichment did neither facilitate, nor prevent the PTSD symptom hyper-arousal in this model (fig. 6.24). Analysing HPC

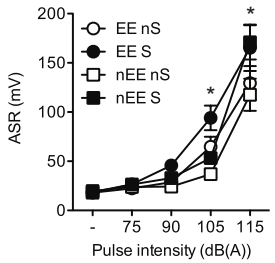


Figure 6.24.: Startle response of mice measured for HPC volume (cf. fig. 6.23C, n = 16 each, except nEE-S n = 15) after either sensitisation (S, black symbols) or no shock (nS, white symbols) and kept under either enriched (EE, circles) or standard conditions (nEE, squares). *: significant effect of sensitisation S vs. nS ($p < 0.05$).

volume, a significant interaction of shock and enrichment was observed ($F(1,32) = 7.30$ $p < 0.05$). Calculating posthoc comparisons showed that shocked, non enriched animals had significant smaller HPC volume than all other three groups of animals ($p < 0.05$, respectively), indicating that shock experience led to HPC shrinkage, which in turn was prevented by enriched housing. However, enriched housing alone did not lead to increased HPC volume (fig. 6.23C).

In summary, enrichment prevented HPC shrinkage after traumatic shock experience, but could not prevent increased hyper-arousal.

7. Pharmacological and optogenetical manipulation of prepulse inhibition

7.1. Prefrontal DR1 and DR2 mediate modulation of prepulse inhibition

7.1.1. Systemic blockage of DR1, but not DR2, increases PPI

To validate the present protocol of prepulse inhibition (PPI) and prepulse facilitation (PPF) measurement in the B6J and BALB/c mouse strains, both strains were treated with low (0.3 mg/kg) and high (1.0 mg/kg) dose of the typical antipsychotic drug haloperidol. In both strains, PPI was strongly increased (i.e. more negative percental ASR change, %ASR) by haloperidol (fig. 7.1).

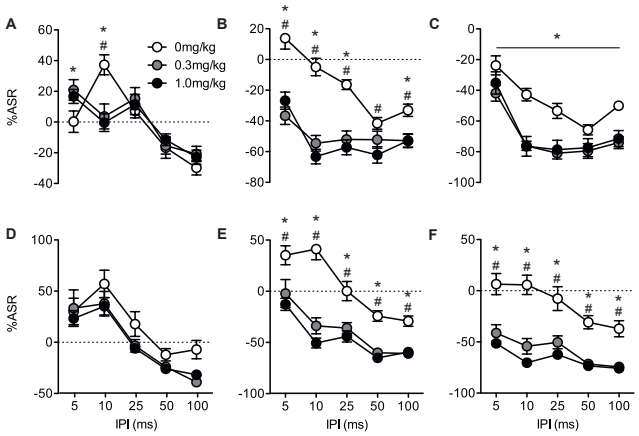


Figure 7.1.: Effects of s.c. haloperidol treatment on percental change of startle (%ASR, mean ± SEM) in BALB/c (A,B,C) and B6J mice (D,E,F) at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle (BALB/c: n = 12, B6J: n = 11); grey circles: 0.3 mg/kg (n = 12 each); black circles: 1.0 mg/kg (n = 12 each). * and #: %ASR changing effect of 0.3 and 1.0 mg/kg vs. veh, respectively ($p < 0.05$).

In detail, haloperidol treatment significantly affected %ASR at 55 dB ($F(8,132) = 6.02$

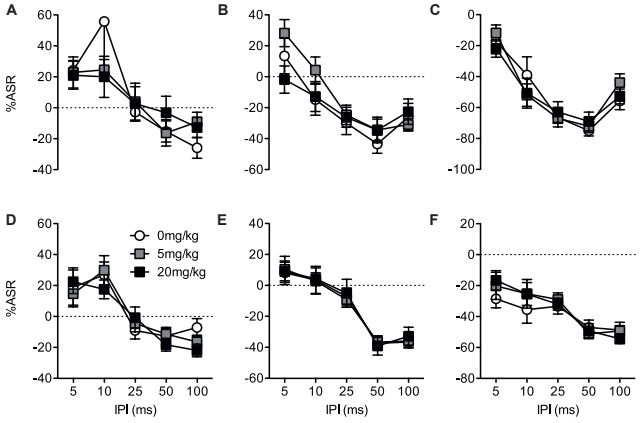


Figure 7.2.: Effects of s.c. sulpiride treatment on percental change of startle (%ASR, mean ± SEM) in BALB/c (A,B,C) and B6J mice (D,E,F) at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle; grey sqaures: 5 mg/kg; black squares: 20 mg/kg (each treatment BALB/c: n = 9, B6J: n = 12).

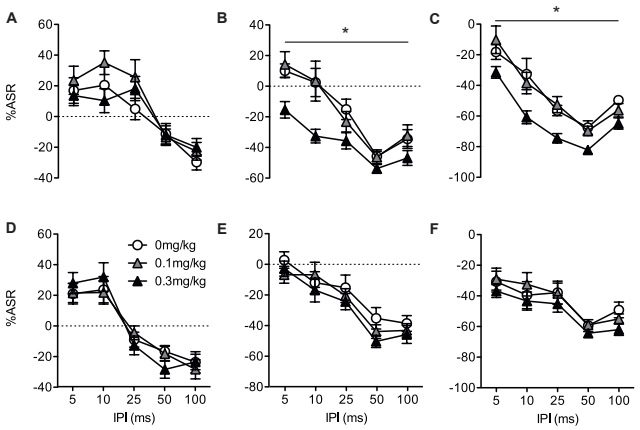


Figure 7.3.: Effects of s.c. SCH23390 treatment on percental change of startle (%ASR, mean ± SEM) in BALB/c (A,B,C) and B6J mice (D,E,F) at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle; grey triangles: 0.1 mg/kg; black triangles: 0.3 mg/kg (each treatment and mouse strain n = 12). *: %ASR changing effect of 0.3 mg/kg vs. veh ($p < 0.05$).

$p < 0.05$), at 65 dB ($F(2,33) = 32.09$ $p < 0.05$) and at 75 dB PP-intensity ($F(2,33) = 11.63$ $p < 0.05$). In B6J mice, %ASR at 55 dB PP-intensity was not significantly affected, while significant changes occurred at 65 dB and 75 dB ($F(8,128) = 5.41$ $p < 0.05$ and $F(8,128) = 2.68$ $p < 0.05$, respectively).

Admittedly, 1.0 mg/kg haloperidol decreased startle response on startle alone trials (i.e. startle pulse without prepulse) in BALB/c mice, indicated by significant interaction of day of measurement (i.e. day of baseline measures without treatment and day of measures after acute treatment) and treatment ($F(2,33) = 3.78$ $p = 0.05$). However, 1.0 mg/kg haloperidol decreased overall activity of the animals, thus probably not startle per se, and a decrease of startle would rather lead to decreased percental PPI scores. Hence, this observation does not question the results described above.

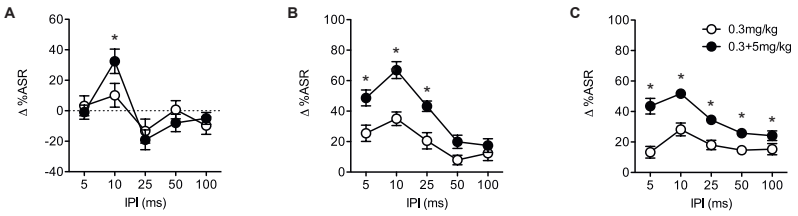


Figure 7.4.: Effects of s.c. SCH23390 (0.3 mg/kg) and SCH23390 + sulpiride (0.3 + 5 mg/kg) treatment on percental change of startle (%ASR) relative to vehicle treated BALB/c mice (Δ%ASR, mean ± SEM) at prepulse intensities of 55 (A), 65 (B) and 75 dB (C) across five different interpulse intervals. Data for SCH23390 treatment alone are the same as displayed in fig. 7.3A,B,C, respectively, but were normalised to vehicle treated animals thereof (cf. Materials and Methods). White circles: SCH23390; black circles: SCH23390 + sulpiride ($n = 12$, respectively). *: facilitating effect of additional 5 mg/kg sulpiride vs. SCH23390 alone ($p < 0.05$). Note that in contrast to other figures, graphs do not show percental startle change (i.e. %ASR), but the calculated difference of %ASR between vehicle treated and SCH23390 or SCH23390 + sulpiride treated animals (i.e. Δ%ASR).

To test for effects of dopamine (DA) receptor (DR) blockage in unchallenged (i.e. no pretreatment with direct or indirect DA-agonist) mice with comparatively low (B6J) or high (BALB/c) cerebral DA levels (cf. George et al., 1995), mice were treated s.c. with the specific DR1-antagonist SCH23390 and the specific DR2-antagonist sulpiride.

Surprisingly, PPI was unaltered after acute injection of the specific DR2-antagonist sulpiride. Neither 5, nor 20 mg/kg sulpiride led to a significant increase of PPI in BALB/c or in B6J mice (fig. 7.2). Contrary, systemic treatment with the specific DR1-antagonist SCH23390 potently increased PPI in BALB/c (fig. 7.3A,B,C). While there was no effect with 0.1 mg/kg SCH23390 (posthoc $p > 0.05$), 0.3 mg/kg SCH23390 significantly increased PPI at 65 dB ($F(2,33) = 6.41$ $p < 0.05$) and 75 dB PP-intensity ($F(2,33) = 6.90$ $p < 0.05$). In contrast, PPI changes in B6J mice after SCH23390 treatment were not significant (all $p > 0.05$, fig. 7.3D,E,F).

While haloperidol strongly affected PPI, sulpiride did not. To address the relat-

ive contribution of DR2-antagonism by sulpiride on PPI, BALB/c mice were further treated with a combination of 5 mg/kg sulpiride and 0.3 mg/kg SCH23390. The combined treatment of sulpiride and SCH23390 increased PPI as did SCH23390 alone (i.e. $\Delta\%\text{ASR} > 0$), but this increase was facilitated by adding sulpiride (fig. 7.4) as indicated by a significantly higher normalised %ASR at 55 dB ($F(4,88) = 2.78$ $p < 0.05$), 65 dB ($F(4,88) = 4.69$ $p < 0.05$) and 75 dB PP-intensity ($F(4,88) = 5.76$ $p < 0.05$). However, combined treatment of SCH23390 and sulpiride increased startle response (i.e. reaction to startle pulse without prepulse), indicated by significant interaction of day of measurement (i.e. day of baseline measures vs. day of measures after treatment) and treatment ($F(1,22) = 9.30$ $p < 0.05$). This is in favour of increased percental PPI scores; although startle response amplitudes of prepulse condition trials were lower in mice of combined SCH23390/sulpiride treatment compared to mice treated with SCH23390 only, this difference was in fact not significant. Even though animals were beforehand not matched for startle amplitudes but prepulse inhibition scores, this finding could interfere with the data reported, weakening the above described effect of facilitated PPI by combination of SCH23390 and sulpiride DR blockage.

7.1.2. Prefrontal blockage of DR increases PPI

The PFC has been shown to play a key role in regulation of PPI and being susceptible to dopaminergic treatment in terms of PPI. To validate the theory of PFC effects on PPI in the present study, prefrontal synaptic transmission was inhibited by increasing inhibitory inputs via infusion of the GABA(A)-agonist muscimol, or by blockage of AMPA-receptors with the specific antagonist NBQX in BALB/c mice. Four mice had to be excluded from analysis of muscimol data due to cannula misplacement.

Local prefrontal infusions of muscimol as well as NBQX led to a pronounced increase of PPI (fig. 7.5). While %ASR at 55 dB PP-intensity were not significantly changed by muscimol or NBQX, significant changes where observed at 65 dB ($F(4,64) = 3.87$ $p < 0.05$ and $F(1,19) = 8.52$ $p < 0.05$, muscimol and NBQX infusions, respectively) and 75 dB PP-intensity ($F(1,16) = 15.98$ $p < 0.05$, muscimol treatment only). However, muscimol infusion increased startle response, as shown by significant interaction of day of measurement (i.e. day of baseline measures vs. day of measures after treatment) and treatment ($F(1,16) = 11.69$ $p < 0.05$). As mentioned above (p. 90) this is in favour of increased percental PPI scores, even though animals were not matched beforehand for startle amplitudes but prepulse inhibition scores. Additionally, also startle response of prepulse condition trials were significantly higher in muscimol compared to vehicle treated mice

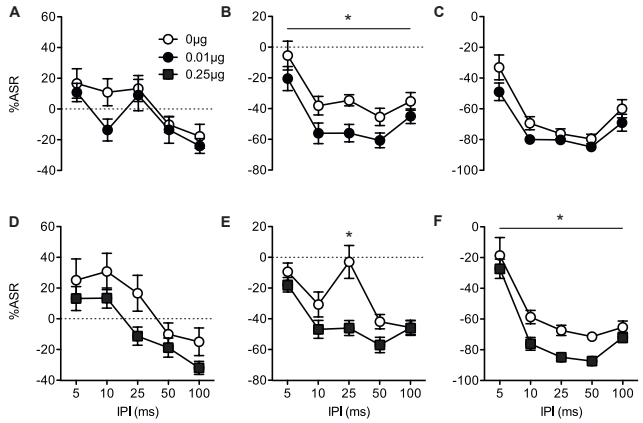


Figure 7.5.: Effects of prefrontal NBQX (A,B,C) or muscimol (D,E,F) infusion on percental change of startle (%ASR, mean ± SEM) in BALB/c mice at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle (NBQX: n = 11, muscimol: n = 9); black circles: 0.01 µg NBQX (n = 10); black squares 0.25 µg muscimol (n = 9). *: %ASR changing effect of NBQX or muscimol vs. veh, respectively ($p < 0.05$).

($F(4,16) = 4.51$ $p < 0.05$). These observations suggest that PPI increase by muscimol might be partly due to its startle enhancing effect.

Since the PFC was found to be involved in PPI of startle in the present experiment, the effects of locally infused sulpiride and SCH23390 were investigated next. Three mice of the BALB/c and B6J stain, respectively, had to be excluded from SCH23390 data and two BALB/c mice from sulpiride data. Exclusion was carried out based on histological brain slices indicating cannula misplacement. B6J did not respond to systemic treatment with sulpirid or with SCH23390. According to the hypothesis of mimicking findings by systemic treatment with prefrontal treatment, it was proposed that B6J would not respond to PFC injections of the used drugs, either.

Contrary, sulpiride as well as SCH23390 infusion significantly increased prepulse inhibition of startle in both, BALB/c and B6J (fig. 7.6 and fig. 7.7). Although significant only at 75 dB PP-intensity ($F(2,28) = 8.28$ $p < 0.05$ and $F(8,120) = 2.28$ $p < 0.05$, respectively), insignificant increase after sulpiride treatment was also observed at 65 dB and slightly 55 dB and short IPI (fig. 7.6), indicating the sensitivity of PPF (i.e. short IPI) to PPI changes. PFC infusion of SCH23390 at a dosage of 0.1 µg led to a small but not significant increase of PPI, again observed at mostly short IPIs (fig. 7.7). 0.5 µg caused a significant increase of PPI at 65 dB ($F(2,26) = 5.83$ $p < 0.05$ and $F(8,116) = 2.35$ $p < 0.05$) and at 75 dB PP-intensity ($F(2,26) = 6.30$ $p < 0.05$ and $F(2,29) = 3.47$ $p < 0.05$)

for BALB/c and B6J, respectively.

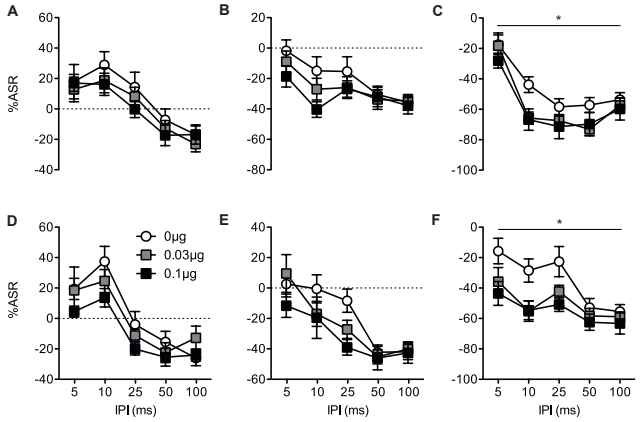


Figure 7.6.: Effects of prefrontal sulpiride infusion on startle percental change (%ASR, mean ± SEM) in BALB/c (A,B,C) and B6J mice (D,E,F) at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle (BALB/c: n = 11); grey sqaures: 0.03 µg (BALB/c: n = 10); black squares: 0.1 µg (BALB/c: n = 11, B6J: each treatment n = 11). *: %ASR changing effect of 0.1 µg vs. veh ($p < 0.05$).

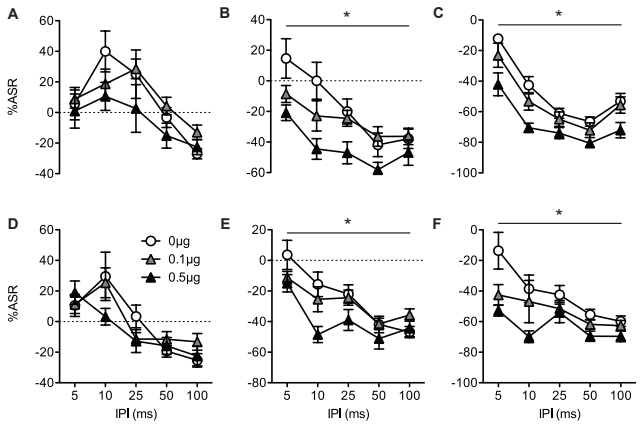


Figure 7.7.: Effects of prefrontal SCH23390 infusion on percental change of startle (%ASR, mean ± SEM) in BALB/c (A,B,C) and B6J mice (D,E,F) at prepulse intensities of 55 (A,D), 65 (B,E) and 75 dB (C,F) across five different interpulse intervals. White circles: vehicle (BALB/c: n = 10, B6J: n = 12); grey triangles: 0.1 µg (BALB/c: n = 10, B6J: n = 12); black triangles: 0.5 µg (BALB/c: n = 9, B6J: n = 8). *: %ASR changing effect of 0.5 µg vs. veh ($p < 0.05$).

7.2. Mimicking pharmacological interference by optogenetic stimulation

7.2.1. PPI and PPF are impaired by 5 and 50 Hz stimulation of the prefrontal cortex

To assess the impact of light driven stimulation of prefrontal cortex (PFC) in ChR2-transgenic mice on PPI and PPF, light flashes of 5 Hz and 50 Hz were applied. Due to apparatus malfunction, one animal had to be excluded from analysis of 5 Hz stimulation.

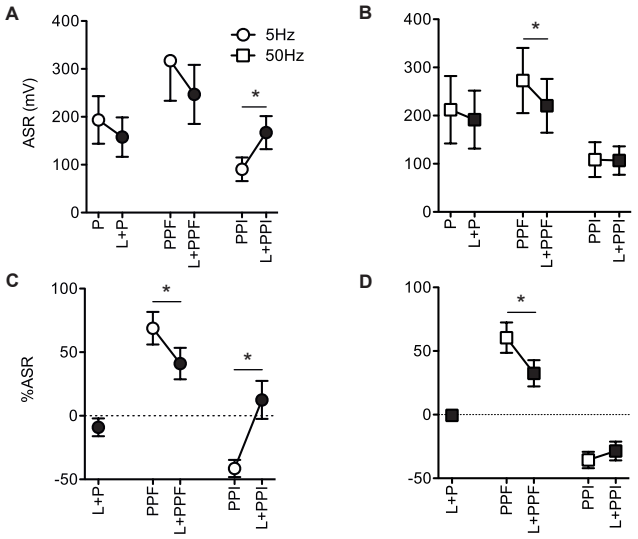


Figure 7.8.: Effects of tonic (5 Hz, n = 13) and phasic (50 Hz, n = 14) light stimulation (L+) of ChR-2 positive prefrontal layer V pyramidal neurons on startle amplitudes (A,B), and PPI and PPF of startle (C,D) in mice (mean ± SEM, respectively). Circles: 5 Hz; squares: 50 Hz; white symbols: no stimulation; black symbols: light stimulation (L+). *: ASR, PPI or PPF changing effect of stimulation (L+) vs. no stimulation ($p < 0.05$).

While light stimulation had no significant effects on ASR amplitudes and on %ASR ($p > 0.05$, respectively), significant effects of 5 Hz stimulation on PPI were observed, regardless whether statistics were calculated on amplitudes ($t(12) = 4.99$ $p < 0.05$) or %ASR ($t(12) = 3.89$ $p < 0.05$). 50 Hz stimulation did not reveal any significant PPI changes ($p > 0.05$), although normalised (i.e. %ASR) values nearly differed significantly ($p = 0.067$). Contrary, significant PPF changes occurred during 50 Hz (amplitude: $t(13) = 3.37$ $p < 0.05$; %ASR: $t(13) = 4.73$ $p < 0.05$) as well as 5 Hz stimulation (%ASR: $t(13) = 2.54$ $p < 0.05$), although significance was failed when calculation did not control

for individual startle amplitudes (i.e. normalising) after 5 Hz stimulation (amplitudes: $p = 0.061$). Interestingly, significant changes due to stimulation decreased PPI, as it decreased PPF, viz. stimulation always led to ASR changes more close to zero percent. Pharmacological manipulation of dopamine receptors in the PFC on the other hand always led to parallel shifts of PPI/F, viz. an increase of PPI and a decrease of PPF (cf. section 7.1.2).

Part IV.

Discussions

8. The startle response in paradigms of anxiety and fear

8.1. Fear potentiated startle in C57BL/6N mice

The present experiments suggest that fear potentiated startle (FPS) is masked by strong unconditioned pre-stimulus effects in C57BL/6N mice. Fear potentiated startle was measured after conditioning mice to light stimuli or sine wave tone stimuli of various duration with electric footshocks of various intensities. None of the parameters applied led to significant higher startle potentiation as had been observed with unconditioned pre-stimuli.

FPS has been successfully measured in BALB/cJ, C3H/HeSnJ, C57BL/6J, CBA/J and DBA/2J mouse strains employing light or sine wave tone stimuli (Falls, 2002). However, visual inputs reach conditioning associated brain areas such as the amygdala only through indirect pathways (Shi and Davis, 2001), while auditory input is channelled to the amygdala directly (e.g. LeDoux, 2000). In fact mice are found to be less efficiently conditioned to light stimuli, confirmed by the present results and also indicated by almost solely application of acoustic stimuli in mouse fear conditioning (cf. fig. 2.1). Interestingly, in mice the performance of visual fear conditioning can be accelerated to auditory levels when visual input is rewired during neonatal development onto structures processing the auditory input (Newton et al., 2004).

Parameters used in the present study are in line with procedures published (for review see Falls, 2002), although the number of tone and shock pairings in the present work (six) was lower than the number suggested by Falls (20 - 30). Thus, it remains to be shown that FPS after a higher number of pairings is also masked by unconditioned tone effects.

However, FPS levels of about 60 - 120 % (cf. fig. 6.2, fig. 6.3 and fig. 6.4) were comparable to the levels reported by Falls (ca. 130 %) and others (e.g. cf. Busse et al., 2004 (ca.

130 %), Heldt et al., 2007 (ca. 60 %), Fadok et al., 2009 (ca. 60 %)) after six tone and shock pairings, and the amount of freezing also indicated sufficient conditioning. Additionally, the unconditioned effects were much higher (ca. 60 - 110 %, cf. fig. 6.2, fig. 6.3 and fig. 6.4) than those reported by Heldt et al. (2000, ca. 15 %) or Falls (2002, ca. 50 %), and even a single footshock to mice of the line subjected in the present work was sufficient to reveal comparable high levels of potentiation (ca. 150 %), even though again not significantly higher than unconditioned potentiation (cf. fig. 6.16).

Closer to protocols found in the literature, conditioning and testing conducted in the same context revealed strong FPS compared to potentiation of unconditioned mice. However, the present data suggest that context conditioning contributed strongly to conditioned potentiation; hence, potentiation to conditioned tone would have been not distinguishable from uncondtioned potententiation equally to the other experiments, if potentiation would have been not facilitated by conditioned context.

Over all the present data show strong unconditioned effects of acoustic stimuli in C57BL/6N mice, potently masking FPS in these animals. Thus, even if FPS would have been observed to a significant level, it is questionable whether these mice can be successfully applied in experiments, where high FPS levels above baseline potentiation (i.e. unconditioned effects) are needed to draw conclusions based on animal behavioural performance.

In contrast, the phenomenon of strong unconditioned tone effects will be studied in the following section to evaluate potential applications in behavioural experiments.

8.2. Tone enhanced startle as a measure of hearing capability, stimulus adaptation and attention

Pre-stimulus facilitation of the startle response has been reported for rats and humans as well as for mice (Falls et al., 1997; Hsieh et al., 2006; Reijmers and Peeters, 1994). In rats as in mice the effect is described that the acoustic startle response (ASR) is increased by steady background noise (Carlson and Willott, 2001; Hoffman and Fleshler, 1963), which is effective even after hours of continuous stimulus presentation (Hoffman and Wible, 1969). In humans, stimuli (pulses) of up to 4 s duration are used to observe an increase in startle. This prepulse facilitation (but cf. p. 27!) is mostly applied to measures of attention (Filion et al., 1993).

In rats, the phenomenon of startle response changes by background sound has been thoroughly studied by Hoffman, Ison and associates (Hoffman and Fleshler, 1963; Hoff-

man and Wible, 1969; Hoffman and Ison, 1980; Ison et al., 1973; Ison and Russo, 1990). While Carlson and Willott (2001) provided a more detailed characterisation of background sound effects in mice, examining interactions of startle eliciting pulse and background stimulus frequency, the present work confirms the work on rats by Hoffman and Wible (1969) in mice, suggesting that pre-stimulus effects are equivalent with background sound effects. The present experiments extend findings by Carlson and Willott (2001) and additionally provide possible applications based on the paradigm of tone enhanced startle (TES). Based on observations that enhancement of startle increases with increasing stimulus intensity (Carlson and Willott, 2001), is reduced during distraction (Filion et al., 1993, 1994), and is observed only during early trials of a test session (Filion et al., 1993, 1994; Graham, 1975), TES is proposed as a paradigm to assess hearing capability, attention, and stimulus adaptation in mice.

Section 6.1 has already demonstrated that the amount of unconditioned effects of pre-stimulus presentation (i.e. TES) can be optimised by choosing suitable parameters of pre-tone and startle eliciting pulse (cf. pp. 62). As a consequence, the paradigm of TES may be applicable not only to the mouse strain tested in this study (C57BL/6NCrl), but also to other strains (cf. p. 79), whereby the optimal parameters may vary between mouse strains. While 9 kHz potently enhanced startle in the present study, Carlson and Willott (2001) found startle inhibited when elicited with noise pulses in a 4 or 12 kHz background in the C57BL/6J strain. Although Carlson and Willott (2001) provided background stimuli as constant background throughout the testing session, the present work suggests stimulus duration $\geqslant 4$ s to be irrelevant for startle potentiation in mice, since there was no difference in tone enhanced startle with 4 s and 20 s in section 6.1.3, and also no differences were found for TES with 20 s or 120 s sine wave tone in section 6.2.2. These data are in line with findings in rats (Hoffman and Wible, 1969). Additionally, while Hoffman and Wible (1969) reported that facilitation persists following termination of the pre-stimulus up to 8 ms before startle pulse presentation, this interval is similarly comparable in mice (< 3 ms, fig. 6.6). Enhancement and inhibition were reported to depend on the startle eliciting pulse intensity (Ison, 2001). Although it only tested for two different intensities (i.e. 105 and 115 dB(A)), the present work cannot confirm this finding, demonstrating that startle with 105 and 115 dB pulses is almost equally enhanced (cf. fig. 6.3 and section 6.1.3).

Measuring hearing capability of an animal usually requires the recording of acoustic brainstem responses (ABR) or othoacoustic emission (OAE), requiring anaesthesia of

the animal. Non invasive techniques are the startle response itself, as well as prepulse inhibition of startle. However, startle is elicited only by intense acoustic pulses far above the auditory threshold, although Willott et al. (1984) demonstrated that the acoustic startle response (ASR) is capable of detecting differences in the neuronal response of the auditory system. Prepulse inhibition on the other hand is capable of testing also very low intensities, but is strongly affected by internal and external states and changes (cf. e.g. Ison et al., 1997). TES offers a third method to assess hearing in a startle based paradigm. Like PPI it is capable at a wide range of intensities, but offers higher robustness, evidenced by only weak susceptibility to even strong sensitisation and its immunity to diazepam or paroxetine treatment. Appositely, Carlson and Willott (2001) found increasing enhancement/inhibition with increased background intensity from 60 to 80 dB, congruent to the present experiment. They also found differences in enhancement/inhibition using the same parameter set when testing mice of different ages, which have been shown to exhibit progressive hearing loss (Johnson et al., 1997). This additionally suggests the applicability of TES in hearing assessment.

The robustness of TES is limited when it comes to attention. In the present experiment, TES was markedly decreased by an additionally introduced light stimulus. This suggests TES to be a tool to measure attentional shifts in animals. TES might be an attentional measure per se, but to control for already existing internal factors that might decrease TES, it is appropriate to measure distraction by a second stimulus on the basis of TES. Although the light stimulus in the present experiment itself inhibited startle, the decrease of TES to almost zero (i.e. unaltered startle), easily exceeding startle inhibition resulting from light presentation, strongly suggests that mainly attentional shift was responsible for the decrease in TES. The possibility of TES as a measure of attention could also explain the finding of increased enhancement by a high dosage of 2 mg/kg diazepam. Diazepam clearly had sedative effects at this dosage and by this could have weakened the animals attention to cues associated with locomotion, thereby shifting attention more to olfactory and acoustic stimuli. This in turn could have resulted in facilitated TES.

While measurement of habituation like processes to long stimuli usually requires fear conditioning to have an appropriate behavioural readout of adaptation (i.e. freezing), TES measures adaptation in naive animals. Adaptation to the pre-stimulus, and thus decrease in startle enhancement, was not masked by habituation to the startle eliciting pulse per se. This was indicated by significant effect of pulse presentation in the pre-stimulus condition, but not in the pulse alone trials of %ASR. Interestingly, no - or limited - adaptation was found to pre-stimulus white noise; the inhibitory effect of white

noise was rather found to increase with ongoing presentation. This may be attributed not to enhancing, but to inhibitory effects of white noise on startle in the subjected mouse strain. However, stimulus quality (i.e. noise or sine wave) severely affects animal behaviour (cf. section 6.3), and white noise seems to be less prone to extinction than sine wave tones (cf. p. 75), suggesting that the lack of stimulus adaptation to white noise pre-stimuli in TES did not result from its inhibitory effect, but was rather due to stimulus quality.

TES is a phenomenon of ongoing stimulus presentation and not stimulus on- or offset relative to startle eliciting pulse onset. This is indicated not only by the present work, but also by the finding that hours of stimulus presentation do not impair the startle enhancing effect (Hoffman and Wible, 1969). According to the present data, TES is rather not a sign of anxiety or fear. Although it was slightly increased by prior strong footshock sensitisation, the anxiolytic drug diazepam had no attenuating effect on TES (i.e. sine wave tone); however, it remains to be shown that no diazepam effect can be observed in sensitised animals (i.e. attenuation of increased startle response in sensitised animals). Also Ison et al. (1997) did not find any effect of diazepam on noise facilitated startle (but cf. Kellogg et al., 1991), and Schanbacher et al. (1996) reported independence of background noise enhancement from amygdala, a brain structure closely related to fear and anxiety behaviour. Stimulus perception is different for noise and sine wave. While adaptation (and thus habituation-like decrease of startle enhancement) was found to sine wave stimuli, white noise was less prone to adaptation. This is in line with the afore mentioned study of Hoffman and Wible (1969) as well as with section 6.3 and work by Mauch et al. (in prep), demonstrating that white noise seems to have a different (higher?) perceptional value than sine wave tones.

Prolonged as well as acute noise has been shown to increase cortisol levels (or corticosterone, respectively) in humans and in rodents (cf. Henkin and Knigge, 1963; Jensen et al., 2010; Spreng, 2004). This is also found for tones of different frequencies (e.g. Borrell et al., 1980). Additionally, recent data indicate that hearing sensitivity is tuned and by this protected against noise-induced hearing loss via corticotropine-releasing hormon (CRH) release in the cochlea (Graham et al., 2010), showing that CRH release can follow immediately after intense acoustic stimulus onset; could TES therefore be a function of CRH release? However, startle is not only enhanced but also found inhibited during presentation of background stimuli of different frequencies. Startle alteration by background was also discussed in terms of anxiety, although overall findings argue against

this hypothesis (cf. above). Also the hypothesis of masking effects (Davis, 1974; Hoffman and Searle, 1965; Ison and Hammond, 1971) is not tenable, considering experiments by Carlson and Willott (2001). For instance startle of 100 dB broadband noise pulses was inhibited by 80 dB 12 kHz background, while it is unlikely that broadband noise of this intensity is masked by such a intense pure tone. Additionally, while a 4 kHz background would rather be suggested to mask startle of 4 kHz pulses, facilitation of startle occurred in this case. A modified masking hypotheses of high frequency cochlear distortions associated with the intense startle stimulus put forward by Gerrard and Ison (1990) is also not supported by the work of Carlson and Willott (2001). According to the hypothesis, high frequency stimuli would be more effectively masked by high frequency background. While this hypothesis could explain the inhibition of startle by 12 kHz background and broadband noise background, the latter did effectively suppress startle of 4 kHz, but not 12 kHz pulses.

Carlson and Willott (2001) put forward a summation hypothesis, suggesting different background sound effects converging at the level of the caudal pontine reticular nucleus (PnC, cf. p. 6). The PnC receives input from divers brain structures, each in itself leading to inhibition or enhancement of the startle response and sensitive to auditory stimuli. Thus, the complex results of startle alteration by pre-stimulus (background stimulus) presentation might be a summation of effects of these stimuli exciting different brain areas that are involved in startle modification.

Taken together, tone enhanced startle (TES) is a phenomenon of long duration (background) acoustic stimuli, preceding startle eliciting pulses. Enhancement and inhibition of stimulation depends strongly on the spectral preferences of the stimulus, while stimulus duration (above a threshold) has no relevance for startle change magnitude. TES shows within session habituation to sine wave tone (but not white noise) stimuli, which makes it a paradigm to measure stimulus adaptation in mice. Additionally TES increases with stimulus intensity, suggesting it to be a tool to assess hearing capability. While TES has been shown to be unaffected by different pharmacological compounds, it is susceptible to distracting stimuli, and maybe a function of attention itself.

8.3. Fear conditioning parameters - the matter of fact

The present work clearly demonstrates the strong impact of stimulus quality (i.e. sine wave or white noise) on animal behaviour in measures of acoustic startle response (ASR),

fear conditioning (FC) and extinction of conditioned fear (ExFC). Stimulus duration, on the other hand, affected neither FC, nor ExFC.

To date, stimuli of different quality have been applied uncritically and parameters and protocols vary considerable between laboratories (fig. 2.1). Showing that the behavioural differences resulting from different stimulus quality may affect the conclusions drawn from the respective experiments, the present findings suggest reinterpretation may be necessary in cases where experiments yielded contradicting results after applying differing stimulus quality.

As in the present experiments, data revealed less freezing behaviour of white noise (wn) conditioned animals during CS memory test in a neutral context following extinction training, indicating that they had deficits in acquisition of extinction memory compared to sine wave (sw) conditioned mice. Contrary, low freezing levels of wn conditioned animals during CS memory test in the conditioned context suggests better extinction memory performance compared to high freezing sw conditioned mice. CS wn onset was accompanied by accelerated movements of the respective animals, sometimes even running and jumping, on day 1 post conditioning. Extinction memory retrieval in the neutral context on day 9 revealed as high freezing levels as shown on day 1, while running and jumping again led to decreased freezing levels in the conditioned context. These observations allow another interpretation of the current data, viz. wn stimuli were sensed more averse than sw, thereby leading to panic-like reactions during FC memory retrieval. This behaviour was facilitated by the presentation of the conditioned context, leading to even lower freezing scores. This example demonstrates how behaviour differently affected by different stimulus quality could interfere with data interpretation, thus demanding careful video analysis, especially when behaviour is scored by means of automated computer algorithms.

While misinterpretations such as the one described above are easily prevented by careful behavioural analysis, other effects of stimulus quality may be more problematic. The present data revealed significant differences in the course of between- as well as within-session extinction of the mice conditioned to different stimulus qualities, suggesting a kind of extinction disability of wn conditioned animals. Hence, studies reporting extinction deficits of some animals while applying white noise stimuli might have rather verified extinction disability to these stimuli than proving extinction deficits in the subjected animals.

The panic-like behaviour triggered by white noise stimuli and the deficits in between-

and within-session extinction and stimulus adaptation (cf. section 6.2.2) in white noise conditioned animals may suggest a higher emotional relevance for wn stimuli. The acoustic environment of mice usually consist of multi-frequent sounds and broadband noises. Therefore it may be that animal behaviour has evolutionary adapted to these kind of stimuli, and that these are rated differently in their ecological importance. Indeed, acoustic noise pulses are found to be more effective in sensorimotor gating (i.e. PPI, cf. section 3; Stoddart et al. (2008); Wynn et al. (2000)), and background noise enhances ASR in rats (Hoffman and Fleshler, 1963). Additionally, differences between white noise and sine wave stimuli are reported for prepulse facilitation (i.e. TES, cf. p. 27, section 6.2 and Hsieh et al. (2006)). However, the prepulse effects reported could result from subjective loudness perception by the subjected animal. Although white noise and sine wave were set to identical intensity, loudness of white noise could have been perceived as higher. This results from summation of the loudness of each excited critical band of the basilar membrane. While pure sine wave tones activate basilar membrane only in one critical band, white noise (20 Hz - 20 kHz) activates all critical bands responding to the respective bandwidth. Interestingly, despite this phenomenon, 20 ms sine wave pulses above 90 dB(A) led to higher ASR than white noise pulses of the same intensity (fig. 6.14). Additionally, white noise and sine wave tones did not simply differ in the magnitude of startle enhancement, but differed categorical in their impact on startle response, as was demonstrated in section 6.3.3 as well as in section 6.2.2. Moreover, freezing levels to unconditioned stimuli of different quality were comparable, and hence freezing behaviour was not affected by different subjective loudness perception. Together these findings render it rather unlikely that differences in perceived loudness to white noise and sine wave tone had any influence on animal behaviour in the present data.

Admittedly, stimuli can gain ecological relevance and rated as important outside evolutionary processes (e.g. reaction time to the sound of skidding tyres, cf. Graham, 1999) and contrary, ecologically important stimuli may not be recognised as relevant innately (cf. Kindermann et al., 2009). It has been shown on the other hand, that when presented with a biological acoustic distractor (such as a frog croak), the processing of any (biological or non biological) visual or auditory cue is disturbed (Suied and Viaud-Delmon, 2009). Additionally, human reaction times to naturally occurring sounds such as roaring of a cat of prey are significantly smaller than to artificial sine wave tones. Applying the temporal envelope of roaring to white noise stimuli, reaction times to these stimuli matched reaction times to natural roaring (Suied et al., 2010), although it remains unclear whether either temporal aspects of the stimulus, or white noise itself led to decrease

of reaction time.

The present results demand a more careful handling of stimulus parameters when it comes to behavioural paradigms. This not only to warrant comparable procedures and handling of animals, but also because parameter-associated behaviour might interfere with data interpretation. The present data indicate that white noise stimuli might activate innate fear associated perception systems, which potentially could be found across mammals (vertebrates). Such innate recognition might strongly interfere with measures of fear and anxiety in animals and animal models of psychiatric disorders, but could also be useful in terms of for instance acoustic warning signals (cf. Graham, 1999).

8.4. Extinction of conditioned fear to context by cue extinction training

The present hypothesis predicted that conditioned stimulus (CS) presentation during extinction training would result also in context extinction - presumably by means of pattern completion in the hippocampus (HPC). Thus, animals that underwent extinction training were expected to show lower freezing levels to the conditioned context than animals which did not. The present data do not support this hypothesis. Extinction trained animals and non-trained animals expressed comparable freezing to conditioned context. This suggests that context extinction either did not occur, or, paradoxically, context extinction did not lead to reduced freezing behaviour to the extinguished context.

In fact there are studies reporting - vice versa - CS extinction through presentation of an associated cue (i.e. present during conditioning, Durlach and Rescorla (1980); Holland and Forbes (1982); Kawai and Kitaguchi (1999); Nakajima and Kawai (1997); Rescorla (1983)). In particular, two studies reported reduced fear to CS after extinction of the context where conditioning to that CS was performed (Marlin, 1982; Stout and Miller, 2004). Already Hall (1996) and Holland (1983) as well as McLaren and Mackintosh (2000) offered theoretical framework to a model predicting this finding. They proposed that presentation of cues associated to the conditioned stimulus, such as the context, could lead to *"retrospective revaluation"* (Stout and Miller, 2004) and, thus, extinction of the actual CS. Perhaps somewhat closer to the mechanism of extinction via pattern completion proposed in the present experiment is the within-compound view by Durlach and Rescorla (1980). According to these authors, associations exist between all components (context, cues, US, i.e. compound) during conditioning, also to the CS. In consequence,

weakening the association between one of these components and the US would lead to weakened associations of all other components and the US.

Assuming that the association of context to US was indeed weakened by CS extinction training in the present experiment, why would this weakened association not lead to decreased freezing behaviour to the context? Bouton and Ricker (1994) demonstrated that extinction considerably depends on the context where extinction training took place. This is indicated also in the present experiment. Extinction trained animals showed high initial freezing levels to the actual extinguished CS in a new context. These levels were comparable to freezing levels of animals which did not undergo extinction training (fig. 6.18D). Freezing to CS in the extinguished mice only decreased after additional presentations of the CS. This decrease is attributed to context dependency of extinction and not to incomplete extinction, proven by movement scores successfully obtained from the startle apparatus which indicate successful training. Thus, context dependency of extinction might have led to initial unaltered freezing to the extinguished (previously conditioned) context. It remains to be shown that repeated context exposure after CS extinction training leads eventually to alleviated freezing to this context in trained, but not (or slower) in non extinction trained animals.

8.5. ASR measures in mouse-models of trait anxiety and PTSD

8.5.1. ASR in mice of high and low anxiety related behaviour

According to the model of HAB/LAB mice, animals of high anxiety related behaviour (HAB) indeed showed highest levels of fear response (i.e. freezing) to a fear conditioned stimulus compared to animals of low (LAB) and normal (NAB) anxiety related behaviour. These differences cannot be attributed to differences in hearing capability or electric footshock susceptibility, demonstrated by measures of tone enhanced startle, baseline startle and movement scores.

Work by Willott et al. (1984) demonstrated that the acoustic startle response (ASR) is capable of detecting differences in the neuronal response of the auditory system and thereby may be applied in measures of hearing capability. The present data are for sure no final proof of equal or unequal hearing capabilities of the mouse lines, but strong differences in startle response with LAB$\gg$HAB at least render it very unlikely that freezing differences were simply due to differences in hearing capability.

Baseline movement scores indicate strong locomotory drive in LAB mice, which might partly account for exceedingly high startle amplitudes as well as for almost not existing freezing during conditioned memory retrieval. That startle amplitudes are not representing hearing capabilities one-to-one is also indicated by tone enhanced startle (TES, cf. section 6.2). Here, LAB animals showed less enhancement of ASR than HAB mice, possibly resulting partly from ceiling effects of the strong startle response observed in LAB. NAB were not found to be susceptible to enhancing tone effects at all. Contrary, NAB animals displayed equally strong susceptibility to electric footshocks as HAB mice. LAB mice reacted with even higher responses, but high movement scores also during phases where no shock was presented relativise these high reactions.

Recently, LAB animals were proposed to be a model for the attention deficit/hyperactivity disorder (ADHD) (Yen et al., 2010). Although attentional deficits might account for low TES as well as poor conditioned memory retrieval, strong startle responses and even lower TES in NAB mice, which do not display exaggerated locomotion, make the explanation of low attention of LAB as implausible as hearing capability or footshock susceptibility alone.

The present experiment demonstrates the usefulness of startle as a non invasive measure to assess differences of perception in animal behaviour. However, inconsistencies in anxiety related behaviour such as high freezing levels but very low startle scores in HAB mice, or in tone perception related behaviour such as good performance in conditioned memory retrieval but absence of TES in NAB animals, hinder a coherent interpretation.

8.5.2. ASR as a measure of hyperarousal in a mouse model of PTSD

By demonstrating that i.c.v. CRH treatment as well as mice of a model of post-traumatic stress disorder (PTSD) display increased acoustic startle response, the present work suggests more profound studies to analyse the role of elevated CRH levels in the mouse model of PTSD are needed. Showing that the PTSD model allows the study of HPC volume loss found in PTSD patients, the present study broadens the spectrum of trauma consequences that can be examined in this model.

Startle scores were much higher in animals of the CRH experiment than in the PTSD experiement (cf. fig. 6.20 and fig. 6.21). Also animals subjected to PTSD protocol and measures of HPC volume (fig. 6.24) displayed lower amplitudes than animals of the CRH experiment, but comparable startle levels as mice of the PTSD experiment. This probably resulted from surgery of the CRH mice as well as injection procedure before startle

measurement, which may lead to a hyper-aroused state of the animals.

Sure enough, increased acoustic startle response (ASR) by artificially elevated intracerebral CRH levels in parallel with findings of elevated ASR in mice of the PTSD model does not necessarily imply altered CRH levels in these mice. However, the tool of local application of CRH or CRH receptor blocker in this mouse model may have implications for understanding the mechanisms leading to PTSD in humans. For instance Keen-Rhinehart et al. (2009) report that elevated CRH levels in the central amygdala lead to increased startle response and a dysregualtion of the hypothalamic–pituitary–adrenal (HPA) axis. Recent findings that neuronal activity in some brain areas is reliably increased by means of elevated cytochrome c activity in animals of the PTSD mouse model (Henes et al., 2009) provides putative target regions to interfere with possible molecular substrates of PTSD, such as CRH.

Blocking the CRH receptor by i.c.v. injection of the specific antagonist αCRH has been shown to affect light enhanced (LES), but not fear potentiated startle (FPS, de Jongh et al., 2003). While FPS is thought to be a measure of fear, LES rather measures anxiety of a subjected animal (cf. p. 13). According to light enhanced startle, background noise is found to enhance startle reactivity in rats and background noise has been reported to increase cortisol levels in humans (for review cf. Spreng, 2004). This raises the question whether TES in mice (cf. section 6.2), as a putative analogue to LES in rats, could be partly a function of increase in CRH (cf. p.101) and, thus, might be a potential measure of (PTSD related) CRH increase. In fact TES was found to be limitedly susceptible to sensitisation with different electric footshock intensities (cf. p. 66). However, the sharp offset of enhancement by tone presentation argues against a neuropeptide effect in TES. Moreover, section 6.2.4 indicates rather attention than arousal to be the cognitive substrate of TES.

Hippocampal (HPC) volume loss has been reported previously in PTSD patients (Bonne et al., 2008; Bremner et al., 2008; Wang et al., 2010). The present experiment shows that decrease of HPC volume is also apparent in a mouse model of PTSD; this can be readily measured by means of processing ultramicroscopic HPC images. As discussed recently by several authors (Bremner et al., 1997; Gurvits et al., 1996; Stein et al., 1997; Winter and Irle, 2004), the present data do not rule out whether HPC volume loss is a function of trauma, or merely a secondary effect of PTSD and its consequences in behaviour and social life, although future experiments based on the present

work may help to resolve this issue. On the other hand, manganese enhanced magnetic resonance imaging (MEMRI) has been described to reveal comparable results in terms of HPC volume changes (Golub et al., 2010). The heavy metal manganese was found to be an excellent contrast medium in MRI; protons that surround paramagnetic metal ions such as manganese display shortened relaxation times of the protonic spin through dipolar interactions of proton and electron spins of the paramagnetic ion (Bloembergen, 1957). In neuronal tissue, manganese ions enter neurons via activated calcium channels (Cross et al., 2007; Drapeau and Nachshen, 1984; Itoh et al., 2008). This manganese can then be measured as a decrease in T_1 spin relaxation time even hours later (Alvestad et al., 2007; Sun et al., 2006), since manganese efflux from cells is very low (Aoki et al., 2004). Although MEMRI might be more elaborative and cost intensive than the present method based on ultramicroscopy, studying an intact HPC and the entire brain, and the possibility of longitudinal studies in animals is clearly advantageous. It might therefore contribute to the hypothesis of predictability of individual vulnerability to PTSD by HPC volume, as put forward by Gilbertson et al. (2002).

However, the present method was also capable of detecting HPC volume increase in animals kept under enriched housing conditions, pointing out general trophic effects of enrichment also discussed by other authors (Van Praag et al., 2000; Goshen et al., 2009). Contrary, when animals were subjected to a behavioural test battery, ultramicroscopic imaging did not detect HPC volume increase in enriched housed mice, while increased HPC volume after such treatment was detected by MEMRI (Golub et al., 2010).

While Golub et al. (2010) also report beneficial effects of enriched environment (and increased HPC volume) to trauma associated contextual fear, the present data suggest amelioration of HPC shrinkage by enriched housing, but hyper-arousal (i.e. startle magnitude) remained unaffected. This demonstrates again the independence of PTSD-like symptoms, shown also by work of Golub et al. (2009) and Pamplona et al. (2010).

The causes of hippocampal shrinkage remain unclear. Golub et al. (2010) suggest a shrinkage of axonal protrusions, indicated by down regulation of the axonal marker GAP43. Measuring the amount of neuronal tissue by means of GFP labelled structures in Thy1-GFP-mice proved to be inadequate, considering the simplicity of the applied image processing technique. More elaborated methods will possibly be more successful in analysing dystrophy in neuronal tissue, also by means of ultramicroscopy studies. Ertürk et al. (2011) already give prospects of what is possible if imaging techniques such as ultramicroscopy and two-photon confocal imaging are combined with tissue clearing

methods, genetic mutants expressing fluorescent dyes and computational techniques.

It is yet unclear whether CRH hypersecretion found in PTSD patients is merely a predisposing factor or occurs only after trauma (Risbrough and Stein, 2006). The same holds true for hippocampal volume which is thought to be a consequence of trauma (or development of PTSD), while studies by Gilbertson et al. (2002) suggest low HPC volume as a risk factor for developing PTSD after trauma.

Although the present work does not causally prove CRH deregulation in the subjected PTSD mouse model, the present data provide the groundwork to study these aspects in future experiments. Additionally, the present experiments confirmed the independency of PTSD-like symptoms, demonstrating that hyper-arousal is not ameliorated by enriched environment, but enriched housing prevents hippocampal volume loss in PTSD animals. Despite the methodological power and flexibility of MEMRI, the present method of ultramicrosopic analysis of HPC provides a fast and cheap tool measuring HPC volume in rodents.

9. Pharmacological and optogenetical manipulation of prepulse inhibition

9.1. Prefrontal DR1 and DR2 mediate modulation of prepulse inhibition

Through the treatment of mice of relative low and high cerebral dopamine (DA) concentrations with specific DA receptor (DR) 1- and DR2-antagonists, the present work demonstrates that PPI enhancement is mediated to a large extent via prefrontal DR1. Contrary, DR2 blockage with sulpiride was less effective in enhancing PPI than blocking DR1 with SCH23390. While systemic sulpirid had no effect, the DR2-antagonist haloperidol potently facilitated PPI in both mouse strains.

The contribution of dopamine (DA) receptors (DR) to PPI mediation and modulation of PPI is undoubted (for review see Swerdlow et al., 2001). On the other hand, published data suggest that DR function in mice is different from rats (Geyer, 2006; Ralph-Williams et al., 2003; Ralph and Caine, 2005), with DR1 being more important than DR2 in mice. Additionally, contribution of either receptor and DR-antagonist effects were reported to depend on pretreatment and were mostly apparent by means of amelioration of disruptive effects of DA agonist administration (direct or indirect, cf. Geyer, 2006). Contrary, some authors reported PPI changes after unchallenged (i.e. no treatment with DA agonist) DR blockage, which led to either a decrease (Ellenbroek et al., 1996; Swerdlow et al., 2005) or increase (Schwarzkopf et al., 1993) of PPI.

The present data rather speak for an inhibitory function of DR1 in modulation of PPI in mice. In all experiments, DR1 blockage led to increased (disinhibited) PPI; only B6J mice did not response to systemic treatment with DR1-antagonist. Since B6J have been shown to have lower concentrations of cerebral DA than BALB/c, it is possible that DR1 exerts more reliably in a milieu of high DA levels, supported by the finding that DR1 has less affinity to DA than DR2 (cf. Creese et al., 1983). Contrary, Ralph et al.

(2001) reported disrupted PPI in DA transporter deficient mice (DAT-ko mice), which are suggested to have higher extracellular cerebral DA levels. Here, PPI was not improved by DR1 blockage with SCH23390, but with DR2-antagonist raclopride treatment. However, SCH23390 treatment had positive effects on other impaired behaviour found to be associated with DAT deficiency. DR1 blockage led to increased PPI in B6J, too, when antagonists were infused into the PFC, additionally questioning the importance of high DA levels for recruiting DR1 at least in this brain region. On the other hand, Mauch et al. (in prep) found increased release of prefrontal DA by means of microdialysis after acute forced swim stress in both BALB/c and B6J mice, while baseline measures confirmed higher prefrontal DA release in BALB/c mice. Since startle measurement itself may serve as an acute stress to the animal (cf. Davis and Sheard, 1974; Groves and Thompson, 1970; Plappert et al., 1999), elevated DA levels in the PFC might favour DR1 action also in B6J mice, while this effect could be masked by actions in other brain areas after systemic DR1 blockage.

The present data draw an ambivalent picture for DR2 function. On the one hand, DR2 blockage by haloperidol potently increased PPI in both B6J and BALB/c mice. This is in line with findings by Ouagazzal et al. (2001), who reported PPI increase after antipsychotic treatment in a variety of mouse strains. Confusingly, DR2-antagonist sulpiride had no effect on PPI in none of the examined strains. Also prefrontal treatment only had minor effects, found at short interpulse intervals and significant only at 75 dB(A) prepulse intensity. Here, inhibition of startle cannot be exclusively attributed to prepulse inhibition since prepulses themselves led to small startle responses, suggesting that also paired pulse inhibition of startle was present (i.e. reaction to first startle pulse inhibits reaction to second pulse by causing a partly refractory state of the startle mediating circuit, but cf. Dahmen and Corr, 2004). In line with the theory of a synergistic function of DR1 and DR2 (cf. Peng et al., 1990; Wan et al., 1996), co-treatment with SCH23390 and sulpiride facilitated the PPI enhancing effect of DR1 blockage with SCH23390 alone. According to the finding of PPI enhancing effects of DR1, but not DR2, the latter rather seems to have auxiliary function in the subjected mice. When K_i values, a measure for receptor affinity, are compared for haloperidol and sulpiride, sulpiride shows an about 3000 times higher affinity to DR2 than DR1, while affinity of haloperidol is only 67 times higher for DR2, thus maybe blocking also DR1 to a higher extent than sulpiride and, hence, mediating the synergistic functions of DR1 and DR2. The PPI enhancing effect of DR1 blockage was indeed facilitated by parallel treatment with DR2-antagonist. However, combined blockage of DR1 and DR2 by SCH23390 and sulpiride increased

startle response itself, which according to calculation of %PPI is in favour of inhibitory scores, thus questioning the finding of potentiated PPI enhancing effect. Additionally, other side effects by either drug as well as concentration issues may account for the contradicting findings by sulpiride and haloperidol treatment, too.

It has been proposed that PPI disruption results from reduced prefrontal DA transmission by causing a disinhibition of descending glutamatergic fibres (cf. Swerdlow et al., 2001). While the present work rather suggests a PPI inhibitory effect of DA transmission (i.e. DR blockage facilitates PPI), it supports the idea of glutamatergic inhibitory efferent pathways, since blockage of excitatory transmission by administration of GABA(A) agonist muscimol as well as AMPA receptor blockage with NBQX led to enhancement of PPI.

To further investigate the interplay of dopamine receptor subtypes by means of prepulse inhibition, the use of short interpulse intervals (IPI) might be of value. The present work indicates that behaviour is much more susceptible to drug treatment in the range of IPI $\leqslant 25$ ms. Most studies try to maximise PPI and only use a small subset of parameters. While at these parameters PPI is thought to be present solely, PPF occurs at short IPI. In fact, it has been proposed that PPI and PPF are two antagonising processes, which are present in parallel during presentation of any prepulse, but parameter sets favour the occurrence of either PPI or PPF (cf. Plappert et al., 2004). This hypothesis is supported by a continuously transition of PPI to PPF by decrease of IPI. Additionally, PPF shows an "inverted U-shaped" function of prepulse intensity and is strongest at intermediate prepulse intensity and short IPI, while PPI is favoured by longer IPI and higher prepulse intensity. Furthermore, PPI is disrupted when the startle eliciting pulse is preceded by two prepulses, one leading to prepulse inhibition and the other to prepulse facilitation when preceding the pulse alone (personal observation). Thus, PPF (i.e. short IPI) is ideally suited to detect changes of underlying PPI, being much more susceptible also to small drug effects which are missed at parameters of strong PPI.

Effects of dopaminergic manipulation in mice have been shown to be very complex (for review see Geyer, 2006). The present work confirms the prominent role of DR1 in prepulse inhibition in mice, where rather DR2 than DR1 seems to exert auxiliary function in prepulse inhibition. However, some contradictions, such as DR type contribution to enhancement of PPI demand further investigation of DR type involvement in the modulation of PPI. The protocol presented here is well suited to study pronounced as

well as small effects resulting from (pharmacological) treatment and suggests the use of a broader spectrum of prepulse parameters in studies of prepulse inhibition and facilitation.

9.2. Mimicking pharmacological interference by optogenetic stimulation

An emerging number of studies using the light driven manipulation of neurons via light sensitive ion-channels has been published since Arenkiel et al. presented the technique of in vivo neuronal light stimulation in 2007. To date, this technique has been used in a variety of studies, including a diversity of fields such as sleep- and Parkinson research, or the role of specific neuron population in brain oscillations or of astrocytes in breathing (Adamantidis et al., 2010; Gourine et al., 2010; Kravitz et al., 2010; Sohal et al., 2009).

The present work demonstrates that optogenetic tools are also easily adapted to measures of the acoustic startle response (ASR). Prepulse inhibition (PPI) as well as prepulse facilitation (PPF) of the ASR was decreased by light stimulation of channelrhodopsin-2 positive layer V pyramidal cells in the prefrontal cortex (mouse line Thy1-YFP-18, cf. Wang et al., 2007). Low frequency 5 Hz stimulation favoured PPI depletion, while PPF was in particular affected by high frequency 50 Hz stimulation, although (insignificant) changes were also observed in PPI and PPF with 50 Hz and 5 Hz stimulation, respectively.

Prefrontal layer V pyramidal neurons have been shown to react to dopamine (DA) or dopamine receptor 1 (DR1)-agonists by means of increased excitability (Wang and O'Donnell, 2001; Chen et al., 2007; Pietro and Seamans, 2010, but see Gulledge and Jaffe, 1998). This effect is blocked by co-application of DR1-antagonists SCH23390 (Wang and O'Donnell, 2001; Chen et al., 2007). In section 7.1.2, the present work demonstrated PPI increase by prefrontal infusion of SCH23390, thereby potentially preventing pyramidal neurons to enter a state of high excitability. Light stimulation on the other hand will have led to a depolarised (i.e. excited) state of these cells (cf. Boyden et al., 2005), proposing a possible mechanism of stimulation driving PPI disruption. Contrary, PPF was always decreased when PPI was enhanced after DR blockage, while PPF like PPI was decreased after light stimulation in the present experiment. Pharmacological treatment was neither specific for a cell type, nor was the drug limited to a small area of action. Thus, comparison between specific light stimulation and receptor, but not cell- and area specificity of pharmacological treatment trivially, but clearly, demonstrates the heterogeneity of involved prefrontal neuronal structures in modulation of PPI and PPF.

Moreover, the effects of light driven excitation of prefrontal layer V pyramidal neurons suggests a new sight on PPF. PPF has been proposed to be simply a summation phenomenon, resulting from addition of the neuron depolarising effects of startle eliciting pulse (P) and preceding prepulse (Hoffman and Ison, 1980). Thus, it was proposed that if only intensity of prepulse (PP) is high enough to depolarise neurons of the startle mediating circuit and interpulse interval is short enough for summation effects, PP + P leads to facilitated startle response (PPF). While Ison et al. (1997) reconsidered their summation hypothesis, showing that also a small (i.e. low intensity), sub threshold increase in background noise leads to pronounced facilitatory effects, Stoddart et al. (2008) are still emphatic for the theory of facilitatory summation. If this would be true, PPI and PPF could not be decreased simultaneously by the same manipulation; a decrease in PPI would have to lead to an increase in (facilitated) startle and vice versa. In the present experiment, PPI and PPF were both decreased by stimulation, but baseline startle was not. Thus, if PPF would simply be a summation of prepulse and pulse in the startle mediating circuit, baseline startle had to be inhibited, too. This clearly proves that PPF is not a function of startle mediating circuits and suggests the prefrontal cortex, and layer V pyramidal neurons in particular, to be a structure involved in prepulse facilitation of startle.

The present work shows that startle measures can be readily manipulated by light driven stimulation of transgenic neurons (i.e. optogenetic stimulation). Prepulse inhibition and -facilitation were decreased by stimulation of layer V pyramidal neurons, discussing a putative target of applied dopamine receptor antagonists (cf. section 7.1.2). While the present results clearly demonstrate that PPF is not a prepulse/pulse summation phenomenon, this proof of concept study opens the door to further neuronal optogenetic manipulation and studies of involvement of cell types in animal behaviour and application in animal models at the Max-Planck-Institute of Psychiatry.

10. Summary and conclusion

The present work successfully established startle reflex measures in mice at the Max-Planck-Institute of Psychiatry in Munich (MPI-P). Applying several paradigms of acoustic startle response (ASR) and contributing to topical issues in animal model research, the present work additionally demonstrats the critical aspect of stimulus quality in behavioural studies. Not only implementing *tone enhanced startle* (TES) as supplemental paradigm of behavioural characterisation, the present thesis introduces optogenetic techniques to manipulations of startle response and its modification in mice.

Fear potentated startle (FPS) is a common paradigm to assess fear in animals and humans (cf. Davis et al., 1993; Hamm and Weike, 2005). This paradigm was applied in a multitude of studies, and parameters and procedures to elicit FPS are well characterised (cf. e.g. Davis and Astrachan, 1978). Also various strains of mice have been shown to be suitable for measures of FPS (cf. Falls, 2002). Yet, the present study did not succeed to establish a protocol for measures of FPS in the C57BL/6NCrl mouse strain (cf. section 6.1), which is commonly used as animal model at the MPI-P. Although being overall in line with procedures applied by others, resulting in adequate fear responses measured by freezing, the present experiments suggest fear potentiation of startle in these mice is masked by strong unconditioned pre-stimulus effects. To establish security learning on the basis of FPS in mice (cf. Falls and Davis, 1997) as a model to evaluate treatment of phobics as it was the aim of the present work, future attempts should therefore employ mouse strains that have been frequently used in measure of FPS and display a strong potentiation, such as DBA/2J mice.

Even though the application of FPS was not successful, the present work took advantage of FPS masking unconditioned pre-stimulus effects. The phenomenon of startle alteration by background sound has been described for rats as well as mice (cf. Hoffman and Searle, 1965; Carlson and Willott, 2001). Studies by Hoffman and Wible (1969) suggest that this phenomenon is equivalent with startle alterations resulting from pre-

stimulus presentation demonstrated for mice, rats, and humans (cf. Falls et al., 1997; Reijmers and Peeters, 1994). This is confirmed by the present experiments (cf. section 6.2), showing that pre-stimulus presentation enhances ASR if presented before startle pulse presentation less than 3 ms, and equally strong enhancement following prestimuli of 4 - 120 s. The present work additionally proposes the paradigm of *tone enhanced startle* (TES) to be a useful tool to assess hearing capability, stimulus adaptation, and attention, capitalising the finding that startle changes increase with increased stimulus intensity (Carlson and Willott, 2001 and fig. 6.5), is stronger early during test session (Filion et al., 1993, 1994; Graham, 1975 and section 6.2.2), and is disrupted by distracting stimuli (Filion et al., 1993, 1994 and fig. 6.13). Future studies may benefit from TES, applying the introduced tools in characterisation of their animal models.

The applicability of TES as a measure of stimulus adaptation was successfully demonstrated, showing that not adaptation, but rather sensitisation, of startle changes occur when presenting white noise pre-stimuli. This raises the question whether acoustic stimulus quality (i.e. white noise or sine wave) affect behaviour not only on levels of reflex modification (i.e. startle response), but maybe also higher brain functions.

The ASR and its modifications was repeatedly shown to depend on stimulus quality (cf. Carlson and Willott, 2001; Gerrard and Ison, 1990; Stoddart et al., 2008). The present work demonstrates, that also learning in terms of fear conditioning and extinction of conditioned fear strongly depend on stimulus quality (cf. section 6.3). The animal's fear response was much stronger to conditioned white noise than conditioned sine wave stimuli, showing less freezing but rather panic like behaviour. Additionally, white noise conditioned animals displayed disability of extinction learning. This may have considerable impact on interpretation of experimental data and, considering the high discrepancy in applied protocols and stimulus parameters in fear conditioning literature (cf. fig. 2.1), may lead to contradictory findings in different laboratories. The present work therefore calls for a discussion about standardising procedures and strongly recommends a careful handling of stimulus parameters applied in behavioural experiments.

The test of the hypothesis that context extinction takes place during conditioned stimulus (CS) extinction training necessitates animal testing in differently arranged enclosures. However, different arrangements such as lighting, smell or shape may not provide enough differentiation, since contextual features also involve properties of the experimental room or procedures of placing an animal into the apparatus. Thus, the

use of the fear conditioning apparatus (cf. p. 35) for conditioning and testing, and the use of the startle apparatus (cf. p. 36) for extinction training was obligatory. On the other hand, the startle response apparatus provides limited possibilities of animal observation and freezing analysis. Although Dr. Kerry J. Ressler provides a manual how to extract freezing data from startle recordings (cf. `http://userwww.service.emory.edu/~kressle/protocols.htm`, *Analysis of SR-generated Freezing and FPS data*), the present work demonstrates that fear response to conditioned stimuli can be readily assessed by directly analysing recorded voltage scores during animal movement.

The hypothesis of parallel context extinction during CS extinction training however is not supported by the present data. Nevertheless, confirmed by the present study, CS extinction learning is highly context specific, and future experiments may solve the question whether context extinction as CS extinction learning is highly context specific, too. This would imply the possibility that context extinction in fact was present in the present experiment, but was not expressed by the animals, because the display of extinguished cue/context necessitates repeated presentation when tested in a context different from that where extinction training took place.

Some of the here and before established paradigms of startle measures were readily applied in two mouse models of mood disorders at the MPI-P.

Mice bred for high, normal, or low anxiety related behaviour, a model of trait anxiety (cf. Krömer et al., 2005), were characterised in their ability of fear memory acquisition. The present data initially suggested that mice related to high anxiety (HAB) indeed acquire fear memory much better than mice related to normal (NAB), or - even worse - low anxiety behaviour (LAB). The present work in fact demonstrates that these differences in fear behaviour do not result from differences in hearing capability or electric footshock sensitivity by applying the paradigms of baseline startle response measures, measures of tone enhanced startle, and measuring animal movement scores.

The mouse model of post-traumatic stress disorder (PTSD) (cf. Siegmund and Wotjak, 2007) has already been shown to resemble several symptoms found in patients, as well as the independence of these symptoms (cf. Pamplona et al., 2010). The present work extended the model, demonstrating that mice of this model also display the symptom of hyper-arousal by means of exaggerated startle responses. Moreover, the present study found altered hippocampal volume in these mice as it is found in PTSD patients, through the method of ultramicroscopic imaging (cf. Dodt et al., 2007). Decreased hippocampal volume was ameliorated by keeping the animals under enriched housing conditions,

suggesting putative treatment strategies in patients. Furthermore, the present work demonstrates that these two additional symptoms of the PTSD mouse model are again independent. Future work on this model may further investigate, which possible treatments could lead to amelioration of not only one, but a majority of symptoms associated with PTSD. It may also address the question, whether hippocampal volume is predictive or symptomatic for PTSD, contributing to an ongoing debate in the research community (cf. e.g. Gilbertson et al., 2002; Winter and Irle, 2004).

Building up a startle lab and establishing startle measures, the paradigm of prepulse inhibition is indispensable. Interfering with the dopaminergic system in animals of relatively high and low cortical dopamine (DA), the present work is conducive to the ongoing characterisation of dopamine receptor (DR) contribution in mediation of prepulse inhibition of startle (PPI). In line with the findings of complex and partially inconsistent actions of DR, the present study found the DR2 differently affected by two different DR2-antagonists, suggesting synergistic effects of DR1 and DR2 activation. The present data clearly show that DR1 blockage leads to increase of PPI in both subjected mouse strains, and that the prefrontal cortex is critically involved in mediation of this effect.

The present work successfully manipulated prepulse inhibition as well as prepulse facilitation of startle (PPI/PPF) by intracerebral light flashes in animals expressing the light sensitive sodium channel channelrhodopsin-2. Through the use of low and high frequency stimulation of prefrontal layer V pyramidal cells, PPI/F were potently disrupted. The present experiment lays the foundation for future experiments applying in vivo optogenetic manipulation at the MPI-P. Furthermore, the present data demonstrate that PPF is in fact not simply a summation effect of prepulse and pulse in the startle mediating pathway, as is proposed for example by Stoddart et al. (2008). Future studies may therefore address the neuronal basis of PPF.

A large part of effort in neuroscience is focused on the question, how the human brain generates and controls behaviour. To understand these processes, it will be eventually necessary to extend the systems theoretical approach, where behaviour is studied by action and reaction, by the reductionistic approach, examining the individual parts responsible for specific aspects and changes of behaviour. The first approach is feasible in humans themselves, and imaging techniques such as proton emission tomography (PET) or magnetic resonance imaging (MRI) nowadays allow partly to study individual aspects of biological processes in humans. However, first and foremost ethical considerations, but

also practical points, limit feasibility of human experiments. To approach this problem,

> [...] it would be useful to study a relatively simple behavior that can be elicited in mammals and that is sensitive to a variety of experimental treatments. (Davis, 1984)

Fulfilling many of these criteria, the startle response has been studied now for at least 100 years and has been applied in various animal models. Nevertheless, as has been introduced (cf. chapter I) and is additionally demonstrated by the present work, startle response measures today is still a contemporary field of research, which has become indispensable in characterisation of animal models of mood disorders.

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Addendum

To assess the frequency of application of different acoustic conditioned stimuli within the last ten years in mouse fear conditioning experiments, MEDLINE database was scanned using PubMed (`http://pubmed.org`) for items *fear [and] conditioning [and] mouse*. MEDLINE is a bibliographic database of life sciences and biomedical information, compiled by *The United States National Library of Medicine* (NLM). PubMed is an internet based open access database, accessing MEDLINE, maintained by the NLM and *The National Institute of Health* (NIH) (cf. `http://www.nlm.nih.gov`).
The scan was performed in April 2010 and included all studies applying acoustic stimuli for fear conditioning in mice, leading back to January 2000. A total of 458 studies were found to match the search criteria. The matching articles are listed below and the results of analysis are displayed in fig. 3.14.

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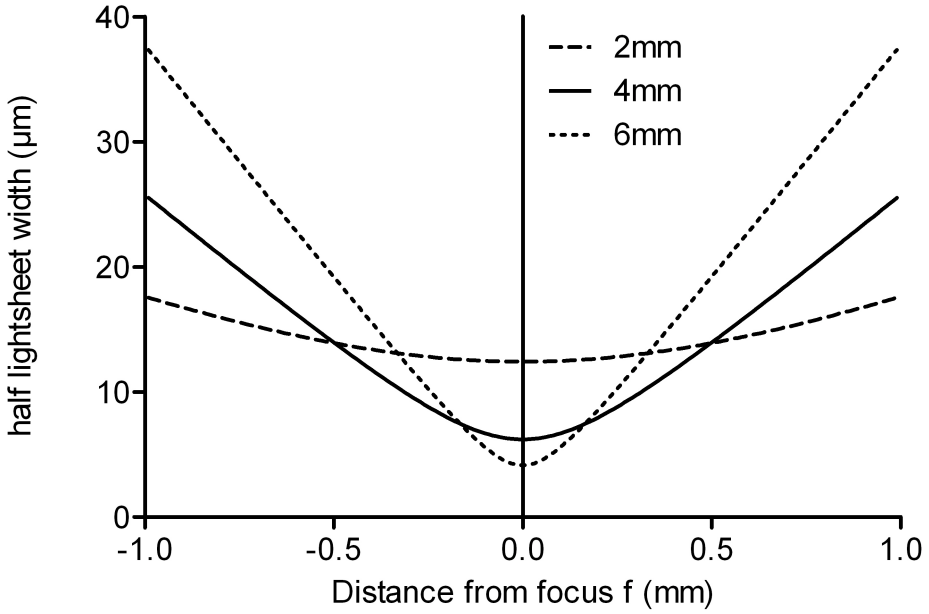
The width of the light sheet (cf. p. 49) $w(z)$ at a point z along the lateral extension of the light sheet was calculated as

$$w(z) = w_0 \cdot \sqrt{1 + \frac{\lambda \cdot z}{\pi \cdot w_0^2}}$$

with

$$w_0 = \frac{\lambda \cdot f}{\pi \cdot w}$$

where w_0 denotes the half of the minimal width of the light sheet and w half of the laser beam width before focusing (slit aperture width). z denotes the lateral light sheet extension, λ the light wavelength, and f the focal point (cf. Dodt et al., 2007). Graphs illustrating $w(z)$ (i.e. half width of light sheet) are exemplary calculated for 2, 4 and 6 mm slit aperture width.



Calculated course of the laser beam (light sheet) passing slit aperture and focus lens. Graphs were calculated for 2 mm (broken line), 4 mm (solid line) and 6 mm (dotted line) slit aperture width. Note that the experimental effective laser beam was less in width, since the equations described above take into account also areas of low light intensity of the Gaussian laser beam profile.

Erklärung

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbstständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

Des Weiteren erkläre ich, daß ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Arbeit liegt weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

Diese Dissertation wurde betreut von Dr. Carsten T. Wotjak.

Ort, Datum	Unterschrift C. P. Mauch