Advanced autonomic and behavioral phenotyping of emotional behavior of mice

Torben Hager
About the thesis
The work described in this thesis was performed at the Department of Functional Genomics, Center for Neurogenomics and Cognitive Research, Neuroscience Campus Amsterdam, VU University Amsterdam, The Netherlands. This work was in part funded by European Union Seventh Framework Programs under grant agreements no. PEOPLE-ITN-2008-238055 (BrainTrain) provided to Dr. Oliver Stiedl.

Publication of this thesis was financially supported by:
Vrije Universiteit Amsterdam, The Netherlands
Biobserve GmbH, Bonn, Germany

About the cover
The cover illustrates the mouse's perspective inside the DualCage while approaching the test compartment (front) and returning to the home compartment (back).
VRIJE UNIVERSITEIT

Advanced autonomic and behavioral phenotyping of emotional behavior of mice

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. F.A. van der Duyn Schouten,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op dinsdag 29 september 2015 om 15.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Torben Hager

geboren te Schwerte, Duitsland
promotor: prof.dr. M. Verhage

copromotor: dr. R.O. Stiedl
Dedicated to Opa Siggi.

Siegfried Hager, 1949
# Table of contents

Nomenclature xi  
List of figures xiii  
List of tables xv  

## 1 General introduction  
1.1 Shortcomings of current behavior studies 2  
1.2 Validities in behavior studies 3  
1.3 The effect of stress on cognition 5  
1.4 Summary 9  
1.5 Aim and outline of this thesis 10  

## 2 Munc18-1 haploinsufficiency results in enhanced anxiety-like behavior as determined by heart rate responses in mice 15  
2.1 Abstract 16  
2.2 Introduction 16  
2.3 Materials and Methods 19  
2.4 Results 24  
2.5 Discussion 31  

## 3 Translational relevance of non-linear heart rate dynamics: findings from behavioral and pharmacological interventions in mice for human autonomic dysfunction 39  
3.1 Abstract 40
Table of contents

3.2 Introduction ................................................. 41
3.3 Material and Methods ..................................... 42
3.4 Results .................................................... 48
3.5 Discussion ................................................ 56

4 Display of individuality in avoidance behavior and risk assessment of inbred mice 63
4.1 Abstract .................................................. 64
4.2 Introduction .............................................. 64
4.3 Materials and Methods .................................... 67
4.4 Results ................................................... 72
4.5 Discussion ................................................ 84
4.6 Supplementary Material ................................... 91

5 Substrain-specific reinforcement-avoidance relations in C57BL/6 mice using an animal-centered fear learning approach 97
5.1 Abstract .................................................. 98
5.2 Introduction .............................................. 98
5.3 Materials and Methods .................................... 100
5.4 Results ................................................... 105
5.5 Discussion ................................................ 112

6 General discussion 121
6.1 Recapitulation of Chapter 2 .............................. 122
6.2 Recapitulation of Chapter 3 .............................. 122
6.3 Recapitulation of Chapter 4 .............................. 123
6.4 Recapitulation of Chapter 5 .............................. 124
6.5 General conclusion and perspective ..................... 125

References 133

Summary 153

Nederlandse samenvatting 157

viii
<table>
<thead>
<tr>
<th>Table of contents</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Publications</td>
<td>161</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>165</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>167</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino)tetralin</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>B6J</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>B6N</td>
<td>C57BL/6N</td>
</tr>
<tr>
<td>CRF</td>
<td>Corticotropin-releasing factor</td>
</tr>
<tr>
<td>CS</td>
<td>Conditioned stimulus</td>
</tr>
<tr>
<td>DC</td>
<td>DualCage (or dark compartment when specified)</td>
</tr>
<tr>
<td>DFA</td>
<td>Detrended fluctuation analysis</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>FC</td>
<td>Fear conditioning</td>
</tr>
<tr>
<td>fps</td>
<td>Frames per second</td>
</tr>
<tr>
<td>HC</td>
<td>Home compartment</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated nucleotide-gated channel</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HRV</td>
<td>Heart rate variability</td>
</tr>
<tr>
<td>HZ</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>icv</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>mACh</td>
<td>Muscarinic acetylcholine</td>
</tr>
<tr>
<td>nACh</td>
<td>Nicotinic acetylcholine</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>oCRF</td>
<td>Ovine corticotropin-releasing factor</td>
</tr>
<tr>
<td>PA</td>
<td>Passive avoidance</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PNS</td>
<td>Parasympathetic nervous system</td>
</tr>
<tr>
<td>PTSD</td>
<td>Posttraumatic stress disorders</td>
</tr>
<tr>
<td>rmANOVA</td>
<td>Repeated Measures Analysis of variance</td>
</tr>
<tr>
<td>RMSSD</td>
<td>Root-mean-square of successive difference</td>
</tr>
<tr>
<td>SAP</td>
<td>Stretch attend posture</td>
</tr>
<tr>
<td>sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDNN</td>
<td>Standard deviation of NN intervals</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNARE</td>
<td>SNAP (Soluble NSF Attachment Protein) receptor</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>SPL</td>
<td>Sound pressure level</td>
</tr>
<tr>
<td>T&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-time</td>
</tr>
<tr>
<td>TC</td>
<td>Test compartment</td>
</tr>
<tr>
<td>TTL</td>
<td>Transistor-transistor logic</td>
</tr>
<tr>
<td>US</td>
<td>Unconditioned stimulus</td>
</tr>
<tr>
<td>USB</td>
<td>Universal serial bus</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
List of figures

1.1 The Yerkes-Dodson law ...................................... 7
2.1 Diurnal heart rate dynamics and activity of munc18-1 heterozygous and wildtype mice .................................................. 26
2.2 Activity and heart rate during novelty exposure ................. 27
2.3 Heart rate responses during retention of fear .................... 29
2.4 Correlation of heart rate and heart rate variability .............. 30
3.1 Linear measures of heart rate and SDNN as a function of treatment .......................................................... 49
3.2 Nonlinear DFA measures $\alpha_{fast}$ and $\alpha_{slow}$ as a function of treatment .................................................. 52
3.3 Comparison of the Euclidian clustering based on linear and nonlinear DFA measures .................................................. 53
3.4 Heartbeat and DFA scaling coefficients of patients evaluated for cardiac infarction .............................................. 55
4.1 DualCage design and experimental procedure. ................. 70
4.2 Training-related behaviors of C57BL/6J mice. *Continued on next page.* .................................................. 73
4.2 Training-related behaviors of C57BL/6J mice. ................. 74
4.3 Behavioral performance during the retention test. *Continued on next page.* .................................................. 76
4.3 Behavioral performance during the retention test. ............. 77
**List of figures**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4</td>
<td>Transfer latencies of B6J and B6N mice. <em>Continued on next page.</em></td>
<td>79</td>
</tr>
<tr>
<td>4.4</td>
<td>Transfer latencies differ between B6J and B6N mice.</td>
<td>80</td>
</tr>
<tr>
<td>4.5</td>
<td>Correlation matrix of behavioral performances.</td>
<td>80</td>
</tr>
<tr>
<td>4.6</td>
<td>Increased variation in retention test measures.</td>
<td>82</td>
</tr>
<tr>
<td>4.7</td>
<td>Substrain-specific differences in circadian activity.</td>
<td>83</td>
</tr>
<tr>
<td>4.8</td>
<td>Re: Boolean map of exploration.</td>
<td>91</td>
</tr>
<tr>
<td>4.9</td>
<td>Home cage re-exploration and door exploration after training. <em>Continued on next page.</em></td>
<td>92</td>
</tr>
<tr>
<td>4.9</td>
<td>Home cage re-exploration and door exploration after training.</td>
<td>93</td>
</tr>
<tr>
<td>4.10</td>
<td>Fear expression and extinction of B6J and B6N mice in classic fear conditioning.</td>
<td>94</td>
</tr>
<tr>
<td>4.11</td>
<td>Fear expression and extinction of B6J and B6N mice in PA.</td>
<td>95</td>
</tr>
<tr>
<td>5.1</td>
<td>DualCage design and experimental procedure.</td>
<td>102</td>
</tr>
<tr>
<td>5.2</td>
<td>Locomotor activity of C57BL/6 mice on day 5.</td>
<td>107</td>
</tr>
<tr>
<td>5.3</td>
<td>Stretch-attend posture during the retention test. <em>Continued on next page.</em></td>
<td>109</td>
</tr>
<tr>
<td>5.3</td>
<td>Stretch-attend posture during the retention test.</td>
<td>110</td>
</tr>
<tr>
<td>5.4</td>
<td>Transfer latencies in the retention test.</td>
<td>111</td>
</tr>
<tr>
<td>5.5</td>
<td>Exploration of TC during retention. <em>Continued on next page.</em></td>
<td>113</td>
</tr>
<tr>
<td>5.5</td>
<td>Exploration of TC during retention.</td>
<td>114</td>
</tr>
<tr>
<td>5.6</td>
<td>Model of the reinforcement vs. avoidance relation.</td>
<td>115</td>
</tr>
<tr>
<td>6.1</td>
<td>Schematic diagram of the emergence of individuality.</td>
<td>130</td>
</tr>
</tbody>
</table>
List of tables

2.1 Overview of behavioral and autonomic fear responses of wild-type and *munc18-1* heterozygous mice in fear retention tests. 33

3.1 Overview of behavioral and pharmacological interventions used to assess heart rate dynamics in mice. 46

3.2 Human heart rate dynamics under various experimental conditions. 54

4.1 Operational definitions of behavioral expressions and measures. 71
1

General introduction
Behavioral neuroscience (or psychobiology) is the study of mental functioning and behavior in relation to other biological processes (Merriam-Webster Learner’s Dictionary. Psychobiology – Def. 1., 2014). In order to investigate these processes in vivo, that is in whole, living organisms, animal models are utilized, while rodent models are advantageous for several (practical) reasons. But importantly, the interpretation of particular measures of the performance on a certain task needs to be done carefully and fulfill certain criteria and validities. The test as well as the animal model itself should fulfill these requirements. Not only the investigation of principles of basic neuroscience is affected by this problem, it also affects the field of drug discovery, which has a worldwide economic cost of over $40 billion per year (Kola and Landis, 2004; Nestler and Hyman, 2010). Of course, the efficacy of an intervention in a rodent model (e.g., an anti-depressant pharmacological intervention) cannot be measured by the amount of observed “anthropomorphized” behavior. A “depressed” mouse, if existing at all, will never whistle less than its normal littermate, like Mickey Mouse did it while operating a steamboat (Disney, 1927). The failure of current animal approaches (from experimental setups via animal models to measures) to unambiguously interpret findings, eventually translate them from one species to the human situation and thereby predict the effect of certain interventions is attributed to a multitude of hypothetical reasons ranging from molecular to behavioral to sociological (Fonio et al., 2012b).

1.1 Shortcomings of current behavior studies

Problems of models and tests to meet certain validities, which will be explained briefly below, and general shortcomings of behavioral studies have been discussed thoroughly in literature (Fonio et al., 2012b; Würbel, 2002, 2009) as well as the potential improvement of methods of analysis (Benjamini et al., 2010; Fonio et al., 2009; Golani, 2012). Not only recently, but already in 1976 Walsh and Cummins (Walsh and Cummins, 1976) listed a wide-spread multitude of effects (like early life experience, enrichment, illumination and other dependent parameters), that beside the intervention of interest of a particular study
(e.g., effects of strain differences or a pharmacological intervention) possibly influence the behavioral performance in a so-called classical test (exemplified by Walsh and Cummins by the open field test). However, as Spruijt et al. nicely formulated it in their recent publication (Spruijt et al., 2014) on reproducibility and relevance of future behavioral research, “The methods of the majority of studies measuring and interpreting behavior of laboratory animals seem to have frozen in time somewhere in the last century.” In the context of behavioral studies, Fonio et al. (Fonio et al., 2012b) encourage researchers to first ask: are we measuring what we intend to measure? And if so, are we measuring this behavior in a useful way? The second question aims at the reliability of the measure. That is, if it is stable over time in a single individual, if it is representative for a group of animals and if it can be reproduced in different animals across different laboratories. Although this might sound trivial, fundamental questions like these should form the basis of science in general (Talpos and Steckler, 2013). However, since each study within a certain field of research premises for slightly different interpretations of validities (e.g., translational research may require amongst others the confidence for costly decision-making towards drug development), general definitions of validities can be found. These interdisciplinary definitions should certainly be handled with care and need to be carefully adjusted to meet the prerequisites of each particular study.

### 1.2 Validities in behavior studies

*Construct validity*, in simply words, can be considered to reflect accuracy by assessing the appropriateness of inferences made on the basis of observations or measurements, or more specific, whether a relevant measure is used to test the intended construct (Cronbach and Meehl, 1955). Construct validity is essential to the perceived overall validity of the test. On the other hand, the extent to which a test is subjectively evaluated as covering the concept it is supposed to measure is referred to as face validity. Thereby it assesses the transparency or relevance of a test as it subjectively appears (Holdon, 2010), although for example homologous biological systems might ultimately indeed be
different when compared to each other (Keeler and Robbins, 2011). Predictive validity refers to the sensitivity and specificity, either in that the test or model makes true predictions with respect to the condition of interest in the translated paradigm, or, more specifically, in that effective interventions are accurately discriminated from effects of other interventions (Cronbach and Meehl, 1955). In the more specific example of translational research, if interventions of interest are treatments whose efficacy needs to be translated (from one species to the other), this kind of validity is also referred to as pharmacological predictive validity (Youn et al., 2012). In this realm of translational research, the criterion of homologous (biological) systems has also been coined etiological validity by Geyer and Markou (Geyer and Markou, 1995), although the etiology, the origin of particularly effective disorders and neurodegenerative diseases is still generally unknown. For instance, rat probe-burrying behavior is attenuated by anxiolotics, although it does not resemble a symptom of Generalized Anxiety Disorder (De Boer and Koolhaas, 2003). While Fonio et al. address the issue of ethological validity (Fonio et al., 2006) by using first-generation-in-captivity wild mice as an ethologically relevant reference, the importance of etiological and especially ethological valid models and test seems to be widely underestimated in behavioral studies (Gerlai, 2002). While behaviorism focuses on behavioral responses in a laboratory setting, ethology is defined as the scientific and objective study of animal behavior under natural conditions (Merriam-Webster Learner’s Dictionary. Ethology – Def. 1., 2014).

Finally, the performance of animals in behavioral studies is prone to potentially unpredictable occurrence of (unspecific) stress. This might affect any of the above-mentioned validities of models and test in an uncontrollable manner. As described in the following section, this might not specifically affect behavioral studies with a focus on emotion (see section “Aim and outline of this thesis” in this chapter) only, but cognition in general, and thus, any measures based on cognition-related performance.
1.3 The effect of stress on cognition

On Good Friday, April 14, 1865, United States President Abraham Lincoln was shot while attending a play at Ford’s Theatre, Washington D.C. Over three decades later, in 1899, Colgrove et al. (Colgrove, 1899) observed that most adults could still explicitly describe events, which occurred on that particular day. Following his descriptions of the recollections of these people, he suggested that strong emotionality could facilitate durable memories of arousing events. More recently, people that have experienced emotionally arousing events of great importance (e.g., the accidental death of Diana, Princess of Wales or the collapse of the World Trade Center on September 11, 2001) could describe similar vivid and long-lasting memories. The powerful fortification of memories, acquired in times of strong emotionality, was first referred to as “hypermnesia” by Stratton et al. (Stratton, 1919) and then as “flashbulb memories” by Brown and Kulik (Brown and Kulik, 1977).

A decade after Colgrove’s description of the influence of emotion on memory, Yerkes and Dodson (Yerkes and Dodson, 1908) studied the effects of different reinforcement properties (in this case: shock intensities) on the rate of learning by mice in a visual discrimination avoidance task. They summarized the interpretations of their experiments in the Yerkes-Dodson law, a principle that states the relationship between arousal and behavioral performance, which can be linear (performance increasing with increasing arousal until saturated) or curvilinear (performance increasing with increasing arousal until breakpoint and then reversing), depending on the difficulty of the task (Fig. 1.1).

However, as Diamond et al. expressed it in their comprehensive review on the temporal dynamics model of emotional memory processing (Diamond et al., 2007), “with its thousands of reference citations in the past century, Yerkes and Dodson may have the dubious distinction to be the most highly cited, but largely unread paper in the history of science”. That is to say, in their work on the performance of mice in a visual discrimination task, Yerkes and Dodson (Yerkes and Dodson, 1908) showed that when mice were trained in a simple task to avoid shock, their rate of learning increased linearly with an increase in
the intensity of the shock. When mice were trained in a more complex visual
discrimination task (decreased visual contrast), their rate of learning was most
efficient in combination with intermediate shock intensity. In conclusion, this
leads to a combination of a dual linear relationship (simple task) with a curvilinear
relationship (complex task). Broadhurst could confirm this relationship of
reinforcement properties and learning performance in rats (Broadhurst, 1957),
also in a visual discrimination task involving different levels of difficulty and
motivation (respectively stress). Thus, these studies, although 5 decades apart,
demonstrated that high levels of stress impaired performance in rodents on a
difficult, but not on an easy task. Other studies on rodents (Mesches et al.,
1999) and humans (Dickman, 2002) have reinforced the notion of the importance of taking into account the difficulty of the task as an intervening variable in arousal effects on performance. However, major figures in the field of
cognitive psychology, like Donald O. Hebb (Hebb, 1955), asserted that the relationship between arousal and performance is exclusively curvilinear. Since
the Hebbian, incomplete illustration of the curvilinear arousal-performance relationship incorrectly came to be known as the Yerkes-Dodson law by later researchers, cognitive psychologists fiercely debated the heuristic value of this Hebbian version, while behavioral neuroscientists often inclined to accept the Hebbian version in their interpretations of brain-emotion interactions (LeDoux,

But, if Hebb was right in his assertion that the arousal-performance relationship is exclusively curvilinear with solely high arousal always impairing the formation of memory, then flashbulb memories should not exist. This also applies to the well-described “weapon-focus” phenomenon (Christianson, 1992), which describes enhanced (memory-) performance under conditions of high arousal. Or even the formation of intrusive memories after exposure to highly stressful, traumatic events, so powerful and durable that they can have long-lasting pathological consequences (e.g., depression or PTSD-like symptoms (Ehlers et al., 2005). On the contrary, research had shown that strong emotionality can enhance memory under certain conditions (McGaugh, 2004).
1.3 The effect of stress on cognition

Figure 1.1: The relationship between arousal (“stress”) and behavioral performance (cognition) known as the Yerkes-Dodson law, based on the original findings of Yerkes and Dodson in 1908 (Yerkes and Dodson, 1908). It takes into account that strong emotionality can enhance performance under simple learning conditions, such as when learning involves focused attention on a restricted range of cues, and impairs performance under difficult (more complex) learning situations, such as in divided attention, multi-tasking, decision-making and working memory tasks. Figure modified from Diamond et al. (2007).
However, to operationally define the subjective measure of “task difficulty” had been quite a challenge for researches in this field during the last 5 decades. And although an ubiquitous answer to this question cannot be found - the very fact of individual differences regarding the perception of stress, difficulty and motivation are contradictory to all-embracing, inter-individual definitions - Easterbrook presented in his landmark paper from 1959 one of the most comprehensive and insightful analysis of how emotion affects cognition (Easterbrook, 1959). In his “cue utilization” hypothesis he noted that strong emotionality “acts consistently to reduce the range of cues that an organism uses, […]”. Depending on the difficulty of the particular task (the use of how many cues are required for proper performance?), this can either be organizing or disorganizing. In other words, excluding irrelevant cues under strong emotionality can be beneficial in tasks that involve focused attention on an isolated cue with minimal cognitive (e.g., decision-making) demands. On the other hand, if a task is complex, involving attention to multiple cues, then performance will deteriorate under conditions of high stress. In conclusion, Easterbrook’s cue utilization hypothesis and the original (dual-linear as well as curvilinear) version of the Yerkes-Dodson law are complementary explanations for the finding that strong emotionality can impair as well as enhance performance depending on the difficulty or complexity of a task.

Additionally, Arnsten (Arnsten, 1998) stated that “stress impairs prefrontal cortex (PFC) function through catecholamine receptor mechanisms […]”. While recent imaging studies have shown that the PFC is critically involved in higher cognitive (executive) functions such as guiding behavior during divided attention (Dannhauser et al., 2005) and working memory (Adcock et al., 2000) tasks, as well as in planning (Rowe et al., 2001) and decision-making (Bechara, 2005). Taken together, this suggests that the extent to which the PFC is involved in the execution of a task and the extent to which activity in the PFC is suppressed by stress might be primary determinants of whether the arousal-performance relation is linear or curvilinear. That is, the more cognitively demanding the execution of a certain task is, the more likely will it be that the performance will suffer under conditions of high arousal.
1.4 Summary

In summary, neuroscience research crucially relies on sensitive and robust methods in behavioral phenotyping. To date, commonly used test systems have several limitations that prevent assessment of a wide spectrum of behaviors in animal models that are relevant to human pathologies. Behavioral phenotypes of rodent model organisms are typically acquired in a novel test environment into which they are transferred by the experimenter. This introduces coercion (stress), which is a confounding factor, particularly when assessing behaviors in which the emotional state of animals is investigated (Hurst and West, 2010). Consequently, classical test systems measure to a large extent the resilience of animals to experimenter-based perturbation of cognitive function (Diamond et al., 2007) instead of intrinsically motivated performance. These tests are typically of short duration. The investigation of processes, whose time scale is days, for example, processes in which adaptation of behavior is relevant, such as in learning and memory (Spruijt et al., 2014), would highly benefit from setups that allow measuring behavior across days (Fonio et al., 2012a). Classical behavior tests are mainly quantified on the basis of a single or a few measures, which limits the analysis of behavioral functions, whose complexity exceeds the characteristics of these measures (Fonio et al., 2012b). For instance, freezing is a sensitive measure of fear, but may be only measurable during the state of fear (Lang et al., 2000) and it is unsuited under conditions of active coping (flight or escape) (Koolhaas et al., 2006). The integration of multiple measures may be particularly advantageous when it concerns learning, memory and emotional states. Attempts to standardize behavioral tests for improved reproducibility across laboratories have failed to a large degree, despite rigorous standardization (Crabbe et al., 1999). The screening of new drugs by the pharmaceutical industry is hampered by the consecutive use of many different tests assays that are labor-intensive and offer only limited throughput (Spruijt et al., 2014). Furthermore, the use of different test assays in order to solve this limitation introduces carry-over effects that depend on the order of the execution of the tests involved (Crawley, 2007). Finally, when it comes to the translational
value of measures from bench to bedside (respectively covering the two-staged process including bed-side to clinical practice) many activity-derived measures including freezing are of relatively limited symptomatic relevance (American Psychiatric Association, 2013) as defensive response in humans (Azevedo et al., 2005). The assessment of measures that characterize real endophenotypes favors behavioral expression based on risk assessment and avoidance. In conclusion, improvement on the abovementioned limitations has the potential to increase general as well as specific validities and the translational value of behavioral studies.

1.5 Aim and outline of this thesis

The aim of the thesis was to characterize emotional memory using classical but also novel behavioral approaches for the characterization of substrain and strain differences as well as effects of genetic interventions in mouse models. This was performed on the behavioral and on the autonomic level. Autonomic assessment occurred using radio-telemetry for remote ECG measurements to determine heart rate in mice and compared to human data. This integrative approach aimed to improve the translational value of basic research, e.g., by reducing the interpretational ambiguity of measures commonly used in behavioral neuroscience.

Chapter 2: Munc18-1 haploinsufficiency results in enhanced anxiety-like behavior as determined by heart rate responses in mice

Heterozygous (HZ) missense mutations in the gene encoding syntaxin binding protein 1 (Stxbp1 or Munc18-1), a presynaptic protein essential for neurotransmitter release (Verhage et al., 2000), causes early infantile epileptic encephalopathy, abnormal brain structure and mental retardation in humans (Saitsu et al., 2008). The aim of Chapter 2 was to characterize HR responses of the murine model under baseline condition including diurnal HR dynamics, during unconditioned and conditioned emotional challenge including extinction of conditioned fear in munc18-1 HZ and WT mice. Novelty served as un-
1.5 Aim and outline of this thesis

Conditioned stressor. Conditioned HR of munc18-1 HZ and WT mice was compared during retention of conditioned fear after auditory delay (hippocampus-independent) and trace fear conditioning (hippocampus-dependent), subsequent extinction of conditioned fear as well as after retraining. Reconditioning was included to determine potential differences in latent inhibition-like effects on relearning which depends on the dorsal hippocampus (Maren and Holt, 2000). These autonomic results were compared with behavioral data based on classical tests (Maroteaux et al. unpublished observations) to draw refined conclusions on the functional consequences of munc18-1 haploinsufficiency.

Chapter 3: Translational relevance of non-linear heart rate dynamics: findings from behavioral and pharmacological interventions in mice for human autonomic dysfunction

To date, a nonlinear characterization of heart rate (HR) dynamics elicited by different pharmacological substances commonly used in cardiovascular research is partially available only in the rat (Beckers et al., 2006). Therefore, the detrended fluctuation analysis (Peng et al., 1995) was used to quantify the physiological state of HR dynamics in freely moving mice upon various behavioral and pharmacological interventions to draw conclusions about autonomic effects and potential pathological consequences that cannot be inferred from changes in HR and its variability. The effects on HR dynamics were compared with those observed in humans under different conditions including pathological states such as heart transplantation to identify similar if not identical functional properties as highly translational measures of cardiac risk based on altered autonomic control irrespective of species-specific absolute HR differences.

Chapter 4: Display of individuality in avoidance behavior and risk assessment of inbred mice

The aim of Chapter 4 was to introduce the development of a novel behavioral approach, able to automatically assess long-term behavioral performance of
mice under semi-naturalistic conditions. Automated analysis of behavior in a home cage design potentially counteracts many of the shortcomings addressed above. Automation reduces tremendously human interference, while granting the monitoring of spontaneous behavior on long time scales. Thereby, adaptation of behavior towards habituated conditions can be observed. Additionally, automation allows high-content recording of a rich set of behavioral measures from which dynamical properties of deterministic organization of behavior can be extracted. The high spatial and temporal resolution of behavioral monitoring under these conditions allows obtaining measures with high translational value such as the stretch-attend posture.

The advantage of uninterrupted long-term monitoring without human intervention in the home cage setup (De Visser et al., 2006; Maroteaux et al., 2012) was combined with the assets of deliberate exploration of an attached environment (Fonio et al., 2009). We developed a flexible modular system (DualCage) consisting of a home cage and attached test compartment to assess multiple behavioral measures based on 3-point tracking. In this system the animal has the choice to deliberately participate in an experiment while the progression of the experiment is determined by the instrumental responses of the animal. This approach might offer more ethologically relevant behavioral studies, under semi-naturalistic conditions taking species-specific characteristics into account (Belzung and Griebel, 2001; Olsson et al., 2003). In Chapter 4 the long-term responses to emotional fear learning of C57BL/6J and C57BL/6N mice were investigated in the DualCage, and compared to their behavioral performance in classic behavioral tests.

**Chapter 5: Effect of different reinforcement properties on emotional learning under semi-naturalistic conditions**

In Chapter 5, the consequences of different reinforcements properties in the DualCage on fear learning were examined, extending the results obtained in Chapter 6. A critical issue here was that the associative strength was modulated by different shock numbers and timing of shock. These experiments allowed determining the consequences of different reinforcement (arousal) on
performance (fear memory) in mice and drawing a refined view of fear learning according to the Yerkes-Dodson law and used behavioral measures. Additionally, a recent study that showed consistent differences in inter-individual fear response magnitudes in isogenic mice (C57BL/6J) based on three unconditioned stressors (Liu et al., 2014). This suggests personality trait-like differences in stress-susceptibility of posttraumatic stress disorder (PTSD) models.

Finally, Chapter 6 summarizes the findings on advanced autonomic phenotyping of emotional behavior of mice serving as models of cognitive and/or emotional dysfunction. Additionally, further perspectives of methodological enrichment and validation of advanced phenotyping in automated, home cage-based behavioral approaches like the DualCage are discussed.
Munc18-1 haploinsufficiency results in enhanced anxiety-like behavior as determined by heart rate responses in mice

Published in:
2.1 Abstract

Heterozygous (HZ) missense mutations in the gene encoding syntaxin binding protein 1 (Stxbp1 or Munc18-1), a presynaptic protein essential for neurotransmitter release, causes early infantile epileptic encephalopathy, abnormal brain structure and mental retardation in humans. Here we investigated whether the mouse model mimics symptoms of the human phenotype. The effects of the deletion of munc18-1 were studied in HZ and wild-type (WT) mice based on heart rate (HR) and its variability (HRV) as independent measures to expand previous behavioral results of enhanced anxiety and impaired emotional learning suggesting mild cognitive impairments. HR responses were assessed during novelty exposure, during the expression and extinction of conditioned tone-dependent fear and during the diurnal phase. Novelty exposure yielded no differences in activity patterns between the two genotypes, while maximum HR differed significantly (WT: 770 bpm; HZ: 790 bpm). Retention tests after both auditory delay and trace fear conditioning showed a delayed extinction of the conditioned HR response in HZ mice compared to WT mice. Since the HR versus HRV correlation and HR dynamics assessed by nonlinear methods revealed similar function in HZ and WT mice, the higher HR responses of munc18-1 HZ mice to different emotional challenges cannot be attributed to differences in autonomic nervous system function. Thus, in contrast to the adverse consequences of deletion of a single allele of munc18-1 in humans, C57BL/6J mice show enhanced anxiety responses based on HR adjustments that extend previous results on the behavioral level without support of cognitive impairment, epileptic seizures and autonomic dysregulation.

2.2 Introduction

Munc18-1 is a neuron-specific protein of the SEC-1 family (Südhof and Rothman, 2009; Verhage et al., 2000). It is a regulator of the formation of the SNARE (SNAP [Soluble NSF Attachment Protein] Receptor) complex, which is essential for presynaptic vesicle docking before fusion for transmitter release.
(Toonen, 2003). Homozygous munc18-1 knockout mice are not viable and die immediately after birth (Verhage et al., 2000), while heterozygous (HZ) munc18-1 mice are viable. HZ missense mutations in the munc18-1 gene are implicated in early infantile epileptic encephalopathy in humans, an early form of epilepsy (Saitsu et al., 2008). Behavioral and cognitive impairments are hypothesized in HZ mice since reduced expression of the munc18-1 gene results in reduced synaptic vesicle release and a smaller readily releasable pool which in turn negatively influences the efficacy of synaptic function (Toonen et al., 2006). Behavioral phenotyping experiments of munc18-1 HZ mice show increased anxiety and impaired emotional learning in fear conditioning and passive avoidance but no spatial learning deficit (Maroteaux et al., prep).

The autonomic nervous system (ANS) mediates physiological adjustments, particularly in response to threatening stimuli, amongst others of the cardiovascular system. This offers useful readouts of the emotional state of an organism, when sheer behavioral measures are difficult to quantify or are subject to interpretational ambiguity (Berntson et al., 1998; Stiedl et al., 2009). A direct measurement of the activity of the ANS in vivo is difficult at least in behavioral studies. Therefore, the dynamics of HR are an indirect but highly sensitive index of ANS function and allow to monitor the change of the emotional state elicited for example by an unfamiliar environment (Stiedl et al., 2009). The ANS influences HR dynamics through its two interdependent subsystems, the sympathetic and parasympathetic nervous system (SNS and PNS, respectively). Specifically, the tonic function of the PNS is essential for the dynamical properties of physiological heartbeat interval fluctuations (Stiedl et al., 2009).

Anxiety disorders are regarded to be the consequence of dysregulated fear mechanisms (Myers and Davis, 2007; Quirk and Mueller, 2008). An increasing number of patients suffer from a range of anxiety disorders including post-traumatic stress disorder (PTSD) (Wittchen et al., 2011). PTSD patients re-experience and persistently avoid stimuli and events they associated with a previous trauma and show increased arousal and impaired fear extinction (Vieweg et al., 2006). Fear extinction is defined as the weakening of the conditioned response to the conditioned stimulus (CS) through non-reinforced exposure to the
Enhanced anxiety-like behavior in *munc18-1* heterozygous mice

CS through new learning (Sotres-Bayon et al., 2006). Animal models based on associative emotional (fear) learning frequently use fear conditioning (Maren, 2011) to investigate the neural circuits of fear and extinction based on behavioral measures such as freezing.

Independent from behavioral expressions, conditioned fear can be assessed by profound HR changes. Recall of fear conditioned to an auditory cue elicits a pronounced tachycardia under otherwise stress-free baseline conditions in the home cage of unrestrained mice, indicating that HR and HRV changes reflect physiological adjustments indicative of associative learning (Stiedl et al., 2009). Fear extinction results in gradually reduced tachycardia to the CS in C57BL/6J mice (Stiedl et al., 1999). Two types of auditory fear conditioning can be distinguished, delay and trace fear conditioning. In delay fear conditioning the CS follows the US without any interval (or CS and US offset coincide), which causes learning that involves amygdala but not hippocampal function. However, in trace fear conditioning a defined time interval separates CS from US. To establish an association between the two stimuli dorsohippocampal and amygadaloid function are necessary (Chowdhury et al., 2005; Misane et al., 2005). Since the cognitive demand is expected to be higher in trace fear conditioning, genotype differences may emerge in delay versus trace fear conditioning depending on the potential hippocampal impairment in *munc18-1* HZ mice.

There is a high comorbidity of cardiac and emotional disorders (Rozanski et al., 1999). Cardiovascular studies in patients suffering from anxiety disorders such as PTSD indicate an increased baseline HR, a decreased HRV (Cohen et al., 1998), a diminished vagal activity and blunted diurnal variation of HRV (Agorastos et al., 2013). This strongly supports a relation between autonomic impairments and affective emotional disorders. HR dynamics as functional readout of brain function due to ANS control is affected by epileptic activity triggering arrhythmias even with lethal outcome in man (Dinan et al., 2008; Langan et al., 2000) and mice (Kalume et al., 2013). The assessment of HR dynamics may help uncover epileptic activity that may not be noticeable at the behavioral level. Since epileptic activity is commonly more prominent
2.3 Materials and Methods

during stressful challenge, HR measurements after challenging conditions may uncover effects that cannot be observed under baseline stressfree conditions.

Therefore, the aim of this study was to characterize HR responses under baseline condition including diurnal HR dynamics, during unconditioned and conditioned emotional challenge including extinction of conditioned fear in munc18-1 HZ and WT mice. Novelty served as unconditioned stressor. Conditioned HR of munc18-1 HZ and WT mice was compared during retention of conditioned fear after auditory delay (hippocampus-independent) and trace fear conditioning (hippocampus-dependent), subsequent extinction of conditioned fear as well as after retraining. Reconditioning was included to determine potential differences in latent inhibition-like effects on relearning which depends on the dorsal hippocampus (Maren and Holt, 2000).

2.3 Materials and Methods

Animals

Male munc18-1 HZ and littermate WT control mice, generated on the 129S1/SV genetic background and backcrossed to C57BL/6JCrI mice (source: Charles River, Netherlands) for more than 30 generations (University Animal Research Center, VU University, Amsterdam), were used in these experiments. In total 17 munc18-1 HZ and 13 WT mice were tested at 9-12 weeks of age with ECG measurements. Body weights of HZ mice (23.2±0.7 g; mean±SEM) was significantly lower (F1,28=5.17, P=0.031) than that of WT mice (25.0±1.0 g) as previously observed (Maroteaux et al., prep). Mice were individually housed in standard cages (Macrolon, Type II short) with both food and water ad libitum. Seven days before the start of experiments mice were accommodated to the behavior facility and its dark-light cycle (lights on from 6 AM to 6 PM). In addition, diurnal activity of 122 mice was measured in the Phenotyper (Maroteaux et al., 2012). Novelty exposure and fear conditioning experiments were performed during the light phase.
Enhanced anxiety-like behavior in \textit{munc18-1} heterozygous mice

\textit{ECG transmitter implantation}

Isoflurane inhalation anesthesia (Isotec 4, SurgiVet; Smiths Medical PM, Norwell, MA, USA) was initiated at 2.5-3\% in O$_2$ and maintained at 1.6\% in O$_2$ for implantation of ECG radiotransmitters (ETA-F10; Data Science, St. Paul, MN, USA). All hair around the surgical area was thoroughly removed. Surgery was performed with disinfected tools (70\% EtOH) on a thermo-plate set to 36°C to prevent hypothermia during anesthesia. The skin in the surgical area was sterilized with iodine. Sterile physiological NaCl solution (0.9\%) was used to prevent dehydration of exposed skin and tissue. A longitudinal cut shifted parallel 4 mm to the left of the rostro-caudal line opened the abdominal skin.

The cut started 2 mm below the height of navel and went rostral for 20 mm. The skin and underlying muscle tissue were separated by removal of connective tissue. Two subcutaneous tracts were created towards the right front leg and towards the left hind leg for placing the ECG electrode wires. A medial second cut in the abdominal muscle tissue starting approximately at the navel in rostral direction for 15 mm allowed to insert the sterile ECG transmitter into the abdominal cavity. The two ECG electrode wires pointed in rostral direction. The muscle tissue was punctured with a sharp forceps to guide the ECG electrodes out of the abdominal cavity for subcutaneous placement. The anode (length \(\sim 3 \text{ cm}\)) was guided towards the right front leg and the cathode (length \(\sim 7 \text{ cm}\)) was placed in a wide anterior loop towards the left hind leg. Re-growth of connective tissue between skin and muscular layers during the recovery period fixed the ECG electrodes in place. The ECG transmitter was then sutured to the abdominal muscle tissue with non-dissolvable thread (size 4/0; Supramid; Braun, Tuttingen, Germany). The intermediate muscle tissue was sewed up with dissolvable surgical thread (size 6/0 Supramid; Braun, Tuttingen, Germany) and the cutaneous layer was closed with metal wound clips (Autoclip 9 mm; Becton Dickinson, Sparks, MD, USA). Thereafter, the wound was thoroughly cleaned with sterile saline. To support post-surgical recovery, 0.5 ml of the nutritious solution Amynin (Lohmann, Cuxhaven, Germany) was injected subcutaneously. Mice were then transferred to clean cages with ster-
ile bedding and nesting material. Mice were allowed to recover for two weeks with daily weight monitoring. Wound clips were removed 1 week after surgery.

**Diurnal heart rate and locomotor activity**

To determine the phenotypical difference under physiological conditions, HR was measured for 20 min every hour for 24 h in the home cage of undisturbed mice. Additionally, home-cage based activity of separate groups of mice was monitored for 24 h in the PhenoTyper (Maroteaux et al. (2012), Noldus Information Technology, Wageningen, The Netherlands). HR and locomotor activity measurements were determined in separate tests, because the larger area of the PhenoTyper (30 cm x 30 cm) does not allow recording of the ECG signal at every position in the cage due to limited signal transmission. However, continuous ECG recording is essential for nonlinear HR analysis. We used the PhenoTyper for activity measurements, because video-tracking in the home cage is not possible without major modifications. Additionally, the ECG signal strength from the radiotransmitter provides only crude information on relative activity that has not been validated. We analyzed activity data 24 h after the initial placement in the PhenoTyper, when novelty-induced effects on activity were absent (Maroteaux et al., 2012), because the home cage also served as habituated environment.

**Novelty exposure**

Behavioral and HR effects of all mice (n = 30) during exposure to novelty were determined in a novel, unfamiliar environment for 34 min. Locomotor activity was monitored in the novel environment (size 36 cm x 21 cm) by photo beams with a spatial resolution of 1.3 cm x 2.5 cm and a sampling rate 10 Hz (TSE-Systems, Bad Homburg, Germany) as described before (Tovote et al., 2004). Before and after each trial the setup used for exposure to novelty was cleaned with 1% acetic acid (in contrast to 70% Ethanol, which was used as a cleaning agent for the setups of fear conditioning). ECG was recorded via the implanted ECG radio-transmitters. This approach had been successfully used in the past.
Enhanced anxiety-like behavior in munc18-1 heterozygous mice

**Auditory delay and trace fear conditioning**

Fear conditioning occurred in a fear conditioning box (TSE-Systems, Bad Homburg, Germany), which consists of a Plexiglas cage (36 x 21 x 20 cm; length x width x height) with a stainless steel grid floor (beam diameter 4 mm, distance 9 mm), through which a single electric foot shock (0.7 mA, 2 s, constant current) was provided as US. A high frequency loudspeaker driven by the TSE control unit generated a tone (10 kHz, 75 dB SPL [sound pressure level], pulsed at 10 Hz) serving as auditory CS. The conditioning box was constantly illuminated (120-350 lx) and white noise (68 dB SPL) served as constant background stimulus except during CS presentation. Before each experiment the conditioning box and the shock grid were thoroughly cleaned with 70% ethanol solution. In contrast to the acquisition phase, the retention tests were performed in the home cage placed under an identical loudspeaker to deliver the CS. Training: During training a mouse was placed in the fear conditioning box which it could explore for 180 s before it was exposed to the CS for 30 s. In the delay fear conditioning experiments the CS was immediately followed by a 2-s US. In trace fear conditioning there was an interval of 30 s between the CS and the US. In delay fear conditioning 8 HZ and 7 WT mice were tested, and in trace fear conditioning 9 HZ and 6 WT mice were tested. The animals were removed from the training environment and returned to the home cage 30 s after the shock exposure. Retention tests: The first retention test was performed 24 h after training in the home cage, followed by 4 more retention tests on a daily basis. Retention tests started with a 180-s pre-CS phase, followed by a 180-s CS phase, in which the tone was replayed, and ended with a 90-s postCS phase. Retraining and additional retention test: Retraining occurred 2 days after the last retention test as during training, except that 3 US exposures occurred at 60-s interval in delay or trace conditioning mode, respectively. One day later a final retention test was performed to measure the fear response to the CS to determine latent inhibition. In latent inhibition learning of the significance of a stimulus is retarded as a result of its previous non-reinforced exposure, resulting in a lower fear response compared to naïve animals (Lubow and Moore, 1959).
2.3 Materials and Methods

**ECG acquisition and data analysis**

The ECG signal of the ECG radio-transmitter was detected by a receiver board (Data Science, RLA1020, St. Paul, MN, USA) placed underneath the home cage or the particular setup, respectively. Via an analog output adapter (Data Science, Option R08, St. Paul, MN, USA) the ECG signal was fed forward into an A/D converter (ADInstruments, MacLab 4S, Spechbach, Germany), which digitized the analog signal and stored it on the hard drive of a PC with data acquisition software (ADInstruments, MacLab Chart v5.5.6, Spechbach, Germany). Off-line ECG analysis was performed as follows. On the basis of the intervals between R-waves (R-R) instantaneous HR values were calculated, while ectopics and artifacts, such as movement artifacts, were automatically detected and manually edited. This editing procedure followed established principles (Tovote et al., 2004). A bradyarrhythmia was defined as an interval that is at least twice as large as the adjacent intervals, i.e., a genuine ‘missing’ beat. Intervals shorter than 70.6 ms (~850 bpm) were identified as tachyarrhythmia, since these are extraordinarily rare considering that the physiological HR limit in C57BL/6J mice is generally around 800 bpm (75 ms RR interval) (Stiedl et al., 2009). HRV was determined by the root-mean-square of successive R-R interval differences (RMSSD). The HR change (ΔHR) at tone onset was calculated from the mean HR in the first minute of the CS phase minus the mean pre-CS HR. In the tone-dependent memory test (duration 7.5 min) in the home cage HR changes were not affected by increased locomotor activity. We observed only small movements and scanning while locomotor activity generally was absent.

To analyze the data recorded during the circadian cycle, subepochs of 20-min were recorded each hour for a whole day (24 h). Subsequently, 8 of the 24 subepochs were analyzed, i.e., 20 min every 3 h starting at 1:00 am (h: 1, 4, 7, 10, 13, 16, 19, 22) of all mice for comparison. The fear retention data were divided into 15 subepochs of 30-s duration of pre-CS, CS and post-CS phase of 180-s, 180-s and 90-s duration, respectively. Novelty data was analyzed in specific 1-min subepochs of the entire 34-min test.
Enhanced anxiety-like behavior in munc18-1 heterozygous mice

Statistical analyses

Analysis of variance (ANOVA) was used for statistical analysis of baseline, US, post-US activity and body weight, which are presented as mean values ± SEM (StatView 5.0.1, SAS Institute, Cary, NC, USA). Parameters that did not fulfill the required criteria for parametric analysis were analyzed using the Mann-Whitney U-test with the factor genotype (WT and HZ) to determine the statistical differences. The data analyzed by non-parametric methods are shown as box plots with the ends of the boxes denoting the 25% and 75% quartiles and the whiskers indicating the upper and lower quartile ± 1.5 times the interquartile range respectively. The lines in the boxes denote the median. An error probability of P<0.05 was accepted as statistically significant. An error probability of 0.1>P>0.05 indicated a trend. Statistical evaluation of data was performed by analysis of variance (ANOVA) and ANOVA for repeated measures (rmANOVA).

2.4 Results

Diurnal heart rate measures

Linear heart rate: Statistical analysis by rmANOVA of HR during the diurnal cycle (8 different time intervals of the day) as a function of genotype indicated a significant difference with lower HR in HZ than in WT mice (F1,8=17.21, P=0.0032; Fig. 2.1A). However, the comparison of mean HR values during single subepochs using an ANOVA showed no significant differences between genotypes. HR values differed significantly across the different time intervals of the day (F1,8=9.50, P<0.0001). There was no genotype × time interaction (F1,7=0.38, P=0.91). Mean HR during the dark phase was higher in WT than munc18-1 HZ mice (F1,8=-3.19, P=0.013; data not shown). Heart rate variability: HRV did not differ between genotypes in a rmANOVA (F1,8=2.57, P=0.15; data not shown). There was a significant effect of time on HRV (F1,7=3.47, P=0.004; data not shown) with the lowest HRV values at 19 h when HR was the highest (see 3.5.). There was no genotype × time interaction (F1,7=0.93,
2.4 Results

Locomotor activity: Analysis of locomotor activity by rmANOVA during the diurnal cycle (8 different time intervals of the day) as a function of genotype indicated no difference between HZ and WT mice ($F_{1,120}=2.34, P=0.13$; Fig. 2.1B). Locomotor activity differed significantly across the different time intervals of the day ($F_{1,7}=127.13, P<0.0001$) with the highest values at 19 h. There was no genotype × time interaction ($F_{1,7}=0.52, P=0.81$). The high level of locomotor activity contributes to the elevated HR values in the first dark phase interval (19 h) which concomitantly resulted in reduced HRV.

Non-linear heart rate dynamics: The scaling coefficient $\alpha$ did not differ between genotypes ($F_{1,8}=0.46, P=0.52$; Fig. 2.1C) despite the HR differences observed across the diurnal measurements. There was no significant time effect ($F_{1,7}=1.06, P=0.40$) and no genotype × time interaction ($F_{1,7}=0.55, P=0.79$).

Novelty exposure

There was no difference in activity between munc18-1 HZ and WT mice (rmANOVA: $F_{1,20}=0.22, P=0.64$; Fig. 2.2A) during novelty exposure. However, novelty exposure resulted in a trend for higher HR ($F_{1,22}=3.58, P=0.072$; Fig. 2.2A) in HZ mice (~790 bpm) compared to WT mice (~770 bpm) as determined by rmANOVA. The comparison of HR values at individual 1-min intervals with higher resolution from 1-5 min as a function of genotype revealed significant differences up to 10 min after the start of the experiment (Fig. 2.2B). Thereafter, the HR difference was maintained but did not reach statistical significance because of increased HRV during the slow recovery of HR towards baseline values with increased parasympathetic tone and decreasing sympathetic tone.

Heart rate responses after auditory delay conditioning

After delay conditioning baseline HR in the pre-CS phase of the first retention test did not differ significantly between the genotypes ($F_{1,12}$=0.003,
Enhanced anxiety-like behavior in *munc18-1* heterozygous mice

**Figure 2.1:** Similar diurnal heart rate (HR) dynamics and activity of *munc18-1* heterozygous (HZ) and wildtype (WT) mice. Mean HR values (A), and diurnal locomotor activity throughout the 24-h test period (B). Scaling coefficients a based on detrended fluctuation analysis of 20-min subepochs on the corresponding times of the day (C). Light was on from 6:00 AM to 6:00 PM. The grey background denotes the dark phase. Error bars show SEM.
Figure 2.2: Locomotor activity and concomitant heart rate (HR) during the 34-min novelty test in 1-min intervals. Locomotor activity in units of distance over time of munc18-1 heterozygous (HZ) and wild-type (WT) mice during novelty exposure for 34 min (A). Heart rate values in units of beats per min (bpm) of HZ and WT mice during the same exposure to novelty (B). Both panels show mean values of representative 1-min intervals; error bars show SEM; *P<0.05; **P<0.01; n=13-17/genotype.
Enhanced anxiety-like behavior in *munc18-1* heterozygous mice

P=0.96; Fig. 2.3A). HZ mice responded with a significantly higher HR than WT mice in the CS phase (F1,12=15.08, P=0.0019; Fig. 3A). In the post-CS phase HR again did not differ between genotypes (F1,12=0.006, P=0.94; Fig. 2.3A). There was no difference in HRV between the two genotypes (F1,12=0.02, P=0.90; data not shown). The tone-induced HR increase from baseline HR (ΔHR) was significantly higher in HZ than that in WT mice (F1,10= 8.10, P<0.05; Fig. 2.3B). ΔHR decreased significantly over the five consecutive retention test days indicating a reduced autonomic change in response to the CS (ΔHR day 1 vs. ΔHR day 5: F1,12=29.38, P<0.01; Fig. 2.3B).

Twenty-four hours after the retention test of day 5, the mice were retrained and another 24 h later a final retention test was performed. Again, there was no difference in the HR responses of the two genotypes (F1,11=4.17, P=0.06; Fig. 2.3B). However, in both genotypes the tone-elicited tachycardia showed no significant difference compared to that of the first retention test (ΔHR day 1 vs. ΔHR day 6: F1,12=0.51, P=0.49; Fig. 3B), but was significantly higher than that of the previous retention test (ΔHR day 5 vs. ΔHR day 6: F1,12=13.19, P=0.003; Fig. 2.3B).

**HR/HRV correlation**

The relationship between HR (RR-intervals) and HRV (RMSSD) was analyzed on the basis of 15 30-s intervals of the first tone-dependent retention test of all mice subjected to either delay or trace fear conditioning to cover a wide dynamical range from baseline to maximum HR. The correlation between the RR intervals and the RMSSD indicated significant linear relations (P<0.0001) in both genotypes, i.e., decreased HRV when HR increased. The steepness of slope of the linear regression of the linear correlations (WT: r= 0.70; HZ: r=0.76) was similar in both genotypes (Fig. 2.4).
2.4 Results

Figure 2.3: Heart rate (HR) responses during retention of fear to an auditory cue after delay and trace fear conditioning and its extinction. HR response of munc18-1 heterozygous (HZ) and wild-type (WT) mice during the first retention test after auditory delay fear conditioning (A). Conditioned tone-induced ΔHR of HZ mice and WT controls during five consecutive retention tests after trace auditory fear conditioning (B). HR pattern of HZ and WT mice during the first retention test after auditory trace fear conditioning (C) and conditioned tone-induced ΔHR of HZ mice and WT controls during five consecutive retention tests after auditory trace fear conditioning (D). The vertical dashed lines in A and B denote the on- and offset of the tone-CS that was presented for 180 s. Data points (A, B) show mean HR of 30-s intervals. ΔHR indicates the CS-induced HR increase from baseline values. RT refers to the retention test 24 h after retraining on day 9. Error bars show SEM; *P<0.05; **P<0.01; ***P<0.001; n=7-9/genotype.
Enhanced anxiety-like behavior in \textit{munc18-1} heterozygous mice

\textbf{Figure 2.4}: Similar correlation between heart rate (HR, additionally shown as RR interval) and its variability (HRV) in \textit{munc18-1} heterozygous (HZ) and wild-type (WT) mice based on 15 30-s intervals of the tone dependent retention test performed 24 h after training (day 1). HRV was determined by the RMSSD measure. Linear regression WT: \( Y = -18.426 + 0.243 \times X \); linear regression HZ: \( Y = -14.313 + 0.204 \times X \). Data points: \( n=195/255 \) for 13 HZ and 17 WT mice, respectively.
2.5 Discussion

HR/HRV correlation

Deletion of one allele of the gene encoding the protein Munc18-1 did not affect neural transmission of the autonomic nervous system that would indicate altered HR dynamics under baseline stressfree conditions during the diurnal cycle by linear and nonlinear methods in freely moving mice. Consequently, the enhanced HR responses during retention of conditioned fear to an auditory cue and to novelty as unconditioned emotional challenge are indicative of increased fear and anxiety in HZ mice. These findings complement and extend the interpretation of recent results of the behavioral characterization of \textit{munc18-1} HZ mice in fear learning tests (Maroteaux et al., prep) as summarized in Table 1. They support the explanation of altered coping style (expression) rather than cognitive impairment (encoding, consolidation and retrieval) underlying reduced freezing and transfer latencies of HZ versus WT mice in fear conditioning and passive avoidance, respectively.

From all linear analyses, only mean HR showed a significant diurnal difference between genotypes. We observed a clear diurnal difference in male backcrossed to C57BL/6JCrI mice that we did not observe in males of the C57BL/6JRj substrain (Centre D’Elevage Janvier) in 2003 (Stiedl and Meyer, 2003a,b). HR and HRV of HZ mice were similar to that of WT mice in the pre-CS phase of the retention tests. The lack of genotype-related differences in the diurnal measurements was confirmed by similar pre-CS HR. However, \textit{munc18-1} HZ mice showed a trend towards lower HR during the diurnal cycle than WT mice despite their lower body weight.

The scaling coefficients calculated across the diurnal phase did not differ between the two genotypes. This indicates absent genotype-dependent differences in autonomic nervous system function impacting on HR dynamics. HR dynamics determined by DFA did not change under baseline stress-free conditions in the home cage within the observed range diurnal range as reported before (Stiedl and Meyer, 2003b) since maximum HR remained relatively low (~700 bpm) and did rise only as consequence of physiological support of be-
Enhanced anxiety-like behavior in *munc18-1* heterozygous mice

havior (locomotion) (Koolhaas et al., 2011) without any emotional contribution. In humans the scaling coefficients drop from ~1.0 to ~0.86 during sleep due to enhanced parasympathetic activity (Meyer, 2002). Since mice have relatively high metabolic needs to maintain their body temperature, the dynamical state during sleep shows only minor changes (Stiedl and Meyer, 2003a). This may be expected in singly housed mice only close to the thermoneutral zone (28-32°C) (Lodhi and Semenkovich, 2009) but not at room temperature (~21°C).

Despite the reported difference in neural transmission in vitro (Toonen et al., 2006) all transmitter systems appear to be similarly affected in vivo with regard to inhibitory versus excitatory transmission, providing for an unaffected homeodynamic state (Meyer and Stiedl, 2006; Peng et al., 1994). Thereby, the autonomic function is kept in its physiological range without genotypic difference. It remains to be clarified, whether the neural activities in vivo differ between genotypes and whether feedback mechanisms are involved in masking a potential functional difference. Considering the before-mentioned distinction of cardiovascular regulation between basic function and emotional adjustment by environmental stimuli, the results of this study combined with previous findings (Maroteaux et al., prep) clearly indicate an enhanced emotional responsiveness in *munc18-1* HZ mice on the basis of HR adjustments.

**Novelty exposure**

Novelty exposure did not result in activity differences between genotypes, but resulted in a higher HR in HZ mice than in WT controls. The difference in HR (~20 bpm) was significant due to the reduced HRV at such high HR (Toyote et al., 2004), supporting a stronger autonomic activation as index of increased anxiety. However, ~20 bpm is -in terms of biological relevance- relatively small with respect to maximum HR of ~800 bpm, and signifies only a 2.5% difference. Nevertheless, this finding shows that it is insufficient to monitor locomotor activity alone, as differences in HR and HRV may emerge in the absence of activity differences.
Table 2.1: Overview of behavioral and autonomic fear responses of wild-type and \textit{munc18-1} heterozygous mice in fear retention tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>Measure / Exp. setup</th>
<th>Result / Conclusion</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contextual fear conditioning</td>
<td>Freezing &amp; activity in FC box</td>
<td>Reduced fear in HZ mice</td>
<td>(Maroteaux, \textit{in prep.})</td>
</tr>
<tr>
<td>New context performance (no tone)</td>
<td>Freezing &amp; activity in new environment</td>
<td>Similar, no fear generalization</td>
<td>(Maroteaux, \textit{in prep.})</td>
</tr>
<tr>
<td>Auditory delay fear conditioning</td>
<td>Freezing &amp; activity in new environment</td>
<td>Reduced fear in HZ mice</td>
<td>(Maroteaux, \textit{in prep.})</td>
</tr>
<tr>
<td>Passive avoidance</td>
<td>Transfer latencies to enter DC in PA box</td>
<td>Reduced fear in HZ mice</td>
<td>(Maroteaux, \textit{in prep.})</td>
</tr>
<tr>
<td>Passive avoidance</td>
<td>Transfer latencies to enter DC in PA box</td>
<td>Faster fear extinction in HZ mice</td>
<td>(Maroteaux, \textit{in prep.})</td>
</tr>
<tr>
<td>Auditory delay fear conditioning</td>
<td>Heart rate in the home cage</td>
<td>Higher fear response in HZ mice</td>
<td>Fig. 2.3A</td>
</tr>
<tr>
<td>Auditory delay fear conditioning</td>
<td>Heart rate in the home cage</td>
<td>Delayed fear extinction in HZ mice</td>
<td>Fig. 2.3B</td>
</tr>
<tr>
<td>Auditory (30-s) trace fear conditioning</td>
<td>Heart rate in the home cage</td>
<td>Higher fear response in HZ mice</td>
<td>Fig. 2.3C</td>
</tr>
<tr>
<td>Auditory (30-s) trace fear conditioning</td>
<td>Heart rate in the home cage</td>
<td>Slower fear extinction in HZ mice</td>
<td>Fig. 2.3D</td>
</tr>
</tbody>
</table>
Enhanced anxiety-like behavior in munc18-1 heterozygous mice

**Fear conditioning**

During trace and delay fear conditioning training HZ did not differ from WT mice in tone-induced activity changes (data not shown). Additionally, as clear tone-induced HR increases occurred in both genotypes, a fear association between the CS and the US in both genotypes is demonstrated. However, the HR response to the tone was higher (~40 bpm) and remained elevated for a longer period (~180 s) in HZ than in WT mice after both conditioning procedures (trace and delay). Subsequently, this resulted in a delayed return to baseline HR in HZ mice. These observations indicate a stronger and longer-lasting fear response of HZ than of WT mice. The HRV data were generally consistent with this result. HRV (RMSSD) decreased profoundly after tone onset, and recovered gradually as HR returned to baseline. This is generally expected since HRV is inversely related to HR (Tovote et al., 2004).

Along successive retention tests the tone-induce HR increase ($\Delta$HR) was generally higher in HZ than in WT mice. In trace conditioning this difference was significant from day 1 to day 5. While in delay fear conditioning the higher $\Delta$HR in HZ mice does not show a consistent significant difference. Even though the HZ mice had a higher $\Delta$HR during the retention tests compared to the WT mice, they also showed extinction of conditioned fear as the $\Delta$HR decreased over the consecutive retention tests. Thus, the HZ mice where capable of both tasks (learning as well as extinction of conditioned fear), suggesting that they do not have a cognitive impairment and no spatial learning deficit (Maroteaux et al., prep), but rather seem to be more anxious than the WT mice. Enhanced anxiety may confound memory recall and expression (Diamond et al., 2007; Kim and Diamond, 2002). Twenty-four hours after retraining the genotypes again showed a tone-induced HR increase in both trace and delay fear conditioning that was (i) significantly higher than in the previous retention test (day 5), but (ii) significantly lower than in retention test 1. To the best of our knowledge, the lower HR increase after retaining versus that of day 1 demonstrates for the first time a latent inhibition-like effect on the basis of conditioned HR responses in HZ and WT mice. The increase in $\Delta$HR was higher in mice after delay conditioning than after trace conditioning, consistent with previous re-
sults of stronger HR responses after delay than after trace conditioning (Stiedl and Spiess, 1997; Youn et al., 2013).

The RR-RMSSD correlation of the two genotypes showed a linear inverse relation between the R-R intervals (HR) and the RMSSD (HRV) as reported before (Tovote et al., 2004). The steepness of slope of the linear regression was similar in HZ and WT mice and points at unaffected neuroautonomic balance in HZ and WT mice. This is in full agreement with unaffected nonlinear HR dynamics. The conditioned HR response to the tone under stress-free conditions in the home cage is indicative of a fear rather than an anxiety response. This is concluded because the behavior of *munc18-1* HZ mice parallels the observation of increased anxiety in DBA/2 mice (Lipkind et al., 2004). These mice also show a lower freezing in fear conditioning (Stiedl et al., 1999) and shorter transfer latency in passive avoidance performance compared to C57BL/6J mice (Baarendse et al., 2008). However, DBA/2 mice show a low HR increase to the conditioned auditory cue in the home cage 24 h after auditory delay conditioning, suggesting that these mice did not form a fear association (Stiedl et al., 1999), whereas *munc18-1* HZ mice have a higher HR response than their WT controls. This demonstrates that despite the aversive experience of a foot shock, the auditory cue itself does not trigger a profound tachycardia as consequence of increased anxiety in mice fail to establish a fear association such as DBA/2J mice. Similarly, shock-exposed C57BL/6N mice, which are known to show generalized fear responses in behavior tests (Radulovic et al., 1998; Stiedl et al., 1999), do not respond with a HR increase to the auditory cue that differs from that of non-shocked controls (Stiedl et al., 2009).

Since we compared the behavioral with the HR responses of these mice under different experimental conditions, i.e., with or without human interference during the fear memory tests in specific setups or the home cage (see table 2.1), it is necessary to perform the entire fear learning test without human interference. Therefore, we developed an automated home cage-based behavioral system (Hager et al., 2012) that allows for testing on extended time-scales without any human interference and inclusion of HR measures. The consequences of these experimental conditions on fear learning, expression and extinction
Enhanced anxiety-like behavior in munc18-1 heterozygous mice

in munc18-1 HZ versus WT mice remains to be determined but we would hypothesize a lower/absent difference between the genotypes if handling serves as additional stressor in HZ mice.

In summary, the tone-elicited tachycardia during expression of conditioned fear after both trace and delay fear conditioning was significantly higher in HZ than in WT mice, whereas baseline HR did not differ between genotypes. These results support the concept of an autonomic separation between basal cardiovascular regulation (basic function), which is attributed to hypothalamic and brain stem regions, and fear-mediated modulation (cognitive function), which is mediated through higher brain regions such as the prefrontal cortex, hippocampus and amygdala (Maren, 2011). In response to environmental stimuli such as emotional challenges higher brain regions are important for adjustment of basal functions.

Conclusions

Munc18-1 HZ mice showed enhanced anxiety based on autonomic (HR) measures that complement and strengthen recent behavioral conclusions (Maroteaux et al., prep). The enhanced anxiety of HZ mice appears to negatively affect fear expression leading to panicky behavior (Koolhaas et al., 2007) only at increased emotional load. This state may have lowered both freezing and transfer latencies (Maroteaux et al., prep), the classical measures of conditioned fear and passive avoidance performance, respectively. These results underscore the importance of independent assessment of emotional states of mice based on autonomic function contrasting with classical behavioral fear measures. It provides further evidence for the necessity of thorough phenotyping of animal models on a broad scale (Urbach et al., 2010) including different experimental conditions devoid of unspecific stress. In contrast to the adverse consequences of deletion of a single allele of munc18-1 in humans, the absence of the phenocopy in our mouse model may be attributed to the genetic background, because we initially observed seizures in HZ mice with mixed C57BL/6J × 129S1/SV background (Maroteaux et al., prep). Therefore, C57BL/6J mice are either able to developmentally compensate for the loss of a single munc18-1 allele and/or additional
mutations need to coincide with the single \textit{munc18-1} deletion as multiple hit model as recently shown in an autism model (Leblond et al., 2012) to replicate the human phenotype in this mouse model.

\section*{Acknowledgements}

We are grateful to Matthijs Verhage and Joke Wortel for helpful comments and discussion. Financial support was provided by the VU University Amsterdam and the Neuroscience Campus Amsterdam (O.S.). T.H. was supported as early stage researcher by the European Union Seventh Framework Programs under grant agreements no. PEOPLE-ITN-2008-238055 (BrainTrain) provided to O.S.
Translational relevance of non-linear heart rate dynamics: findings from behavioral and pharmacological interventions in mice for human autonomic dysfunction

Hager, T., Agorastos, A., Meyer, M., Ögren, S., and Stiedl, O. submitted to *Pharmacological Research*
3.1 Abstract

The beat-by-beat fluctuation of heart rate (HR) in its temporal sequence (HR dynamics) provides information on the control of the heart mediated by the autonomic nervous system (ANS) and its dysregulation in pathological states. The main aim of this study was to compare linear and nonlinear HR measures, including the detrended fluctuation analysis (DFA), based on ECG recordings by radio-telemetry in C57BL/6N mice. This comparison was conducted following different behavioral and pharmacological interventions altering ANS control to characterize pathological states. It included administration of various drugs affecting cardiovascular function through different peripheral and/or central mechanisms including activation of receptors implicated in human psychopathologies. Thereafter, the comparison of mouse and human DFA measures was used to assessment its translational value. Under physiological conditions HR dynamics constitute a self-similar, scale-invariant, fractal process with persistent intrinsic long-range correlations resulting in physiological DFA scaling coefficients of $\alpha \approx 1$. Altered DFA scaling coefficients ($\alpha \neq 1$) indicated compromised HR dynamics as pathological condition. This was mediated by parasympathetic blockade and parasympathetic overactivation, underscoring the importance of the vagal system. Sympathetic overactivation but not inhibition compromised HR dynamics. The DFA scaling coefficients ($\alpha \approx 1$) were similar in mice and humans under comparable physiological and pathological conditions. Functional denervation by human heart transplantation resulted in HR dynamics that mimicked that of mice with parasympathetic blockade underscoring the importance of tonic vagal control on physiological HR dynamics in these two species. DFA provides an important translational measure that reliably identifies pathological HR dynamics unrecognized by linear measures such as increased HR variability.
3.2 Introduction

The beat-by-beat fluctuation of heart rate (HR) is regulated by the modulation of the myogenic pacemaker systems of the heart through the parasympathetic (PNS) and the sympathetic nervous system (SNS), which constitute the autonomic nervous system (ANS). Both systems are generally interdependent to maintain blood flow in the body with proper blood pressure to support physiological and metabolic demands ranging from posture changes via physical activity to emotional challenges. Thus, internal and external sensors affect the regulation through feedback systems acting on different time scales from fast baroreflex feedback to slow endocrine changes in the complex cardiovascular regulatory system (Guyton et al., 1972).

The high comorbidity of affective disorders and cardiovascular disease, i.e. particularly the elevated risk of cardiovascular failure after emotionally challenging events (Steptoe and Brydon, 2008), indicates a crucial role of stress-induced adjustments by the sympathetic-adrenal medullary system (Kvetnansky et al., 2009). The fear circuitry largely overlaps with the central autonomic network (Ter Horst et al., 1996). Even insular cortex forebrain stimulation results in arrhythmogenesis through altered central ANS function (Oppenheimer et al., 1991). Mutations in the human KCNQ1 gene, which encodes a cardiac and forebrain-specific delayed rectifying potassium channel, link epileptic seizures and arrhythmias with sudden unexplained (cardiac) death. This finding indicates the dual arrhythmogenic potential of an ion channelopathy through increased neuronal excitability in the brain and prolonged QT syndrome in the heart (Goldman et al., 2009).

Clinically, reduced HR variability as index of reduced regulatory capacity is considered an increased cardiovascular risk factor (Shaffer et al., 2014) based on diagnostic measures derived from the time or the frequency domain (Camm et al., 1996). However, two functional properties of HR dynamics, non-stationarity and interdependence, formally prohibit the use of linear analysis (Meyer and Stiedl, 2003). Non-stationarity is the drift-like behavior of HR and interdependence is the correlation of heartbeat intervals in its temporal
sequence under physiological conditions. Nonlinear measures provide useful information on the dynamical state with superior discrimination of physiological versus pathological changes (Youn et al., 2013). The clinical significance of nonlinear (fractal) analysis of HR dynamics in humans for the assessment of cardiovascular risk has been demonstrated in a number of studies (Goldberger et al., 2002; Meyer and Stiedl, 2003). Various nonlinear dimensionless measures of the dynamical properties of HR may serve as valuable diagnostic tool (Vandendriessche et al., 2014). Despite freely available software modules for nonlinear analyses (see www.physionet.org) its use is quite limited largely due to its complexity (Aubert et al., 2009). To date, a nonlinear characterization of HR dynamics following treatment with different pharmacological substances commonly used in cardiovascular research is partially available only in the rat (Beckers et al., 2006).

The aims of this study were firstly to compare linear (HR and its variability) and nonlinear HR measures (detrended fluctuation analysis; Peng et al. (1995)) after various behavioral and pharmacological interventions. This included drugs acting at central receptors that are implicated in affective disorders, e.g., depression and post-traumatic stress disorder, since they show high comorbidity with cardiovascular risk. Euclidian clustering was used to identify similarities and differences across interventions based on linear and nonlinear measures. Secondly, the translational relevance of nonlinear measures was assessed by comparing the HR measures obtained from mice with those from humans studied under different conditions. This included pathological states such as heart transplantation, in order to identify similar or even identical functional properties with that elicited by pharmacological interventions in mice.

### 3.3 Material and Methods

**Subjects**

The experiments were performed with a total of 176 male C57BL/6N mice (Charles River, Germany and The Netherlands) obtained at an age of 8 weeks. They were individually housed in standard type II Macrolon cages with free
access to food and water and were kept on a 12-h darklight cycle with lights switched on at 7 a.m. Mice were 11–13 weeks of age at the time of testing, performed during the light phase to minimize the effects of physical activity. All animal experiments were ethically approved by local ethics committees and performed in accordance with the European Council Directive (86/609/EEC) and are in accordance with the ARRIVE guidelines (Kilkenny et al., 2010). Human ECG data were obtained from ethically approved health monitoring of patients in the clinics.

**ECG surgery, recording, and processing**

ECG signals of mice were recorded by radio-telemetry using miniature ECG radio-transmitters (TA10EA-F20 and ETA-F10, Data Sciences, St. Paul, MN, USA) implanted into the abdominal cavity of mice with the ECG electrodes placed subcutaneously in lead II position as described before (Hager et al., 2014b; Stiedl and Spiess, 1997). Experiments were performed 14-21 days after surgery, when mice were fully recovered. All ECG recordings lasted for 18 min providing $\sim 10^4$ beats/mouse under physiological conditions.

The ECG signal emitted by the radio-transmitter was detected by a receiver (RLA1020, Data Sciences) and converted to an analog signal (ECG Output Adapter Option RO8, Data Sciences). This signal was digitally recorded (LabChart 7.1, PowerLab, ADInstruments, Spechbach, Germany) at 4 kHz sampling rate and stored. The digitized ECG was analyzed offline (HRV 1.4 for LabChart, ADInstruments) to obtain discrete time points corresponding to the successive R-wave maxima. Ectopic (bradycardic) beats, typically 1 in $10^4$ beats, were fitted by a third-order autoregressive model to the beat interval data stream using multiples of the interquartile distance as detection threshold and replaced by linear-spline interpolation (Meyer and Stiedl, 2006). This is important since incorrect RR intervals including ectopic beats impair the quality of nonlinear detrended fluctuation analysis (DFA) (Tarkiainen et al., 2007). The need of thorough (and timeconsuming) data pre-processing as recommended by the HRV Task Force (Camm et al., 1996) is a fundamental prerequisite prior to final data analysis. Lack of compliance with these standards will nega-
Translational relevance of non-linear heart rate dynamics

tively affect the quality of results or may not provide the necessary data quality for non-linear analyses. Short-term ECG recordings (15-40 min) in humans were acquired at a sampling rate of 1200 Hz by a dual-channel miniature ECG recorder-amplifier system (Meyer, 2002). Human HR data were processed as described for mice.

HR (in beats per min; bpm) and the standard deviation of the NN intervals (SDNN; in ms) served as linear measures. DFA was performed as previously described (Stiedl and Meyer, 2003b) with the scaling value $\alpha$ theoretically ranging from absent correlations, i.e. white noise with $\alpha=0.5$, via long-term correlations, i.e. 1/f-noise with $\alpha \approx 1.0$, to short-term correlation, i.e. Brownian noise with $\alpha=1.5$.

**Drugs and administration**

All drugs and dosages used are provided in table 3.3. These drugs, their dosages and administration routes were selected on the basis of previous studies (Stiedl et al., 2005; Youn et al., 2013). All drugs were freshly dissolved on the day of use. They were injected subcutaneously (sc) into the scruff of the neck or intracerebroventricularly (icv) into the lateral ventricles using bilateral symmetrical brain cannula implanted 5 days prior to the experiment (Stiedl et al., 2005; Tovote et al., 2004). Central drug injection was necessary because the neuropeptides neuropeptide Y (NPY; 36 amino acids) and ovine corticotropin-releasing factor (oCRF; 41 amino acids) do not cross the blood-brain barrier. Drug administration was performed during a brief ~30- 90-s isoflurane anesthesia period as used by us in many studies before (Stiedl et al., 2005; Tovote et al., 2004; Youn et al., 2013). This brief anesthesia is necessary in mice to avoid the unspecific ‘stress’ of the restraining procedure required for drug injection that leads to tachycardia and hyperthermia in the awake state (Olivier et al., 2003). During anesthesia ECG was measured in the narcotized state on a heat pad at 37°C to avoid hypothermia, which will additionally alter ANS control thereby affecting the dynamical properties (Mertens et al., 2008).
Experimental conditions

Experiments were performed in the home cage of mice, except when the effects of anesthesia, novelty and restraint stress were tested. Novelty measurements were performed in a novel cage into which mice were transferred (Stiedl et al., 2004). For restraint stress tests, mice were immobilized, i.e., fixed with the ventral side up on the ECG receiver. Sleep was determined on the basis of minimal and only transient ECG amplitude changes during the 18-min ECG recording (Stiedl and Meyer, 2003a) during the middle of the light phase in the home cage mice when activity of C57BL/6N mice is the lowest (Hager et al., 2014a). Only mice that provided ECG data as controls, during novelty, restraint and sleep were subsequently tested in a single pharmacological drug to avoid sensitization and other potential interactions with ECG measures through repeated drug treatment.
## Table 3.1: Overview of behavioral and pharmacological interventions used to assess heart rate dynamics in mice.¹

<table>
<thead>
<tr>
<th>Condition / Drug</th>
<th>Drug action / function</th>
<th>Dose</th>
<th>Injection</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Baseline (incl. saline &amp; aCSF)</td>
<td>/</td>
<td>ip, sc, icv¹</td>
<td>23</td>
</tr>
<tr>
<td>Novelty</td>
<td>Behavioral stressor</td>
<td>/</td>
<td>/</td>
<td>8</td>
</tr>
<tr>
<td>Restraint</td>
<td>Behavioral stressor</td>
<td>/</td>
<td>/</td>
<td>10</td>
</tr>
<tr>
<td>Sleep</td>
<td>Lowest resting state condition</td>
<td>/</td>
<td>/</td>
<td>11</td>
</tr>
<tr>
<td>6-OH-Dopamine</td>
<td>noradrenergic &amp; dopaminergic neurotoxin: peripheral sympathectomy</td>
<td>200 mg/kg*</td>
<td>ip</td>
<td>17</td>
</tr>
<tr>
<td>Anesthesia</td>
<td>ketamine/xyalazine (NMDA receptor antagonist/α₂ receptor agonist)</td>
<td>130/13 mg/kg</td>
<td>ip</td>
<td>11</td>
</tr>
<tr>
<td>Atropine</td>
<td>mACH receptor antagonist</td>
<td>2 mg/kg</td>
<td>ip</td>
<td>13</td>
</tr>
<tr>
<td>Dobutamine</td>
<td>β₁ receptor agonist</td>
<td>15 mg/kg</td>
<td>ip</td>
<td>10</td>
</tr>
<tr>
<td>DSP-4</td>
<td>noradrenergic neurotoxin</td>
<td>100 mg/kg</td>
<td>sc</td>
<td>6</td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>nACH receptor antagonist</td>
<td>15 mg/kg</td>
<td>ip</td>
<td>10</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>α receptor agonist</td>
<td>3 mg/kg</td>
<td>ip</td>
<td>13</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>α₁ receptor agonist (hypertensive)</td>
<td>15 mg/kg</td>
<td>ip</td>
<td>8</td>
</tr>
<tr>
<td>Robinul (glycopyrrolate)</td>
<td>peripheral mACH receptor antagonist</td>
<td>0.8 mg/kg</td>
<td>ip</td>
<td>12</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>vasodilator (antihypertensive)</td>
<td>0.18 mg/kg</td>
<td>ip</td>
<td>12</td>
</tr>
</tbody>
</table>

Continued on next page
<table>
<thead>
<tr>
<th>Condition / Drug</th>
<th>Drug action / function</th>
<th>Dose</th>
<th>Injection</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sotalol</td>
<td>peripheral $\beta$ receptor antagonist</td>
<td>2 mg/kg</td>
<td>ip</td>
<td>12</td>
</tr>
<tr>
<td>Sotalol + Atropine</td>
<td>$\beta$ and mACh receptor antagonists (both)</td>
<td>2 mg/kg</td>
<td>ip</td>
<td>11</td>
</tr>
<tr>
<td>Zatebradine</td>
<td>Sinus node (HCN channel) inhibitor</td>
<td>2 mg/kg</td>
<td>ip</td>
<td>11</td>
</tr>
<tr>
<td>ovine CRF</td>
<td>preferential CRF$_1$ receptor agonist</td>
<td>210 ng/mouse</td>
<td>icv</td>
<td>12</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>NPY$_{1-5}$ receptor agonist</td>
<td>500 ng/mouse</td>
<td>icv</td>
<td>10</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>5-HT$<em>{1A}$/5-HT$</em>{7}$ receptor agonist</td>
<td>0.5 mg/kg</td>
<td>sc</td>
<td>8</td>
</tr>
</tbody>
</table>

1 pooled data from all injection sites; *tested 7 days after the third 6-OH-dopamine injection of 40/80/80 mg/kg given at 24-h intervals; $^4$tested 10 days after the second DSP-4 injection of 50 mg/kg given at 24-h interval
Translational relevance of non-linear heart rate dynamics

Human data

To compare HR dynamics of mice with that of humans, we included human
HR data from previous experiments (Meyer, 2002). Based on DFA analyses,
the nonlinear properties underlying HR dynamics were directly comparable in
these two species. Data was included from humans with heart transplantation,
during sleep, with congestive heart failure and from different postures and di-
urnal phases.

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) or by Welch ANOVA
in case of inhomogeneity of group variances of data as determined by Lev-
ene’s statistic (JMP 5.0.1a and StatView 5.0.1, SAS Institute, Cary, NC, USA).
An error probability of P<0.05 was generally accepted as statistically signifi-
cant. Due to many comparisons, the P-values were corrected by the minimum
positive false discovery rate following a previously reported procedure (Verho-
even et al., 2005). The threshold was set at 5% to correct for the inflated risk
of type I errors. Hierarchical clustering dendrograms were plotted based on
the Euclidean distance of either the individual linear measures HR and SDNN
(weighed 1:1) or the nonlinear measures $\alpha_{fast}$ and $\alpha_{slow}$ (weighed 1:1), to deter-
mine similarities of functional consequences of different pharmacological and
behavioral interventions.

3.4 Results

Effects of behavioral states and pharmacological interventions on linear heart rate measures in mice

HR (F$_{19,69}$=115.82; P<0.0001) and SDNN values (F$_{19,68}$=44.04; P<0.0001) in-
dicated highly significant differences between experimental groups (Fig. 3.1A,
B). Based on the two values, cluster analysis was performed to determine the
similarity of treatment effects on HR and SDNN based on Euclidian dendro-
gram with identical weight for both measures (Fig. 3.1C).
3.4 Results

**Figure 3.1:** Linear measures of heart rate (A) and SDNN (B) as a function of treatment according to their Euclidian clustering (C). Clustering is based on the two parameters (A, B) weighed with equal contribution. Blue values and labels indicate behavioral interventions. HR (A) and SDNN values (B) indicate the generally inverse relation between these two measures. Values are based on 18-min ECG recordings in mice. Asterisks denote significant differences versus control group values: *P<0.05, **P<0.01, ***P<0.001.
Under physiological conditions in the home cage (awake, no physical activity) mean HR of mice was \(~570\) bpm and an SDNN of \(~560\) (Fig. 1A, B). Novelty exposure resulted in HR increase to maximum physiological levels (\(~790\) bpm) and concomitantly decreased SDNN (\(~2.4\) ms). Restraint stress led to a lower absolute HR (\(~730\) bpm) with slightly higher SDNN (\(~3.3\) ms). Isoproterenol, atropine and robinul increased HR and concomitantly decreased SDNN compared to control group values (Fig. 3.1A, B).

Many drug treatments (e.g., nitroprusside, dobutamine, DSP-4, 6-OHDA, sotalol and atropine, sotalol; see Tab. 3.3) did not significantly affect linear HR measures or had only mild effects (Fig. 1A, B). In contrast, zatebradine, oCRF, phenylephrine, sleep, 8-OH-DPAT, hexamethonium and anesthesia generally decreased HR and increased HR variability (Fig. 3.1A, B). The most extreme bradycardia was elicited by anesthesia (mean HR \(~200\) bpm).

Overall, the different treatments resulted in a wide range of HR values from very high (novelty) to very low (anesthesia) with SDNN values that generally were inversely related to HR (Fig. 3.1A, B). An exception was the very low SDNN value by NPY (\(~6.8\) ms). Phenylephrine (\(~39.4\) ms) 8-OH-DPAT (\(~28.7\) ms) and anesthesia (\(~25.4\) ms) substantially increased SDNN values that were higher than that observed during sleep (\(~14.9\) ms).

The cluster analysis of the linear measures (Fig. 3.1C) ranked most treatments from novelty exposure to sotalol in one subgroup and provided a second group ranging from anesthesia, hexamethonium, 8-OH-DPAT, NPY, phenylephrine, ovine CRF to zatebradine. The first group shows HR values in upper range with low HR variability and included the baseline values of the control group. The second group contains interventions resulting in lower HR values with generally increased HR variability.

**Effects of behavioral states and pharmacological interventions on nonlinear heart rate measures in mice**

The \(\alpha_{fast}\) values (F\(_{19,69}=64.87; P<0.0001\)) and the \(\alpha_{slow}\) values (F\(_{19,68}=8.43; P<0.0001\)) indicated highly significant differences between experimental groups (Fig. 3.2A, B). Based on the two values, cluster analysis was performed to
3.4 Results

determine the similarity of treatment effects on $\alpha_{fast}$ and $\alpha_{slow}$ based on Euclidean dendrogram with identical weight for both measures (Fig. 3.2C). Under normal physiological conditions in the home cage the scaling coefficients $\alpha_{fast}/\alpha_{slow}$ were close to 1 (Fig. 3.2A,B). The scaling coefficient was shifted towards $\alpha_{fast}=1.5$ by robinul and atropine (± sotalol) indicating shift from long-term to short-term correlation (Brownian noise). On the other side, hexamethonium, anesthesia, and isoproterenol shifted the dynamical properties towards a random pattern (white noise) with $\alpha_{fast}=0.5$.

Novelty exposure resulted in reduced long-range correlation of $\alpha_{slow} \sim 0.83$ (Fig. 3.2). Restraint stress led to a lower shift of DFA values from physiological values (Fig. 3.2). The atropine-induced shift of $\alpha_{fast}=1.5$ is indicative of lack of parasympathetic cardiac control. The $\beta_{1/2}$ agonist isoproterenol induces tachycardia along with a breakdown of short-range ($\alpha_{fast}=0.68$) and longrange correlations ($\alpha_{slow}=0.75$) due to combined sympathetic and concomitant parasympathetic (baroreflex) activation (enhanced sympathovagal antagonism) with a dominant sympathetic activation.

Nonlinear cardiovascular measures indicated a different picture of effects. DFA was shifted towards $\alpha_{fast}=1.5$ by robinul, atropine, and sotalol and atropine, which all block the vagal system. Many interventions, from DSP-4 to novelty, did not alter $\alpha_{fast}$ in comparison to that of the control group. Ovine CRF, zatebradine and restraint stress lowered $\alpha_{fast}<1.0$. Phenylephrine and isoproterenol resulted in a drop of $\alpha_{fast} \sim 0.8$. Anesthesia and hexamethonium produced a further drop of $\alpha_{fast} \sim 0.5$. This pattern was complemented by elevated $\alpha_{slow} \sim 1.2$ by robinul, a significant drop of $\alpha_{slow} \sim 0.8$ by 8-OH-DPAT, oCRF and isoproterenol. In contrast, anesthesia resulted in an extremely amplified range of $\alpha_{slow}$ from 0.8-1.2 and hexamethonium increased $\alpha_{slow} \sim 1.4$.

Comparison of the Euclidian clustering (Fig. 3.3) indicated substantial differences between linear (NN and SDNN) and nonlinear measures ($\alpha_{fast}$ and $\alpha_{slow}$) with the conclusions on pathological HR dynamics often available on the basis of linear measures, e.g., as indicated for isoproterenol.
Figure 3.2: Nonlinear DFA measures $\alpha_{\text{fast}}$ (A) and $\alpha_{\text{slow}}$ (B) as a function of treatment according to their Euclidian clustering (C). Clustering is based on the two parameters (A, B) weighed with equal contribution. Blue values and labels indicate behavioral interventions. Partial (yellow boxes) to complete (red boxes) decoupling of physiological regulatory systems (autonomic nervous control) occurred irrespective of mean heart rate. Values are based on 18-min ECG recordings in mice. Asterisks denote significant differences versus control group values: *P<0.05, **P<0.01, ***P<0.001.
Figure 3.3: Comparison of the Euclidian clustering based on linear (NN and SDNN from Fig. 3.1C) and nonlinear DFA measures ($\alpha_{fast}$ and $\alpha_{slow}$ from Fig. 2C) with an indication of the alignment of the measures according to sympathetic (SNS) and parasympathetic nervous system (PNS) tone as indicated by arrows (↑,↓) and its positional differences that are largest as indicated by red lines between the two dendrograms. The abbreviations of drug treatments and behavioral interventions (blue font) follow that shown in Figures 3.1 and 3.2.
Translational relevance of non-linear heart rate dynamics

Heart rate dynamics in humans

Physiological HR dynamics in humans show DFA values close to 1 (Tab. 3.2) irrespective of the activity. When analyzing nighttime intervals, a drop in the scaling coefficient becomes obvious during sleep ($\alpha=0.86$), whereas the daytime periods are more similar to the overall 24-h values. Furthermore, the scaling coefficient was lower in children ($\alpha=0.75$) than in adults. Pathological HR dynamics was identified in congestive heart failure patients. The group-averaged DFA exponent $\alpha=1.24$ differed significantly from $\alpha \sim 1$ as determined from normal healthy subjects (Tab. 3.2).

<table>
<thead>
<tr>
<th>Condition/Treatment</th>
<th>n</th>
<th>DFA $\alpha \pm$ SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy adults (unsteady state)</td>
<td>17</td>
<td>1.01 $\pm$ 0.11</td>
</tr>
<tr>
<td>Healthy adults (steady state, 24 h)</td>
<td>14</td>
<td>0.99 $\pm$ 0.07</td>
</tr>
<tr>
<td>Healthy adults (unsteady state, 24 h)</td>
<td>9</td>
<td>0.99 $\pm$ 0.04</td>
</tr>
<tr>
<td>Healthy adults (4 h day time)</td>
<td>9</td>
<td>0.96 $\pm$ 0.05</td>
</tr>
<tr>
<td>Healthy adults (4 h night time)</td>
<td>9</td>
<td>0.86 $\pm$ 0.04</td>
</tr>
<tr>
<td>Healthy children</td>
<td>8</td>
<td>0.75 $\pm$ 0.08</td>
</tr>
<tr>
<td>Congestive heart failure (unsteady state)</td>
<td>20</td>
<td>1.24 $\pm$ 0.16</td>
</tr>
<tr>
<td>Heart transplantation (steady state, supine):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2 years after transplantation</td>
<td>13</td>
<td>1.48 $\pm$ 0.11</td>
</tr>
<tr>
<td>&gt; 2 years after transplantation</td>
<td>17</td>
<td>1.02 $\pm$ 0.16</td>
</tr>
</tbody>
</table>

A straightforward approach addressing the significance of autonomic cardiac control for the fractal dynamics of HR is facilitated by studies in recipients of a cardiac transplant. The DFA value $\alpha=1.5$ in heart transplant recipients less than 2 years after transplantation indicate that the denervated human heart exhibited dynamics paralleling Brownian noise ($\alpha \sim 1.5$), i.e., short-term correlation. In contrast, DFA values approached $\alpha \sim 1.1$ more than 2 years after transplantation. To potentially identify of patients at high risk for lethal outcome in the absence of ECG waveform alterations DFA was applied to ECG data of a group of 323 patients admitted to the hospital for the evaluation of acute chest pain. In DFA plots based on 15-min ECG recordings two distinct regions with different slopes were observed separated at a breakpoint (Fig. 3.4). Out of a
total of 323 patients 13 died for cardiac reasons within a period of 3 months. Furthermore, 9 patients, on admission to intensive care, were identified as high-risk candidates for cardiac death on the basis of highly abnormal HR dynamics with scaling properties approaching white noise ($\alpha=0.5$) or Brownian noise ($\alpha=1.5$).

Figure 3.4: Heartbeat interval time series (left panels) and DFA scaling coefficients (right panels) of five patients evaluated for cardiac infarction due to acute chest pain that were identified as high-risk patients for cardiac death based on the strong deviation of DFA coefficients for $\alpha_{\text{slow}}$ and/or $\alpha_{\text{fast}}$ from the physiological value of $\sim1$. Three patients (b113, b141 and b159) were with admitted to the intensive care unit (ICU). Two patients (b100 and b137) were sent home with telemetric ECG recording, but in retrospect should have been admitted to the hospital (modified from Meyer (2002)).
3.5 Discussion

The analysis of linear and nonlinear HR measures demonstrated a dissociation of the interpretation of physiological and pathological conditions in mice. The nonlinear DFA measures identified pathological conditions despite presumably beneficial HR variability increase. The DFA measure provides for a direct comparison of the dynamical properties of the heartbeat interval fluctuations of mice and man with similar changes based on similar functional interventions. This study demonstrated that the HR dynamics under physiological baseline state are determined by proper autonomic control and depend on the proper vagal tone, i.e. neither absent nor increased, in both mice and humans irrespective of differences in species-specific absolute HR.

Pharmacological effects

The acute pharmacological interventions tested here yielded clear-cut effects on autonomic regulation. The pharmacological blockade of muscarinic acetylcholine receptors by atropine underscores the importance of tonic vagal (inhibitory) control for the dynamical properties under physiological conditions as well as the changes observed under stressful conditions. These dynamical states are mimicked by anesthesia in mice and heart transplantation, resulting in functional denervation of the heart in humans, and demonstrate similar functional principles across species irrespective of absolute HR values.

Parasympathetic and sympathetic relevance

As in humans heart dynamics in mice is under tonic PNS control. This is not new (Stiedl et al., 2009; Uechi et al., 1998). However, Gehrmann et al. (2000) stated “the lack of HR increase in conscious mice after parasympathetic blockade or sympathetic stimulation further supports the supposition of predominant sympathetic activity or low vagal tone under physiological conditions”. However, this conclusion is incorrect because the reported baseline HR of 714 bpm represents an elevated HR in C57BL/6J mice with a maximum HR of approximately 800 bpm (Hager et al., 2014b; Stiedl et al., 2009; Uechi et al., 1998).
contrast, resting state HR in C57BL/6J and C57BL/6N mice in the home cage is normally the range of 500-600 bpm (Stiedl et al., 2009). Therefore, the HR increase by atropine to ~665 bpm under stress-free conditions as shown here and reported earlier (Uechi et al., 1998) was undetectable in the study by Gehrmann et al. (2000) due to the substantially elevated HR. The atropine-induced HR increase parallels the HR increase in human heart transplant recipients, indicating tonic PNS function for lowered HR under resting state conditions with similar dynamical consequences, i.e. absent short-range correlation.

The role of the sympathetic system under basal stress-free conditions is low. Sympathetic inhibition by the peripheral β antagonist sotalol exerted no effects on HR dynamics. Noradrenergic depletion by 8-OH-dopamine and DSP-4 both did not alter the dynamical properties (Yamamoto et al., 1995) and linear measures (Van den Buuse et al., 2001) in rats. However, sympathetic over-stimulation by phenylephrine and isoproterenol did alter HR dynamics. These effects are in part due to baroreflex activation to counteract high blood pressure by slowing HR through vagal activation as particularly indicated by the phenylephrine-mediated high SDNN values (Uechi et al., 1998).

**Unspecific and specific stress**

The experimental conditions determine the basal behavioral/emotional state that in turn affects HR dynamics of mice. This is evident from any short-lasting (>10 min) experiments involving handling, since HR in mice easily increases to values in the range of 750-800 bpm due to enhanced sympathetic activation and parasympathetic withdrawal (Stiedl et al., 2004). Thus, conventional short-lasting tests are unsuited to assess baseline HR dynamics in mice since novelty, which increases locomotor activity (Tovote et al., 2005), and emotional states such as fear and anxiety substantially elevate basal HR as shown here. Therefore, home cage-based experiments or tests in habituated environments are essential for studies in mice to determine autonomic response adjustment to defined stressors. The high sensitivity to fluctuating environmental conditions in mice may partly be due to the limited experiential history as a consequence of commonly impoverished housing conditions (Stiedl et al., 2004). Avoidance
of any challenging condition therefore requires home cage-based experimental approaches (Hager et al., 2014a). Only highly aversive conditions such as restraint stress elevated HR to values above 700 bpm. Here, a mild change of HR dynamics occurred towards that of white noise due to the withdrawal of the parasympathetic tone and enhanced sympathetic tone. The blood pressure increase during restraint stress (Gross and Luft, 2003) may be higher than during novelty exposure, thereby activating the baroreflex to slightly lower HR when compared with HR during novelty exposure.

The specific responses to stressors are of utmost importance since hyper- and hyporesponsiveness may be used as indicator for altered autonomic function in response to emotional challenge that can be affected by the baseline state (Tovote et al., 2004; Youn et al., 2013). Challenge-induced response differences without baseline differences can be affected by the serotonin transporter-linked polymorphic region (Agorastos et al., 2014) and by long-term SSRI treatment in healthy controls (Agorastos et al., 2015). This demonstrates that the challenge-induced HR response magnitudes in psychopathology can be attenuated because of altered adaptive capacities (Koolhaas et al., 2011). Furthermore, a recent study in isogenic C57BL/6J mice shows consistent differences in inter-individual fear response magnitudes based on three unconditioned stressors suggesting personality trait-like differences in stress-susceptibility of post-traumatic stress disorder (PTSD) models (Liu et al., 2014). Unlike the assumption that altered autonomic responsiveness may be reflected in the response to challenging conditions (Berntson et al., 1998), it is evident that nonlinear methods may increase the sensitivity to detect changes in basal diurnal HR dynamics as shown in PTSD (Agorastos et al., 2013).

**Heart rate dynamics during sleep**

Sleep in mice resulted in a profound HR decrease and SDNN increase without significant change of DFA scaling coefficients. This contrasts with results in humans, where sleep increases PNS activity which results in a significant $\alpha$ decrease (see Tab. 3.2; Meyer (2002); Penzel et al. (2003)). The lack of altered HR dynamics during sleep in mice is attributed to the higher basal
metabolic needs to maintain body temperature than in humans (White and Seymour, 2005), particularly when mice are housed individually. The normal variation of circadian body temperature fluctuation (range ±1°C) reported in individually housed C57BL/6J mice (Tankersley et al., 2002) does not alter the dynamical properties as shown here. Enhanced PNS activity, as observed in human sleep, may occur during sleep in group-housed mice. Hugging together may support lower metabolic rates (Späni et al., 2003) without a profound body temperature drop.

**Nonlinear approaches**

Nonlinear dynamics methods are powerful tools to study any kind of oscillatory measure as commonly observed in time series analysis of physiological processes, e.g., EEG and ECG waveforms, neuronal channel activity, neuronal responses and human gait alterations (Losa et al., 2002, 2005). The DFA results demonstrate the role of the parasympathetic system for short-term correlation that is shifted towards Brownian noise by atropine ($\alpha_{fast}=1.5$). Pathological conditions, such as heart transplantation in humans, further indicate the importance of the autonomic control for physiological HR dynamics. The return of DFA values to $\alpha=1.1$ more than 2 years after transplantation suggests recovery of autonomic cardiac control over time (Meyer et al., 1996) as recently supported by a 10-year longitudinal study (Cornelissen et al., 2012). This finding can be replicated by atropine treatment and anesthesia in mice, indicating that the same functional principles across species underlie the fully translational DFA measure. Thus, the qualitative assessment of HR dynamics is favored by nonlinear analyses, because linear measures lack a clear indication of health versus disease state, e.g., as indicated by the mild HR increase and SDNN reduction of the peripheral mACh receptor blockade by robinul (Holschneider et al., 2002) but strongly impaired HR dynamics.
Translational relevance of non-linear heart rate dynamics

Translational value

The general view that decreased HR variability, as index of attenuated parasympathetic and/or increased sympathetic function, is a predictor of increased cardiac risk (Huikuri and Stein, 2013; Stein and Kleiger, 1999) cannot be unequivocally supported. Decreased HR variability by pharmacological interventions (e.g., NPY, sotalol) did not result in pathological HR dynamics. In contrast, substantially increased HR variability indicated partially dysregulated ANS control of HR dynamics. This occurred through central CRF1 receptor activation by ovine CRF (Stiedl et al., 2005) and 5-HT1A receptor activation by 8-OH-DPAT (Youn et al., 2013). The adverse consequences of acute postsynaptic 5-HT$_{1A}$ receptor stimulation by 8-OH-DPAT on HR dynamics with reduced HR but increased HRV in mice (Youn et al., 2013) mimics the pathological symptoms of selective serotonin reuptake inhibitors (SSRIs) at high doses as observed in humans (Unterecker et al. (2012); Zareba (2007); see FDA warning on the Celexa at http://www.fda.gov/drugs/drugsafety/ucm297391.htm). Thus, abnormal states of HR dynamics with increased HR variability include overactivation of both branches of the ANS (Meyer and Stiedl, 2006; Stiedl et al., 2009; Youn et al., 2013) as identified exclusively by nonlinear measures.

The current results underscore the translational properties of DFA scaling values independent from species-specific HR differences in mice and humans. Furthermore, normothermic pigs exhibited HR dynamics of $\alpha \sim 1$, whereas hypothermia (28°C) resulted in HR dynamics of $\alpha \sim 1.5$ (Meyer, 2002) signifying the loss of parasympathetic tone similar to that observed in heart transplant recipients within the first two years after transplantation. This has also been shown before in humans (Goldberger et al., 2002), rats (Beckers et al., 2006) and rabbits (Balocchi et al., 2002), pointing towards a more general principle in these mammalian species under physiological conditions, i.e. without severe stressors and during normothermia. The importance of tonic PNS activity for the dynamical (fractal) properties was also revealed by atropine treatment in humans (Yamamoto et al., 1995).

In conclusion, we demonstrated the crucial role of tonic parasympathetic function for the longrange correlation underlying HR dynamics in both mice
and humans. We provide evidence for superior functional assessment of altered HR dynamics by nonlinear analysis. Altered parasympathetic function, commonly blunted vagal tone, is a major cardiovascular risk factor (Thayer and Lane, 2007) particular in association with affective disorder such as PTSD (Agorastos et al., 2013). Since DFA scaling is able to forecast cardiovascular risk in the absence of ECG alterations in humans (Meyer, 2002), altered autonomic control in the absence of genuine heart disease is a likely cardiovascular risk in these patients. The potential underlying mechanisms are unknown but include epileptic-like neuronal activity altering ANS function (Dinan et al., 2008). A better mechanistic understanding of the functional coupling of the brain with the heart can predominantly be achieved by interventions with high spatio-temporal control in animal models to unravel the neural mechanism involved in the top-down control of the ANS for tonic parasympathetic function and dysfunction. Parasympathetic connectivity of the prefrontal cortex is well described (Ter Horst et al., 1996). Impaired rational control of emotionality, presumably arising from prefrontal cortex hypofunction (Hänsel and von Känel, 2008; Thayer and Brosschot, 2005), is implicated in psychopathology. HR dynamics as readout of brain function may provide an improved understanding of the comorbidity of affective disorders, such as PTSD and depression, with cardiovascular disease for refined/novel therapeutic strategies including safety pharmacology aspects. Mouse models can substantially improve our understanding of the role of specific receptors in the central autonomic neurocircuitry when translational nonlinear measures of HR dynamics are used under physiological conditions.

Acknowledgements

Financial support was provided by the Max Planck Society (M.M.), the VU University Amsterdam and the Neuroscience Campus Amsterdam (O.S.). T.H. was supported as early stage researcher by the European Union Seventh Framework Programs under grant agreements no. PEOPLE-ITN- 2008-238055 (Brain-Train) provided to O.S.
Display of individuality in avoidance behavior and risk assessment of inbred mice

Published in:
4.1 Abstract

Factors determining individuality are still poorly understood. Rodents are excellent model organisms to study individuality, due to a rich behavioral repertoire and the availability of well characterized isogenic populations. However, most current behavioral assays for rodents have short test duration in novel test environments and require human interference, which introduce coercion, thereby limiting the assessment of naturally occurring individuality. Thus, we developed an automated behavior system to longitudinally monitor conditioned fear for assessing PTSD-like behavior in individual mice. The system consists of a safe home compartment connected to a risk-prone test compartment (TC). Entry and exploration of the TC is solely based on deliberate choice determined by individual fear responsiveness and fear extinction. In this novel ethological assay, C57BL/6J mice show homogeneous responses after shock exposure (innate fear), but striking variation in long-lasting fear responses based on avoidance and risk assessment (learned fear), including automated stretch-attend posture quantification. TC entry (retention) latencies after foot shock differed >24 h and the re-explored TC area differed >50% among inbred mice. Next, we compared two closely related C57BL/6 substrains. Despite substantial individual differences, previously observed higher fear of C57BL/6N versus C57BL/6J mice was reconfirmed, whereas fear extinction was fast and did not differ. The observed variation in fear expression in isogenic mice suggests individual differences in coping style with PTSD-like avoidance. Investigating the assumed epigenetic mechanisms, with reduced interpretational ambiguity and enhanced translational value in this assay, may help improve understanding of personality type-dependent susceptibility and resilience to neuropsychiatric disorders such as PTSD.

4.2 Introduction

Individuality is commonly defined as the collection of divergent behavioral and physiological traits among individuals and develops when unique environment-
tal influences act on the genome, following complex routes, to produce pheno-
notypic diversity (Champagne, 2013). Consequently, enrichment experiments
demonstrate that experience contributes to the development of individuality and
affects behavioral performance (Rosenzweig and Bennett, 1996). Emergence
of individuality is under intense investigation, as it is considered central in
the development of several neuropsychiatric disorders. Personality-type dif-
ferences are suspected to be predictive of disease incidence, progression and
recovery, for instance in major depression and posttraumatic stress disorder
(Zovkic and Sweatt, 2013). With some exceptions (Freund et al., 2013), dis-
play of individuality and personality type dichotomies have been hard to model
using animal models. In classical behavior tests individual variation, the quan-
titative measure for observed differences of a particular phenotype, is consid-
ered a disadvantage since it negatively affects statistical power and replicabil-
ity (Button et al., 2013). However, animal models offer unique advantages to
study individuality due to the availability of well-characterized inbred popu-
lations. Furthermore, systematic analysis of individuality offers the potential
to exploit behavioral extremes that could serve as improved disease/disorder
models (Borsini, 2012; Lathe, 2004).

Commonly used test systems are not tailored to measure inter-individual
differences and are in fact often unsuited to assess individual variation. First,
behavioral phenotypes of rodent models are typically acquired in a novel test
environment. This introduces coercion (stressor), which is a confounding fac-
tor, particularly when the emotional state of animals is investigated (Hurst and
West, 2010). Hence, most experiments are confounded by the susceptibility
of animals to experimenter-based perturbation of cognitive function (Diamond
et al., 2007), instead of investigating intrinsically motivated performance to
challenging conditions. Second, these tests are typically of short duration. The
assessment of individuality clearly benefits from measuring behavior on appro-
priate time-scales (Fonio et al., 2012a,b). Third, classical behavior tests mainly
quantify a single measure, which limits the analysis of complex behaviors and
individuality. Integration of multiple measures may be particularly advanta-
geous when it concerns learning, memory and emotional states (Koolhaas et al.,
Display of individuality in inbred mice

2006). Additionally, freezing - the classical behavioral fear measure in mice - is of limited symptomatic relevance in humans (American Psychiatric Association, 2013; Azevedo et al., 2005). Although freezing is a sensitive measure of fear, it was claimed that it may be only measurable during the state of fear (Lang et al., 2000) and it is unsuited under conditions of active coping (flight or escape). The progression of other unambiguous fear-related measures that can only be acquired when the mouse is observed over longer time intervals might yield information that is more relevant to the human phenotype.

Automated analysis of behavior in a home cage design solves many of the limitations outlined above. Various solutions exist for automated tracking and analysis (De Visser et al., 2006; Fonio et al., 2009; Kas et al., 2008, 2011; Urbach et al., 2014). We developed a flexible modular system (“DualCage”) that consists of a home cage (HC) and attached test compartment (TC). This system combines the advantage of uninterrupted long-term monitoring without human intervention in the home cage setup (De Visser et al., 2006; Jhuang et al., 2010; Maroteaux et al., 2012; Steele et al., 2007; Viviani et al., 2011; Voikar et al., 2010) with the assets of deliberate exploration of an attached novel environment (Fonio et al., 2009). In the DualCage system the animal has, to a certain extent, the choice to deliberately participate in an experiment, and the progression of the experiment is determined by the instrumental responses of the animal. This offers improved ethological relevance, i.e., the responses are biologically meaningful, by taking species- and strain-specific characteristics into account (Belzung and Griebel, 2001; Olsson et al., 2003). The assessment of multiple behavioral measures based on the 3-point tracking in the DualCage enabled us to delineate risk assessment and avoidance (Augustsson and Meyerson, 2004; Blanchard et al., 2011). The stretch-attend posture (Grant and Mackintosh, 1963) is an important activity-independent behavioral expression in the context of risk assessment for detection and analysis of threat stimuli (Blanchard et al., 2011; Stankowich and Blumstein, 2005). An extended memory test with a duration of 2 days allowed revealing inter-individual differences in fear responsiveness through different coping styles (Koolhaas et al., 2007) in isogenic mouse lines (Freund et al., 2013) with enduring conditioned fear re-
4.3 Materials and Methods

Animals

Mice were obtained at an age of 8 weeks and individually housed upon arrival at constant 12-h dark-light cycle under controlled temperature (21±1°C) and humidity (55±10%) conditions with ad libitum access to food and water. Experiments started after an acclimation period of 1-2 weeks to the housing facility with shifted 12-h dark-light cycle (lights off at 2 p.m.). The illuminance during the light phase was approximately 40 lx, whereas red light was provided during the dark phase. Mice were 9-11 weeks old during the experiment. Data from 24 male C57BL/6Jicoc and 27 male C57BL/6NCrl mice (Charles River, Netherlands) were analyzed in these experiments. All studies involving animals were approved by the animal research committee of the VU University Amsterdam according to Dutch regulations and comply with the European Council Directive (86/609/EEC).

DualCage

The automated home cage environment (DualCage; commercially available as HomeCagePlus©, Biobrave, Bonn, Germany; Fig. 4.1A,B) consists of a home compartment (HC; 30 × 25 × 23 cm, width × depth × height) attached to the test compartment (TC) separated by a sliding door (5 × 6 cm, width × height). A control unit with USB-TTL I/O connection provided for hardware control.

A separation of home and test compartment is important to exploit a conflict situation between novelty-seeking and avoidance behavior of mice at dis-
tinct times after training to investigate short and long-term fear memory similar to the passive avoidance test (Ögren and Stiedl, 2010). Two cameras tracked the behavior in a region of interest of 320 × 264 pixels for each compartment. The rear zone (10 cm) of the HC was excluded from the body length analysis, because here the nest site of mice was located in all cases which, based on the curled body posture and cover by nesting material, confounded the correct body length measurements. Few shocked mice that initially built their nest close to the door “moved” it to the rear wall predominantly upon reopening of the door in the retention test indicating an active coping strategy to increase the distance to the TC. By using a 2-channel frame grabber both camera images were merged resulting in one video image for data analysis. Data were assigned to different groups after the export of batch-processed raw data based on customized software (Viewer©, Biobserve GmbH, St. Augustin, Germany). Short-term artifacts in the 3-point-tracking, caused by a frame-to-frame inversion of nose and tail tracking position, were automatically detected and corrected by a low-pass filter within a period of less than 400 ms (10 frames) in the offline analysis and replaced with neighboring values. Long-term artifacts caused by an inversion of nose and tail at low frequency could not be detected in this automated manner. These artifacts occurred during episodes of rearing and sleep and were irrelevant to the behavior responses analyzed here. Body length tracking was not confounded during crucial behaviors such as risk assessment based on stretch-attend postures in the door region. Cages for tests with or without shock exposure were randomly chosen among 10 available DualCages and alternated across test batches according to k-permutation.

The behavior was monitored by two cameras using specific tracking software (Viewer©, Biobserve). A Viewer© software script controlled all hardware actions in an operant fashion. The behavior was monitored for 7 days during light and dark phases (Fig. 4.1C) and the video was stored digitally. Mice were not handled during the whole experimental time. Infrared diodes provided for a constant illumination undetected by mice. Specific camera filters allowed optimal tracking throughout the circadian cycle. The digitally recorded video material (recorded by the Viewer© tracking software at ~25 Hz) was crucial
Materials and Methods

for quality control of automated tracking data and recheck of specific behavioral responses. Posture analysis was performed on the basis of a threepoint detection algorithm (Viewer©, Biobserve) that allowed recognizing the nose tip, the center of gravity and the tail base of a mouse.

Experimental training sequence and behavioral measures

The experimental sequence is depicted in Fig. 4.1C. Initially, 1 day after placement in the HC, baseline HC behavior was monitored for 3 days. Contextual fear conditioning training was initiated after the onset of the dark phase of day 5 by automatically opening the door to the TC compartment. Upon full entry of the TC each mouse was confined for 30 s and then exposed to a single shock (US: unconditioned stimulus; 0.7 mA, 2 s, scrambled) whereas control mice did not receive a shock. After US offset, the door was opened and upon HC return, the door was closed again for ~24 h until it was reopened 1 h after the onset of the dark phase of day 6 and then remained open for 2 days. The time to open the door was chosen according to the highest level of mobility as indicated by circadian activity (De Visser et al., 2006) and running-wheel activity of B6J mice (Rosenwasser and Fixaris, 2013; Viviani et al., 2011).

All behavioral measures were based on 3-point tracking of mice. The circadian activity of mice was monitored based on the center of gravity. Operational definitions of all measures are provided in Table 1. The progression of HC and TC exploration was determined by the Boolean map of exploration (Fig. 4.8). Both TC and HC area were segmented into 5 x 5 pixel zones so that the total number of zones (~3000) corresponds to 100% of accessible area of each compartment. The first visit of each zone determined by the nose tracking point of a mouse was cumulatively quantified in a binary manner (visited/not visited). Thereby, a mouse that fully explores the TC or HC will eventually reach the maximum value of 100% explored area. In addition, visits of the door area (Table 1) were determined for a half-circular zone with a maximum distance of 3 cm from the door on the basis of the nose tip of mice. The body length of mice was determined as a function of distance to the door. These values were plotted
Figure 4.1: DualCage design and experimental procedure. (A) Picture of the DualCage (frontal top view) with the two head stages (HS1 and HS2) video-tracking the mouse in both home (HC) and test compartment (TC). Please note that no bottle was provided in the TC (×) in the present experiments. (B) Merged video frames of both cameras monitoring HC and TC with the software-controlled door being open. The three body points, tail base, center of gravity and nose tip that are depicted on the mouse, were used for tracking. (C) Experimental sequence along the 7 days of an experiment with the different tests and the dark (D1-D7) and light phases (L1-L7) indicated. The solid gray bar indicates the access (open door) to the TC. Fearconditioned but not control mice were subjected to a single foot shock 30 s after the TC entry during training (D5).
4.3 Materials and Methods

for the first 15 min of the retention test and a linear fit was used to determine
the slope.

**Table 4.1:** Operational definitions of behavioral expressions and measures.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Door approach</td>
<td>Nose tip in a half-circular door area (distance to door max. 3 cm) in the HC</td>
</tr>
<tr>
<td>Peeking</td>
<td>Tail base and center of gravity in HC or TC, nose tip in the other compartment</td>
</tr>
<tr>
<td>Partial entry</td>
<td>Tail base in HC or TC, center of gravity and nose tip in the other compartment</td>
</tr>
<tr>
<td>Full entry</td>
<td>All three body points tracked in one compartment</td>
</tr>
<tr>
<td>Exploration</td>
<td>Percentage of Boolean map area* explored by the nose tip within HC and TC</td>
</tr>
</tbody>
</table>

**Data analysis**

Behavioral differences were examined on the basis of analysis of variance (ANOVA), repeated measures ANOVA or nonparametric comparison by Mann-Whitney U-test when appropriate. Transfer latencies were plotted as cumulative incidence of transfer and compared by Cox regression Jahn-Eimermacher et al. (2011). For better comparability, we determined the $T_{50}$ values, i.e., the values when 50% of the mice entered the TC. Pearson’s $r$ rank correlation coefficients (z-score transformation) were computed using algorithms as described by Press et al. (1988) in the correlation matrix. An error probability level of $P \leq 0.05$ corrected by false discovery rate analysis was accepted as statistically significant throughout the study. To correct for potentially inflated type I error due to multiple comparisons $P$-values were corrected by the minimum positive false discovery rate (FDR). We followed a previously reported procedure (Verhoeven et al., 2005) with a threshold set at 5% detecting one potential type I error in the correlation matrix of extracted measures (Fig. 4.5). Analyses were performed using StatView 5.0.1 and JMP 5.0.1a (SAS Institute, Cary, NC, USA). Non-parametric data are presented as box plots with the ends of the box...
denoting the 25% and 75% interquartile range and the whiskers providing the upper and lower quartile ± 1.5 times the interquartile range, respectively, while the line in the box denotes the median.

4.4 Results

*Exploration and immediate stress responses are homogeneous among C57BL/6J mice.*

To assess variation in long-term fear responses of individual mice, B6J mice were placed in the HC of the DualCage 1 h before onset of the dark phase. Thereafter, mice habituated to the HC for three days. The door to the test compartment (TC) was opened 1 h after the onset of the dark phase of day 5 to start fear conditioning training. All mice entered the TC spontaneously with a latency of less than 2 min (see Fig. 4.4A). After the full TC entry the door closed to confine the mouse in the TC. Thirty seconds after closing, either a shock (unconditioned stimulus) or no shock was given (control). Thereafter the door was reopened. All mice (shocked and non-shocked) returned to the HC immediately (Fig. 4.2A,B; HC return time 12.1±2.0 s, mean±SEM) without significant difference between groups and the door to the TC was closed.

After training, mice that had received a foot shock were significantly less active in the HC than non-shocked mice (Fig. 4.2C). This difference emerged immediately in the HC (F₁,22=22.03; P<0.0001) and lasted up to 1.75 h after shock exposure (F₁,22=8.12; P=0.0093). Activity measurements in 1-s bins from 10 s before to 10 s after the 2-s shock exposure in the TC during training were used to identify the significant shock-induced activity increase (∼5-fold compared to basal activity; F₁,22= 233.50 and 61.11, respectively; P<0.0001) compared with non-shocked control mice. There was no difference in the shock-induced activities of B6J and B6N mice suggesting similar pain perception and response (data not shown). Shocked mice re-explored their HC after return from the first TC visit with a slower progression and slightly lower total area than control mice (Fig. 4.2D,C). Especially shock-exposed mice spent significantly less time in the door area than non-shocked mice (Fig. 4.9D).
Figure 4.2: Training-related behaviors of C57BL/6J mice. Continued on next page.
Figure 4.2: Continued from previous page. Training-related behaviors of C57BL/6J mice indicate fast TC entry (low anxiety) and post-shock activity suppression (unconditioned stress). Typical activity patterns of (A) a non-shocked (noUS) and (B) a shocked mouse (US) with its distance to the door in HC and TC from 30 min before to 60 min after the TC entry. The negative deflection denotes the brief TC entry during training ± shock exposure. (C) Mean activity of non-shocked (noUS; n=11) and shocked mice (US; n=13) throughout the training day with the dashed vertical line indicating when the door to the shock compartment was opened. The small dark gray block indicates the time of the two panels shown above (A,B). The bar on the top denotes the range of significant activity differences ($F_{1,22} \geq 5.29; *P<0.05$). The door was opened $X=0$ min/h or as indicated by an arrow, gray background areas denote dark phases. After HC return from the TC after training, there was a slower home cage re-exploration in shocked than in non-shocked C57BL/6J (D) mice as determined by the Boolean map of progressive exploration.

These observations indicate that naïve habituated mice show low variation in their latency to enter the novel environment (TC), in their latency to return to the HC, and in their typical fear-induced activity suppression after shock exposure, consistent with corresponding measures in classical fear assays (Fig. 4.6).

Posture differences are powerful indicators of avoidance behavior.

Twenty-four hours later (day 6; Fig. 4.1C), again 1 h after the onset of the dark phase, the door reopened and remained open for 48 h to determine fear memory (retention) and extinction performance. During the first hour of the dark phase of day 6, 23-24 h after the shock, shock-exposed B6J mice did not differ from non-shocked B6J mice in locomotor activity (Fig. 4.3A,B,H) providing no indications of generalized fear when the door was still closed. However, when the door was reopened, nonshocked mice immediately re-entered the TC (Fig. 4.3A), whereas shocked mice did not (Fig. 4.3B). Opening of the door instantly triggered increased alertness in shocked mice indicated by changes of body posture, avoidance of the door area, and reduced exploratory behavior. Non-shocked mice had a relatively constant body length (mean ~7 cm) irrespective of their location in either HC or TC (Fig. 4.3A). In contrast, shocked mice showed freezing in a crouched posture (reduced body length) in the rear
part of the HC and infrequent approaches towards the door, increasing their body length due to the stretch-attend posture (maximum ~10 cm) (Fig. 4.3B). The relation of body length versus nose position in HC and TC (Fig. 4.3C,D) indicated highly significant body posture differences (slope differences: U=143; P<0.0001) in shocked versus non-shocked mice in the first 15 min after reopening the door (Fig. 4.3E). Especially when approaching the door, shocked mice showed a significantly increased body length (Fig. 4.3D). In contrast, no body length difference existed between the two groups during training (Fig. 4.3F).

**Individuality among C57BL/6J mice emerges in long-term fear responses.**

In the first 15 min after reopening of the door, non-shocked mice spent considerable time in the TC, while shocked mice generally avoided the TC providing for a significant difference (U=104; P<0.001; Fig. 4.3G). The locomotor activity of shocked mice was initially reduced and increased only gradually, while non-shocked mice showed the opposite activity pattern. The statistical differences between the two groups disappeared 2.5 h after the door was reopened (Fig. 4.3H), indicating that the threat of the open door induced a long-lasting suppression of locomotor activity in shocked mice.

The time elapsed between opening of the door and the first full entry into the TC (the transfer latency) was short during training and quantified as the time when 50% of the mice entered the TC (T_{50}=64.8 s; Fig. 4.4A). In the retention test, all non-shocked mice entered the TC again with a short delay (T_{50}=21.6 s; Fig. 4.4A). Shocked mice showed substantially increased transfer latencies (T_{50}=1.8 h; Fig. 4.4A) with considerable inter-individual variation. One out of 13 mice did not enter the TC within 48 h. Upon TC entry all non-shocked mice explored the TC completely as indicated by Boolean map analysis (see Fig. 4.8), with low individual variation (Fig. 4.4B) and in a very short time (T_{50}~4.4 min; Fig. 4.4F). In contrast, shocked mice showed large interindividual variation of their progression of TC exploration (Fig. 4.4B). In general, TC time spent in the TC (Fig. 4.4D,G) and entries (data not shown) were largely confined to the dark phase and were significantly correlated (Fig. 4.5). High variation was confined to retention latency and subsequent progression of TC
Figure 4.3: Behavioral performance during the retention test. Continued on next page.
4.4 Results

Figure 4.3: Continued from previous page. Retention test behaviors indicate increased risk assessment and avoidance in fearconditioned versus control C57BL/6J mice. The position of the nose tip is plotted together (top panel) with the body length (lower panel) in HC and TC for 5 min before and 15 min after opening the door to the TC for (A) a non-shocked (noUS) and (B) a shocked mouse (US). The non-shocked mouse maintained an intermediate body length irrespective of position in HC and TC. In contrast, the shocked mouse alternated between the rear end of the HC and door approaches (including one partial TC entry) with highly variable body length. Short body length values of mice, particularly before door opening, are related to rearing. The changes in distance to door correlated with locomotor activity (data not shown). (C) The plot of body length versus distance to the door of the non-shocked mouse showed a relatively homogeneous intermediate body length irrespective of its position in both HC and TC, whereas the shocked mouse (D) showed a more irregular pattern with reduced body length at larger distance from the door and increased body length towards the door. (E) The slopes of the least square linear fits during the first 15 min with reopened door showed a significant difference between non-shocked and shocked mice. (F) In naïve mice, the body length did not differ during the TC entry in the training. (G) Significant difference in the time spent in the TC during the first 15 min after door opening. (H) Mean activity of noUS and US mice throughout the retention test day with the dashed vertical line indicating when the door to the shock compartment was opened. Time (X=0 min) indicates opening of the door. The dark gray block indicates the time of the two panels shown above (A,B). The bar on the top denotes the 2.5-h range of significant activity differences (F_{1,22} \geq 5.35; *P<0.05). noUS: n=11; US: n=13; ***P<0.0001 (Mann-Whitney U-test).
exploration, and was not observed for any other measure in shockexposed mice (Fig. 4.6).

**Correlation analyses of behavior data in C57BL/6J mice do not predict individual performance differences.**

Since we observed considerable individual variation among shocked B6J mice, we performed correlation analyses across a number of derived measures to determine whether measures of activity during the first days, and specific training measures, predicted postshock behavior. For example, no correlation existed between shock activities and transfer latencies in B6J mice (Fig. 4.5). Although many activity measures were significantly correlated across different days (Fig. 4.5), indicating high individual consistency, these measures did not predict whether individual mice would show short or long transfer latencies in the retention tests.

Thus, transfer latencies were certainly not merely reflecting general differences in activity. Interestingly, delayed retention latency was significantly correlated with a slower TC compartment exploration during training (Fig. 4.5).

**Irrespective of individual differences, known fear differences remain between substrains.**

To test how individual variation among isogenic mice relates to differences between substrains, we compared the performance of B6J with that of B6N mice. Fear and extinction differences based on freezing have been reported previously between these highly related substrains in classical tests (Siegmund et al., 2005; Stiedl et al., 1999). In both substrains, activity was maximal during the first hour of DualCage exposure (novelty) at the end of the light phase (Fig. 4.7). After the onset of the dark phase, the activity of B6N mice continued to decrease, whereas the activity of B6J mice increased. During all four dark phases of days 1-4 the maximum activity of B6J mice was significantly higher than that of B6N mice. Significant activity differences emerged with a 30-min
Figure 4.4: Transfer latencies of B6J and B6N mice. Continued on next page.
Figure 4.4: Continued from previous page. Transfer latencies differ between fear-conditioned C57BL/6J (B6J) and C57BL/6N (B6N) mice with stronger avoidance (fear) in B6N than in B6J mice followed by similar TC exploration (fear extinction). (A) Transfer latencies for the first full TC entry based on the cumulative incidence of transfer plot of pooled non-shocked (noUS) and shocked (US) mice during training, and retention test of noUS and US mice indicating a significant difference between shocked B6J and B6N mice in retention. Each vertical step with the amplitude of 100/n (%) denotes the response of one individual. T\textsubscript{50} indicates when 50\% of the mice of each group entered the TC. Note that 1 out of 13 B6J and 3 out of 15 B6N mice did not re-enter the TC within 48 h. Boolean map of progressive TC exploration of non-shocked (noUS) and shocked (US) B6J mice (B) indicated an instant onset and fast exhaustion of TC exploration in all mice after the first full entry and delayed onset of exploration with generally slightly lower slope except for four B6J mice with delayed progression (#denotes low values of one mouse). (C) The TC exploration in non-shocked B6N mice (no US) was similar to that of B6J mice. Shocked B6N mice showed a delayed TC entry but relatively fast TC exhaustion. The cumulative time spent in the TC in B6J (D) and B6N mice (E) shows similar progression after its start and corresponds to the explored TC area. Quantitative comparison of both substrain performances is shown for the halftime (T\textsubscript{50}) to explore 50\% of the TC after its first full entry (from B and C) in the retention test (F) that depends on shock exposure but not substrain. The total time spent in the TC (G) during the first (d6) and the second day (d7) of the retention test (from D and E) are lower in shocked mice with higher variation. Arrows denote the opening of the door; gray background areas denote dark phases. B6J noUS: n=11; B6J US: n=13; B6N noUS: n=12; B6N US: n=15.
4.4 Results

Figure 4.5: Correlation matrix of behavioral performances of shocked C57BL/6J mice. High correlation of activity data across days but lack of correlation with increased retention test transfer latencies in shocked C57BL/6J mice. Correlation matrix of different behavioral measures based on Pearson’s linear correlation. The order of measures follows the timeline of the DualCage measures from baseline via training to retention test. Black and white dots indicate significant correlations after FDR correction. The exemplified correlation plot shows that the retention latency is positively correlated with the halftime ($T_{50}$) of TC exploration. In contrast, no significant correlation was found in baseline and training measures with the retention latency (framed vertical column). act.: activity, D: dark phase, HC: home compartment, max.: maximum, TC: test compartment, train.: training, US: shock.
delay after light offset and lasted for approximately 5 h during the first half of the dark phase (Fig. 4.7).

Figure 4.6: Increased variation particularly in the retention test measures of shocked C57BL/6J mice in DualCage but not passive avoidance experiments. Normalized (relative) variation across all DualCage measures versus passive avoidance measures in non-shocked (no US) and shocked (US) C57BL/6J mice. Normalized variation was determined by the inter-quartile range (IQR) divided by the median as non-parametric analog of the coefficient of variation. Higher variation occurred in the retention test measures with particularly higher variation in measures from shocked mice in the DC than from shocked mice in passive avoidance. act.: activity, BC: bright compartment, d: day, DC: dark compartment, HC: home compartment, max.: maximum, TC: test compartment, train.: training, US: shock.

Despite general differences in locomotor activity, the latencies to enter the TC during training in the naïve state did not differ between B6J ($T_{50}=64.8$ s) and B6N mice ($T_{50}=76.2$ s; $P=0.328$; Fig. 4.4A). Shock-exposure resulted in a similar activity reduction in B6N mice (data not shown) as in B6J mice (Fig. 4.2C). However, shocked B6N mice showed a slower HC re-exploration (Fig. 4.9B) than B6J mice (Fig. 4.2D and Fig. 4.9A) as compared for three specific times (1 h, 6 h, 11 h) after training on day 5 (Fig. 4.9C). There was no difference in the time spent in the door area in the HC on day 5 after training.
Figure 4.7: Substrain-specific differences in circadian activity. C57BL/6N (B6N) mice showed reduced circadian activity during the initial dark phases of days 1-4 (D1-4) compared to C57BL/6J (B6J) mice. Similar initial activity immediately after placement in the DualCage in the first h of day 1 followed by activity drop of B6N (n=27) but activity increase of B6J mice (n=24). B6N displayed significantly lower activity values ($F_{1,49}$ ≤ 4.08; *P<0.05) than B6J mice during the first half of the dark phases on days 1-4 as indicated by black horizontal bars on the top.

compared to shocked B6J mice (Fig. 4.9D) indicating no avoidance difference before the door was opened. During the 24-h period after training, door approaches in the HC did not differ between shocked B6J and B6N mice but were lower than in non-shocked mice of both substrains with B6N mice showing high variability (Fig. 4.9D).

The latencies to re-enter the TC in the retention test did not differ between non-shocked B6J ($T_{50}=21.6$ s) and B6N mice ($T_{50}=28.8$ s; P=0.42; Fig. 4.4A), as expected on the basis of similar training latencies. In contrast, shocked B6N mice showed significantly longer transfer latencies ($T_{50}=23.4$ h) than B6J mice ($T_{50}=1.8$ h; P=0.034; Fig. 4.4A). The total time spent in the TC increased similarly in B6J and B6N mice from retention test day 1 to day 2 (Fig. 4.9C) with higher variance in shocked than in non-shocked mice. The increase of explored TC area and TC exploration time served as indices of fear extinction. Non-shocked B6J and B6N mice showed a similarly short halftime ($T_{50}=4.6$ min) to explore the TC after its first entry in the retention test (Fig. 4.4B,C,F). However, in contrast to B6J mice, only 12 out of 15 shocked B6N mice re-explored the TC. These B6N mice showed a delayed TC exploration (Fig. 4.4B) resulting in $T_{50}=24.8$ min (Fig. 4.4F) that was significantly longer than that

83
of non-shocked B6N mice (P<0.0001), but similar to the TC exploration of shocked B6J mice (Fig. 4.4F) with comparable TC time (Fig. 4.4G) suggesting similar extinction in both substrains.

In conclusion, both B6J and B6N mice exhibit substantial individual differences in their avoidance responses, but this does not preclude the detection of significant fear expression differences, such as increased fear of B6N versus B6J mice as previously observed.

### 4.5 Discussion

Here we describe substantial individual differences in avoidance behavior to re-enter the TC during deliberate choice as identified by high-content behavioral monitoring using a new fear learning approach, the DualCage. We show considerable inter-individual variation specifically in long-lasting fear responses based on risk assessment and avoidance. Despite large inter-individual variation, the DualCage discriminated fear responsiveness between two genetically closely related C57BL/6 substrains, similar to classical fear conditioning tests (Bryant et al., 2008; Radulovic et al., 1998; Siegmund et al., 2005; Stiedl et al., 1999).

The DualCage approach combines a number of important advances that in combination are essential for valid high-content behavioral phenotyping. We exploited ethologically valid behavior exclusively motivated by intrinsic novelty-seeking to explore the TC. This intrinsically motivated behavior was unfettered from human intervention and other unspecific stressors. While classical fear learning tests rely on one core measure such as freezing and transfer latency, we used multiple measures to analyze the behavioral performance with a focus on avoidance as reliable and unambiguous emotional measure in the DualCage. To match the test duration with the time scale of the examined physiological processes (Fonio et al., 2012a) to determine valid inter-individual variation, the retention test lasted for 48 h instead of 10 min as adhered to in classical tests. We observed substantially extended transfer latencies with a median of $\sim$1.8 h, demonstrating that the standard passive avoidance test
duration, with a cut-off time of maximally 10 min (Baarendse et al., 2008), only grasps a small fraction of the dynamic range of the avoidance behavior. Short test durations result in more homogeneous distribution of avoidance performances (Baarendse et al. (2008); Fig. 4.6) probably due to truncated data. The long test duration increased the bandwidth of responses fostering the characterization of individual differences despite their genetic identity, similar experimental history (Caldij et al., 2011) and lack of human interference. This is only possible based on the refined/novel behavior assay development (Choi and Kim, 2010), whereas classical tests have a number of shortcomings in measuring and interpreting behavior (Spruijt et al., 2014). Classical tests therefore need complementation by high-content studies as performed here to reduce the interpretational ambiguity.

In many affective disorders, avoidance behavior is a core symptom (American Psychiatric Association, 2013), while freezing is a core measure of emotionality in rodents. Freezing assessment is essential for studies of the fear circuitry (Maren, 2011) and pharmacological and/or optogenetic modulation of memory processes (Goshen et al., 2011), but so far has limited symptomatic value in humans as indicated by only a few reports on freezing (Azevedo et al., 2005; Hagenaarsa et al., 2014). Avoidance requires higher cognitive functions involving the hippocampus (Ambrogi Lorenzini et al., 1996; Baarendse et al., 2008) and the prefrontal cortex (Barraclough et al., 2004). Inappropriate risk-taking is linked to increased impulsivity and attributed to impaired executive function due to cortical hypofunction (Paulus, 2007). Avoidance is used here as unambiguous analog of the human endophenotype for improved studies of animal models (De Mooij-van Malsen et al., 2009).

Our study indicates the presence of individuality of genetically identical mice (Freund et al., 2013), with the extremes of avoidance and risk assessment behavior, resulting in short or extremely long/absent transfer latencies. This classifies B6J mice as low (bold, pro-active) or high fear (shy, reactive) individuals (Koolhaas et al., 2007), respectively. The absolute variation in transfer latencies and TC exploration of shock-exposed mice exceeded that of any other measure acquired in the DualCage (Fig. 4.6) providing evidence for specifically
altered emotional responsiveness. This avoidance variation, serving as index of individuality in coping styles within substrains, clearly suggests epigenetic modulation of fear expression (Caldij et al., 2011; Wong et al., 2005; Zovkic and Sweatt, 2013).

Statistically, the results of this study indeed suggest that the power to detect significant strain or treatment-dependent differences is reduced due to the observed inter-individual variation, and therefore, larger group sizes are necessary (Button et al., 2013). However, this in turn might also lower the risk of type 1 errors (false positives) potentially reducing the replicability problem (Benjamini et al., 2010; Button et al., 2013).

Individuality in rodents can emerge during development as a consequence of intrauterine position, nutrition and social interaction, imprinting errors, maternal stress and disease, and early postnatal interactions such as handling (Lathe, 2004) and maternal experience (Siegmund et al., 2009). Additionally, random events, such as residual segregation, individual differences in molecular states or changes in the epigenetic state of a genome in general, dynamically interact with each other, so that ontogeny possibly amplifies particular functional consequences in one individual while not in the other. Individual alterations (e.g., in the structure and function of the nervous system) in turn also affect the responsiveness to environmental stimuli (Dias and Ressler, 2014), such as fear responses. Since individual response variation increased here only after aversive experience, this suggests an important role of learning and memory on individuality. Similar to the findings of Siegmund et al. (2009), the prediction of high or low avoidance behavior was not possible on the basis of baseline behavior during habituation in the HC and after contextual fear conditioning (training and consolidation). Such prediction would be extremely valuable to identify individuals at risk for early treatment to counteract the potential etiology of PTSD but may require additional independent measures such as EEG (Machida et al., 2013) and/or heart rate (Stiedl et al., 2009) or cortisol levels as claimed in human studies (Shalev et al., 1998; Yehuda et al., 1998). Isogenic C57BL/6J mice respond to three unconditioned stressors with consistent heart rate response magnitudes further indicating inter-individual fear
expression differences on the autonomic level (Liu et al., 2014). Furthermore, we have preliminary evidence for altered avoidance responses depending on rearing/housing conditions (unpublished observation). Thereby, it is possible to bias the avoidance response based on the experiential history (e.g., postnatal stress) of mice to enrich the fraction of PTSD-like responders independent of the genetic background (Molet et al., 2014). However, this is cannot be resolved in this study due to the unknown rearing conditions of mice at the breeder.

Human studies have indicated individual susceptibility differences as important determinant of treatment efficacy in affective disorders (Borsini, 2012). Inter-individual differences in emotional responsiveness serve as translational model for PTSD in humans (Holmes and Singewald, 2013) displaying variation in proneness to stressors (Daskalakis et al., 2013; Yehuda and LeDoux, 2007). Consequently, a full appreciation of epigenetic variation affecting individual responsiveness is imperative for increased validities of animal models on a genetic, molecular and pharmacological level as ultimate prerequisite for treatment efficacy of personalized medicine.

In contrast to the variation in emotional responsiveness, the activity measures showed low variance within the B6J strain that persisted throughout the retention test. Furthermore, unlike in classical behavior tests (Stiedl et al., 1999), B6N mice covered significantly less distance than B6J mice throughout the first part of the dark phase during habituation (Fig. 4.7), indicating a strong genetic effect (heritability $h^2 \sim 0.47$ for the 4-h dark phase activity starting 1 h after onset of D2-D4) attributable to few SNP differences between the two substrains that only emerged under these experimental conditions. The low variance of locomotor activity, that even persisted across the retention test, suggests low epigenetic influence on basic behavioral expressions such as locomotion under these experimental conditions.

The variance in avoidance during the retention test observed in B6J mice was also observed in B6N mice with more individuals showing persistent avoidance. However, there was no difference in extinction between B6J and B6N, based on the $T_{50}$ measure to explore the TC after its first full entry (Fig. 4.6D).
This is inconsistent with fear extinction in classical tests (Siegmund et al., 2005; Stiedl et al., 1999), as replicated by us (Fig. 4.10), and extended to passive avoidance experiments (Fig. 4.11). Fear-conditioned heart rate responses to an auditory cue in B6J and B6N mice resulted in similar extinction rates, when retention tests were performed in the home cage without human intervention (Stiedl et al., 1999). It is plausible that extinction rates are similar between substrains when there is no negative impact of unspecific stressors (handling, novelty) on behavioral performance. Extinction learning might be particularly susceptible to unspecific stressors and generalized fear (Radulovic et al., 1998; Stiedl et al., 1999) resulting in cognitive impairment (Diamond et al., 2007). Our experiments did not provide indications of generalized fear of B6N mice before the TC was accessible during the retention test, whereas these mice exhibited increased fear generalization in classical tests (Radulovic et al., 1998; Stiedl et al., 1999). Thus, generalized fear observed in B6N mice might be a consequence of unspecific stressors such as human handling and novelty in classical fear assays that is expected to affect subsequent emotional and cognitive responses (Diamond et al., 2007). However, we are dealing here with prolonged (2-day) within-session fear extinction, whereas most fear conditioning experiments deal with short (e.g., 3-9 min) within-session extinction combined with across-session extinction (tested once daily for several days). Prolonged fear extinction is more ethologically relevant than shortterm across-session extinction as relatively artificial procedure. It remains to be tested whether stronger avoidance responses or signs of generalized fear can be incubated by prolonged periods of consolidation as in fear conditioning (Pamplona et al., 2011) and whether different timing of extinction sessions affects remote avoidance responses as reported in fear conditioning (Golub et al., 2009) and spontaneous recovery of fear. In general, there is limited information on extinction in operant fear learning tasks (Ögren and Stiedl, 2010) as opposed to classical fear conditioning.

Finally, in some mice the persistent avoidance of the TC for 2 days indicated long-lasting (tonic) rather than transient (phasic) emotional state (Sylvers et al., 2011). While this may be a semantic issue (McNaughton, 2011), concep-
tually, the open door to the TC serves as specific threat (fear) rather than being a diffuse threat (anxiety) as previously defined (Lang et al., 2000; Sylvers et al., 2011). Furthermore, we are convinced that the decision-making process involved in re-entering and subsequent re-exploration of the TC makes this operant conditioning assay, offering animals a choice, distinct from classical fear conditioning. Furthermore, while freezing can be elicited and expressed on a subconscious level (LeDoux, 2014), we here deal with conscious fear and risk assessment based on deliberate choice to enter the TC with the involvement of the prelimbic prefrontal cortex in the expression of conditioned contextual fear (Burgos-Robles et al., 2009; Kim et al., 2013).

In conclusion, we provide evidence for substantial individual differences in fear expression in both isogenic mouse substrains B6J and B6N based on fear responses in the DualCage. Individual avoidance differences emerged only after severe emotional challenge and with long-term persistence indicating its validity as PTSD model (Daskalakis et al., 2013). This study highlights the importance of inter-individual differences on valid and unambiguous interpretations of emotional behavior in genetically identical organisms. High avoidance performances have been implicated in increased susceptibility for anxiety disorders fostering the exploitation of behavioral extremes for disease/disorder modeling. This approach brings a new quality to the study of the phenotype to parallel the study of the genotype (Houle, 2009).

Acknowledgements

We thank Drs. Sven Ove Ögren, Jaap M. Koolhaas and Jamie Peters for helpful comments on the manuscript. We are grateful to VU University fine mechanics and electronics departments for initial developmental support. We thank Dr. Christian Gutzen and colleagues from Biobserve for thorough hard- and software support. T.H. and S.N.M. were supported as early stage researchers by the European Union Seventh Framework Programs under grant agreements no. PEOPLE-ITN- 2008-238055 (BrainTrain) provided to O.S. and Dr. Christian Gutzen. S.v.d.S. is supported by the Netherlands Scientific Organization
(NWO/MaGW: VIDI-452-12-014). A.B.S., S.v.d.S. and M.V. were supported by a consortium grant from Agentschap NL (NeuroBasicPharmaPhenomics, FES-0908). Finally, we mourn the loss of our colleague R.F.J.
4.6 Supplementary Material

**Figure 4.8:** Re: Boolean map of exploration. The matrix of 5 x 5 pixels/zone (exemplified here for the test compartment) was used to quantify the area explored in each compartment (given in % of the total area) based on the nose position of a mouse. Revisiting the same area does not increase the total area. This allows determining the exhaustive exploration of the whole test compartment.
Figure 4.9: Home cage re-exploration and door exploration after training. Continued on next page.
Figure 4.9: Continued from previous page. Home cage re-exploration and door exploration after training in C57BL/6J and C57BL/6N mice. Upon HC return from the TC after training, there was a slower re-exploration in shocked (US, right panels) than in non-shocked (noUS, left panels) C57BL/6J (A) (as shown in Fig. 4.2D) and C57BL/6N mice (B) as determined by the Boolean map of progressive exploration (Fig. 4.8) with significantly lower maximum explored area. B6N mice did not differ from B6J mice when not exposed to shock (noUS), whereas shocked B6N mice (US) exhibited a profound delay in HC re-exploration compared to B6J mice. (C) Box plots show the group comparisons of explored HC area (from A,B) of non-shocked (noUS) and shocked (US) B6J and B6N mice after training at specific times as indicated. (D) The cumulative time spent in the door region of the HC after training in the TC show reduced door time in shocked versus non-shocked mice and suggest an increased attractor function in non-shocked C57BL/6N mice.
Figure 4.10: Slightly lower fear responses and significantly faster extinction of C57BL/6J versus C57BL/6N mice in context-dependent fear retention tests. Fear-induced changes were assessed in classical fear conditiong. Mice were subjected to a single shock (closed circles) or no shock (open circles) during training. Fear responses were assessed on the basis of inactivity (A), activity (B) and exploration area (C) on 4-5 consecutive days (d1-d5) 24 h after training in 180-s test sessions. Delayed extinction was observed in 6N mice. *P<0.05, **P<0.01, ***P<0.001 between groups. Values indicate mean±SEM, n/group=9-10. The fear conditioning experiments were performed as previously described (Stiedl et al., 1999).
Figure 4.11: Lower transfer latencies and faster extinction of C57BL/6J versus C57BL/6N mice in passive avoidance retention tests. Latencies were assessed in classical passive avoidance experiments with a cutoff time of 600 s. Mice were subjected to a 0.7 mA/2 s US (shock) after the first dark compartment (DC) entry during training (T) on day 1. B6N mice showed significantly longer dark compartment transfer latencies than B6J mice during retention test 1 (R1) on day two. On day 3, mice were subjected to a forced exposure (FE) to the dark compartment for a total of 8 min (minus the time spent in the DC during R1). From day 4 on, additional retention tests (R2-R8) were performed at 24-h interval. Mice of both substrains not subjected to US re-entered the DC immediately upon door opening in all retention tests (median transfer latency >25 s). The long upper whiskers of the box plots in B6N mice denote a subpopulation of mice (3 out of 12) that showed substantially delayed DC transfer latencies indicative of delayed fear extinction. Mice were placed in the bright compartment (1000 lx) and the latency to enter the dark compartment (10 lx) was determined. Both compartments were identical in size to that of the DualCage. For detailed passive avoidance information see (Baarendse et al., 2008). n=12/group; *P<0.05 based on Mann-Whitney U-test.
Substrain-specific reinforcement-avoidance relations in C57BL/6 mice using an animal-centered fear learning approach

Hager, T., Golani, I., Verhage, M., and Stiedl, O. *in preparation*
\section*{5.1 Abstract}

The Yerkes-Dodson law states that high arousal (‘stress’) due to challenging conditions can enhance performance in simple learning tasks but impair performance in complex tasks. Here, we investigated the relationship between the reinforcement strength and contextual fear learning of C57BL/6J and C57BL/6N mice. Different numbers of foot shocks (none, 1x, 3x or 5x) were used as reinforcement. Fear was tested by exploiting deliberate choice of mice during two days in the ‘DualCage’. The DualCage allows for discriminating between specific and unspecific stressors to correctly interpret performances, consequently facilitating the understanding of ‘stress’-induced dysregulation of memory processes. C57BL/6J mice show significantly increased contextual avoidance upon strong reinforcement. In C57BL/6N mice strong reinforcement resulted in two subgroups showing either significantly reduced or increased contextual avoidance, respectively. These results confirm the hypothesis of impaired fear learning by strong reinforcement in C57BL/6J mice while C57BL/6N mice exhibited either impaired fear learning or PTSD-like persistently increased contextual fear. However, the exploratory behavior of C57BL/6N mice that show impaired fear learning after strong reinforcement indicates increased generalized fear. The minimization of unspecific stressors in the DualCage allowed for the investigation of effects on emotional learning solely based on specific stress. This underlines the importance to recognize that differences in cognitive tests can emerge due to the differential impact of specific and unspecific stressors on emotional states (anxiety) particularly in complex tasks involving higher cortical functions.

\section*{5.2 Introduction}

The Yerkes-Dodson law states that high arousal can enhance learning performance in simple tasks and impair learning performance in more complex tasks (see also Yerkes and Dodson (1908); Diamond et al. (2007)). It is debated whether contextual fear conditioning represents a ‘simple’ or a ‘more com-
plex’ learning task (Diamond et al., 2007). It has been reported that contextual fear conditioning is enhanced by pre-exposure to aversive or stressful conditions such as restraint before training (Blank et al., 2002) and by increasing the reinforcement strength such as shock intensity in fear conditioning (Milanovic et al., 1998) and passive avoidance (Ögren and Stiedl, 2010). This reinforcement-performance relation is as predicted for simple learning tasks, i.e., performance increases in a linear manner with increasing arousal until saturation (ceiling effect). However, Todorovic et al. (2007) reported impairment of conditioned fear of mice that were subjected to 1-h immobilization stress immediately before training, suggesting a curvilinear reinforcement-performance relation, as predicted for complex tasks (Yerkes and Dodson, 1908).

To determine impairing effects of high reinforcements levels on fear learning, the assessment of fear must be based on measures that are selective for specific (conditioned) fear. Conditioned fear is generally assessed on the basis of freezing. However, specific fear and concomitant generalized (Radulovic et al., 1998) or sensitized fear (Siegmund and Wotjak, 2007) cannot be discriminated based on freezing. Thus, generalized fear may mask the expected impairment in contextual fear conditioning following increased reinforcement. Furthermore, freezing is a general fear response in rodents (Blanchard and Blanchard, 1988) that is also triggered as innate response (LeDoux, 2014), e.g., by olfactory stimuli such as alarm pheromones (Brechbühl et al., 2008). Unspecific stressors such as handling also elicit freezing.

Fear conditioning and passive avoidance both characterize fear learning with commonly similar outcomes. Contextual fear conditioning and passive avoidance learning both depend on multisensory processing of contextual stimuli involving hippocampal N-methyl-D-aspartate receptor function in C57BL/6J mice (Baarendse et al., 2008; Stiedl et al., 2000). Nevertheless, there are a few specific differences between the two tasks. Fear conditioning is a classical conditioning task based on freezing as active suppression of behavior. Although fear conditioning has been essential to identify the neural circuitry for learned fear responses (Maren, 2011), correct interpretation of behavioral performance depends on individual coping styles. In contrast, passive avoidance depends on
an instrumental response, which is the go/no-go decision to enter the compartment in which the unconditioned stimulus (e.g., foot shock) was experienced, suggesting decision-making. It is expected that this decision-making process involves the prefrontal cortex more than freezing.

Our recently developed DualCage assay (Hager et al., 2014a) opens the possibility to resolve the issue of simple or complex task based on an instrumental fear response. Locomotor activity independent measures of fear such as the stretch-attend posture (Grant and Mackintosh, 1963) and avoidance can be longitudinally assessed under conditions of minimized unspecific arousal. Furthermore, the directionality towards the threat is included in this analysis. In contrast, under this experimental condition, freezing cannot be assessed unambiguously, specifically during phases of low activity, rest and sleep.

To determine the relation between fear learning and reinforcement strength in the DualCage, we conducted experiments with different number of shock exposures in two C57BL/6 substrains of mice. These substrains differ in their fear responsiveness in classical fear conditioning (Siegmund and Wotjak, 2007; Stiedl et al., 1999) as recently confirmed in the DualCage (Hager et al., 2014a). We hypothesized that the avoidance response in the contextual fear memory test in the DualCage follows that of a complex cognitive task. Based on this hypothesis it is predicted that contextual avoidance is impaired by strong aversive reinforcement. We further expected that C57BL/6N mice show higher susceptibility to stronger reinforcement than C57BL/6J mice due to their inherent fear responsiveness.

5.3 Materials and Methods

Animals

Mice were obtained from Charles River (Leiden, The Netherlands) at an age of 8 weeks and individually housed upon arrival in type II cages (Technilab-BMI, Someren, The Netherlands). All mice had unlimited access to food and water before, during and after the experiments. In the housing facility as well as in the experimental facility a constant 12-h dark-light cycle (lights off at
2 p.m.), controlled temperature (21±1°C) and humidity conditions (55±10%) were provided. The illuminance during the light phase was approximately 40 lx, whereas red light was provided during the dark phase, i.e., also during training. After an acclimation period of 1-2 weeks to the housing facility experiments were initiated, hence mice were 9-11 weeks old during the experiment. Data from 18 male C57BL/6Jco (B6J) and 19 male C57BL/6NCrl (B6N) mice were collected and analyzed in this study. These were compared to data from 24 male B6J and 27 male B6N mice of our previous study (Hager et al., 2014a). All experiments were approved by the animal research committee of the VU University Amsterdam according to Dutch regulations and comply with the European Council Directive (86/609/EEC).

**DualCage**

The automated home cage environment (DualCage; commercially available as HomeCagePlus©, Biobserve, Bonn, Germany; Fig. 5.1a,b) consists of a home compartment (HC; 30 × 25 × 23 cm, width × depth × height) attached to the test compartment (TC; same dimensions as HC) separated by a sliding door (5 × 6 cm, width × height) as previously described (Hager et al., 2014a). A control unit with USB-TTL I/O connection provided for hardware control.

The behavior in a region of interest of 320 × 264 pixels for each compartment was tracked by two cameras equipped with infrared filters. Infrared diodes provided for a constant illumination inside the compartments undetected by the mice, allowing optimal tracking throughout the circadian cycle. By using a 2-channel frame grabber both camera images were merged resulting in one video image for data analysis. The behavior was monitored by specific tracking software (Viewer III©, Biobserve) for 7 days during light and dark phases (Fig. 5.1c). The extracted track information and the uncompressed video were stored digitally. The video material (recorded at ~25 fps) was crucial for quality control of automated tracking data and recheck of specific behavioral responses. A Viewer III© software script controlled all hardware actions on-line in an operant fashion. Mice were not handled during the whole experimental time.
Figure 5.1: DualCage design and experimental procedure. (a) Photo of the DualCage (frontal top view). The two head stages (HS1 and HS2) contain a camera for video recording of events in both home (HC) and test compartment (TC). Sliding panels on the front and back can contain different applications, such as a water bottle and a food dispenser. (b) Merged still frames of both cameras monitoring HC and TC. The three body points depicted on the mouse, tail base, center of gravity and nose tip, are used for tracking. Please note that a food dispenser was provided here in the panel on the most left side of the HC. This food dispenser is not present in the photo (a). (c) Experimental sequence along the 7 days (d1-d7) of an experiment with the different tests across dark (D1-D7) and light phases (L1-L7). TC access (open door) is indicated he solid gray bar. Mice were exposed to different reinforcement conditions in the TC during training (D5).
Experimental training sequence and behavioral measures

Figure 5.1c shows a schematic representation of the experimental sequence. After transfer of mice from their home cage into the DualCage, the first 24 h included novelty exposure. Therefore, baseline behavior in the HC was monitored for three days (d2–d4). One hour after the onset of the dark phase (D5) of day 5 (d5) the door was opened by the Viewer III© software, thereby giving access to the TC. The deliberate entering of the TC initiated contextual fear conditioning training. Upon full entry (all 3 body points in the TC) each mouse was confined (door closed) for 30 s in the TC before the specific training sequence began. Depending on the particular reinforcement property assigned to each experimental group, mice received either 1, 3 or 5 electric foot shocks (US: unconditioned stimulus; 0.7 mA, 2 s, scrambled). In the case of 3 or 5 shock exposures (3xUS and 5xUS, respectively), foot shocks were separated by 30-s intervals providing for massed training. Massed training was used since after 1 shock exposure, individual B6J and B6N mice already showed persistent avoidance of the conditioning context (Hager et al., 2014a). Spaced training was avoided because of the risk of persistent avoidance since novelty-seeking served as exclusive motivation to re-enter the TC.

After the offset of the shock the door was opened and upon HC return of mice, closed again for ~24 h. Thereafter, it was reopened 1 h after the onset of the dark phase of day 6 (d6) and then remained open for 2 days (retention test, d6–d7). The time to initially open the door, and thereby providing access to the TC during training and retention, was chosen according to the highest level of mobility as indicated by circadian activity (De Visser et al., 2006; Loos et al., 2014) and runningwheel activity of B6J mice (De Visser et al., 2007).

All behavioral measures were based on 3-point tracking of mice (body points: head, center of gravity of the body and tail base). The circadian activity of mice was monitored based on the center of gravity. Transfer latencies from TC to HC during training and retention were extracted by determining the delay between the time when access to the TC was provided and the first full entry occurred. Latencies were visualized by inverted survival statistics, i.e., plotted as cumulative incidence of transfer as a function of time for each group.
To describe group performances in a quantitative manner, $T_{50}$ times were extracted, indicating the time when 50% of the group transferred into the TC. The body length of mice, based on the cumulative distance between all three tracked body points, was determined as a function of distance to the door. To extract only relevant information several filters were applied on the body length. First, body length information was only extracted if all three body points were more than 5 cm away from all walls of HC or TC except for the separating wall in the middle, thus defining a relevant zone of approximately 50 x 20 cm (width x depth; see Fig. 5.3a,b). This excluded possible artifacts caused by rearing. Second, a band-pass filter was applied, so that only body lengths between 3 and 10 cm were included, since body lengths outside this range were considered as artifacts (e.g., caused by rearing or nesting material covering parts of the mouse confounding precise directional information). Third, body length information was only extracted if the mouse showed directionality in its movement towards the TC. Directionality was based on the direction of the nose tip in relation to the remaining two body points towards defined target zone in the back of the TC. Movements towards the door had to be directed within an angle of 70° towards this TC target zone. The directionality-dependent filters prevented the inclusion of body length information extracted during episodes of behavior not relevant for the analysis (e.g., moving away from the TC or passing by the door without orientation towards the TC). This directionality is assumed to stem from olfactory, tactile and visual information processing forming a spatial map of the HC.

The overall relation between body length and horizontal position was quantified by a linear least square fit of the relevant data, respectively by the slope of this fit with a slope close to 0 representing a constant body length disregarding the position of the mouse. Additionally, to quantify and compare the occurrence of relevant changes in body length during specific episodes, the count of extracted data points after filtering was determined.

The progression of HC and TC exploration was determined by the Boolean map of exploration as used before (Hager et al., 2014a). Areas of interest were segmented into 5 x 5 pixel zones with the total number of zones corresponding
5.4 Results

to 100% of the accessible area. The first visit of each zone, determined by the nose body point of a mouse, was cumulatively quantified in a binary manner (visited/not visited). Thereby, a mouse that fully explored the accessible area eventually reached the maximum value of 100% explored area. For better comparability of group data we extracted the \( T_{50} \) time, i.e., the time required by each individual to explore 50% of the area of interest after the first full entry (HC and TC).

**Data analysis**

The significance of differences in locomotor activity was tested with analysis of variance (ANOVA). Nonparametric comparison by Mann-Whitney U-test was used to test significance differences between group performances that were tested negatively for homoscedasticity. Group differences of transfer latencies were statistically compared by Kaplan-Meier estimates and Cox regression, respectively (Jahn-Eimermacher et al., 2011).

Graphical and statistical analyses were performed using R v3.1.1 (R Development Core Team, 2008). Non-parametric data are presented as box plots with the ends of the box (colored, solid line) denoting the 25% and 75% interquartile range. The whiskers (black, dashed line) providing the upper and lower quartile ± 1.5 times the interquartile range, respectively, while the thick black line in the box denotes the median.

**5.4 Results**

**Basal substrain differences**

To test our hypothesis that contextual fear memory in the DualCage is impaired by strong reinforcement, we analyzed the behavioral performance of two C57BL/6 substrains during an experimental sequence of in total 7 days (Fig. 5.1c; see Hager et al. (2014a)). The observed circadian activity differences between B6J and B6N mice during novelty and baseline reconfirmed
previous findings of lower activity of B6N mice (d1–d4; F1,96=24.98; P<0.05; data not shown).

Training performance

Comparison of training latencies between B6J (T50=71.2 s) and B6N (T50=86.8 s), confirmed no difference in the initial motivation to enter the TC between genotypes (P=0.81; data not shown). All mice immediately returned to the HC as soon as the door was reopened and did not show freezing in the TC immediately after shock exposure. A profound activity suppression of B6J mice was observed in the time range of 1.75 h after single shock exposure (F1,22>8.12; P<0.0093). This suppression was significantly enhanced in both substrains by stronger reinforcement using 3 and 5 shocks (B6J: noUS vs. 3xUS, 2.75 h: F1,18>9.30; P<0.018; noUS vs. 5xUS, 2.75 h: F1,20>6.38; P<0.021; B6N: noUS vs. 1xUS, 0.75 h: F1,25>8.13; P<0.0086; noUS vs. 3xUS, 1.75 h: F1,20>5.97; P<0.0239; noUS vs. 5xUS, 2.25 h: F1,21>8.13; P<0.0342; Fig. 5.2a,b).

Retention test performance

Stretch-attend postures upon TC access

The stretch-attend posture, when the head extends towards a threat (extending the sensory range) while trying to stay at a ’safe’ distance with the remaining body parts, is a behavioral expression of fear (Grant and Mackintosh, 1963) displayed here during the approach of the TC. Relevant body length information in relation to the horizontal position in HC and TC showed characteristic patterns depended on the treatment mice received during training (Fig. 5.3a,b). Mice that received no shock during training re-entered the TC fast in the retention test and showed a relatively constant body length versus their position in the DualCage (Fig. 5.3). In contrast, mice that were exposed to 1xUS, 3xUS and 5xUS initially avoided the TC as indicated by a decreased count of relevant body length data points (Fig. 5.3c,d) and had a significantly increased body length when approaching the door to the TC. Thus, the TC served as aversively conditioned context (group comparison of slope of fit: B6J: noUS vs. 1xUS:
5.4 Results

Figure 5.2: Locomotor activity of C57BL/6 mice on day 5 (training). Mean locomotor activity (cm/15 min) based on the center of gravity of mice throughout the training day with light gray bars in the background indicating the 12-h dark phase and dark gray bars indicating when access to the TC was granted. (a) C57BL/6J and C57BL/6N mice (b) showed lower locomotor activity with increasing number of shock exposures during training. Groups exposed to 3xUS and 5xUS were compared to the performance of nonshocked (noUS) and 1xUS mice from a previous study (Hager et al., 2014a). The horizontal colored lines in the upper left corner of each panel denote the time range after US offset and HC return during which the activity of the respective color-coded group differed significantly (P<0.05) from activity of the non-shocked control group.
Substrain-specific reinforcement-avoidance relations

P<0.001; B6N: noUS vs. 1xUS: P<0.001; Fig. 5.3e,f). More precisely, mice that received more than a single shock show deviant patterns in the relation of body length and position as specifically described below. The group comparison of B6J (Fig. 5.3c,e) and B6N mice (Fig. 5.3d,f) reveals substrain-specific reinforcement-response curvelike patterns. B6J mice showed a trend towards the lowest count of relevant body length data points after exposure to 3 shocks during training (Fig. 5.3c). This trend in B6J mice correlated with the slope of body length during door approach, which showed the highest values after mice received 3xUS during training (Fig. 5.3e) and decreased as soon as more than three shocks were provided (3xUS vs. 5xUS: P=0.003). The count of body length data points of B6N mice (Fig. 5.3d) was less correlated with the slope of fit and showed an increased variation in individual performances compared to B6J mice. B6N mice reacted with the steepest body length slope for the door approach already after 1xUS exposure (Fig. 5.3g). In contrast to B6J mice, B6N mice did not showed a reduced body length during door approach when 5 shocks were provided in comparison to either 1 or 3 shocks (Fig. 5.3f).

**Latency to re-explore the shock compartment**

B6J mice exposed to a single shock showed substantially increased transfer latencies (T_{50}=1.8 h; P<0.001) compared with non-shocked B6J mice (T_{50}=21.6 s; Fig. 5.4a). Statistical analysis (Coxregression) revealed no significant difference in the transfer latencies of B6J mice that were exposed to 3xUS (T_{50}=3.8 h) as compared to 1xUS mice (P=0.96). In contrast, the transfer latencies of 5xUS mice (T_{50}=0.6 h) were significantly shorter compared to 1xUS and 3xUS mice (P<0.001; Fig. 5.4a). The latencies of all shocked mice required a drastically expanded temporal dimension (X-axis in h) for the inverted survival plot.

The performance of B6N mice during the retention test also demonstrates significantly prolonged transfer latencies of 1xUS mice (T_{50}=23.4 h) as compared to non-shocked mice (T_{50}=28.8 s; P<0.001; Fig. 5.4b). However, B6N mice exhibited significantly shorter transfer latencies (T_{50}=2.4 h) than 1xUS B6N mice (P=0.033; Fig. 5.4b) after exposure to 3xUS. This contrasts with the similar avoidance performance of B6J mice after 1 shock and after 3 shocks.
Figure 5.3: Stretch-attend posture during the retention test. continued on next page.
Substrain-specific reinforcement-avoidance relations

Figure 5.3: Continued from previous page. Stretch-attend posture during the first 15 min of the retention test. The analysis of the body length relative to the position in home compartment (HC) and test compartment (TC) revealed significant differences depending on the reinforcement during training. The body length of representative individual mice for the non-shocked (noUS) (a) and 3xUS group (b) is plotted against the distance from the door. Each data point represents body length and positional information extracted during the first 15 min of the retention test on day 6. Only data points were extracted that met the requirements of the above-described filters (zone, band-pass, directionality of movement and head directionality; see Material and Methods). The red line indicates the linear least square fit of the body length versus position data. The mouse exposed to three shocks (3xUS) showed no transfer into the TC during the first 15 min of the retention test (b). Different numbers of points, showing directional moves towards the TC, were quantified in the different groups of C57BL/6J (c) and C57BL/6N mice (d). The numbers of points dropped significantly with higher numbers of shock exposures versus nonshocked controls (3xUS, 5xUS vs. noUS). Body length slopes are plotted for groups of C57BL/6J (e) and C57BL/6N mice (f). ***P<0.001.

Interestingly, a further drop in the latencies to enter the TC after 5xUS was observed only in a subpopulation of B6N mice (n=5), whereas the remaining mice (n=5) did not reenter the TC anymore (1xUS B6N vs. 5xUS B6N: P=0.40; 3xUS B6N vs. 5xUS B6N: P=0.165).

Test compartment re-exploration

Upon the first full TC entry most mice of both substrains explored the TC completely as shown by Boolean map analysis (Fig. 5.5a,b). This is indicated by the maximum height (explored TC area in %) the Boolean progression curves reach during the 48 h of the retention test (d6–d7). The slope of the curves describes the speed of re-exploration of the TC. Horizontal episodes in these curves indicate no visits of previously unexplored TC areas during this time or no TC visit until full TC exploration has occurred. This could be observed in several individuals, but did not correlate with the training procedure they were exposed to. Only in the subpopulation of 5xUS B6N mice that re-explored the TC, a trend towards an increased amount of episodes of no further TC exploration could be observed. Thus, despite significantly different transfer la-


**Figure 5.4:** Transfer latencies of C57BL/6 substrains in the retention test. Inverted survival statistics (Kaplan-Meier estimator) showing the cumulative incidence of transfers from HC to TC during the retention test (d6-d7). Each vertical step with the amplitude of 100/n (%) denotes the response of one individual. $T_{50}$ times (dashed horizontal line) indicate when 50% of the mice of each group entered the TC. Comparison of group responses by means of Cox-regression revealed significant differences depending on the treatment mice received during training (d4). (a) Retention latencies of C57BL/6J mice. The display of latencies of 1xUS, 3xUS and 5xUS mice requires an X-axis with time in hours. (b) Retention latencies of C57BL/6N mice. Similar to the performance of C57BL/6J mice, retention latencies of shocked mice were significantly prolonged compared to non-shocked mice. Notably, C57BL/6N mice exposed to 3xUS during training showed significantly shorter latencies than 1xUS mice, whereas in C57BL/6J mice there was no difference between these two groups. Exposure to 5 shocks resulted in either relatively short latencies or complete avoidance in C57BL/6N mice.
tendencies, once a full TC entry could be observed, it was usually followed by a relatively fast and exhaustive TC exploration irrespective of the treatment during training. Nonetheless, in consideration of the sensitivity of the T$_{50}$ times to individual episodes of no TC exploration, the quantitative comparison of group responses (Fig. 5.5c,d) revealed significant differences depending on the treatment received during training. These differences were most obvious in both substrains in the comparison of nonshocked and 1xUS mice (B6J: noUS vs. 1xUS: P<0.001; B6N: noUS vs. 1xUS: P<0.001), but also in the comparison of 3xUS and 5xUS B6N mice (3xUS vs. 5xUS: P=0.73).

5.5 Discussion

Main findings

Here we describe substantial differences in learned fear expression between two closely related mouse substrains and a robust impairment of this expression at high reinforcement strength. The impairment of contextual fear memory by relatively high levels of ‘stress’ (Koolhaas et al., 2011) resulting from multiple shock exposure indicates an increased complexity of contextual fear learning in the DualCage (Diamond et al., 2007). This contrasts with the majority of classical fear conditioning studies that almost exclusively report fear facilitation (Blank et al., 2002). Furthermore, we observed a higher susceptibility of B6N mice to memory impairment as a result of increased shock exposure (3xUS) than in B6J mice (5xUS). These results confirm our hypotheses that contextual fear learning classifies as a complex cognitive task with lower conditioned avoidance of B6N than of B6J mice following high reinforcement. These findings comply with the Yerkes-Dodson law for complex tasks (Yerkes and Dodson, 1908).

Despite the observed inter-individual differences in fear expression in the retention test, this finding suggests that classical fear conditioning experiments investigating reinforcement properties in conditioned fear may use the wrong measure (freezing) in the wrong setup (novelty) for the wrong duration (commonly ≤10 min) as recently reported in open field experiments (Fonio et al., 2011).
Figure 5.5: Exploration of TC during retention. Continued on next page.
Substrain-specific reinforcement-avoidance relations

**Figure 5.5**: *Continued from previous page.* Exploration of TC during retention of C57BL/6 mice. Analysis of progressive TC exploration during the retention test by means of Boolean maps indicated an instant onset and fast exhaustion of TC exploration in all mice after the first full entry. Different reinforcement properties during training mainly affected transfer latencies and transfer frequency but had only marginal influence on the progression of TC exploration of (a) C57BL/6J and (b) C57BL/6N mice. This results in a slightly lower slope of the Boolean progression. Light-gray vertical bars denote the dark phases of d6–d7. For quantitative comparison of the performance of both substrains (c,d) the progression of TC exploration was normalized to the corresponding TC entry. The $T_{50}$ time was significantly elevated in groups exposed to 1-5 shocks versus non-shocked mice. Despite the relative short latency in a subpopulation (n=5) of C57BL/6N of the 5xUS group, the exploration of the TC was very slow with a median $T_{50} > 2$ h. Note that $T_{50}$ data are unavailable from 5 mice that showed only partial (peeking) but not full TC entry.

2012a,b). This conclusion favors home cage-based phenotyping with high sensitivity/resolution of learned fear assessment based on avoidance learning as exemplified in the DualCage.

**The Yerkes-Dodson law**

The Yerkes-Dodson law states that high arousal can enhance learning performance in a simple task and impair learning performance in a more complex task (Yerkes and Dodson, 1908). Although it is difficult to operationally define the complexity of a task, Easterbrook’s cue utilization hypothesis (Easterbrook, 1959) provides a basis. It states that with increased arousal, the amount of cues that can be processed is reduced. A more complex task requires attention to multiple cues as commonly used in contextual fear conditioning (Maren, 2011), including higher cognitive functions such as decision-making as exploited here. Thus, performance is more likely decreased by the influence of high levels of ‘stress’ (Joëls et al., 2006). A curvilinear relation is observed in simple tasks based on simple associations with low cognitive demand, in which the performance of individuals is less susceptible to the influence of ‘stress’. The comparison of the performance of experimental groups from two substrains exposed to different levels of ‘stress’ during training indicates a biphasic relation.
of ‘stress’ and performance (or more specifically, arousal and avoidance) that characterized complex tasks. This can be observed through measures quantifying learned contextual fear based on transfer latencies (Ögren and Stiedl, 2010) and stretch-attend postures during approach behavior (Grant and Mackintosh, 1963). The overall group performance in relation to the assumed state of arousal of B6J and B6N mice is summarized in Figure 5.6. In this modified model from Yerkes and Dodson, relatively low levels of ‘stress’ (1xUS) facilitate the performance of B6N mice as compared to B6J mice.

![Figure 5.6: Model of the reinforcement vs. avoidance relation in C57BL/6 substrains based on the Yerkes-Dodson law. The avoidance (performance or ‘cognition’) as a function of reinforcement (arousal or ‘stress’) is shown here in relative dimensions for the two C57BL/6 substrains. A biphasic arousal-performance relation was observed in C57BL/6J mice. C57BL/6N mice showed an increased susceptibility to increased reinforcement strength resulting in lower retention latencies (avoidance) at relatively moderate level of reinforcement (3xUS) compared to C57BL/6J mice. After strong reinforcement (5xUS), the responses indicated two subpopulations with either relatively low or complete avoidance. The latter classifies as PTSD-like (hypermnesic) phenotype. The mode, height, width and other performance characteristics of this function are affected by genetic and environmental factors such as experience.](image-url)
On the other hand, medium levels of ‘stress’ impair the performance of B6N mice while the performance of B6J mice is facilitated. The increased susceptibility to ‘stress’ of B6N mice as well as the relatively increased variance in fear responses confirms previous findings on the learning performance of these two substrains (Hager et al., 2014a).

The bimodal distribution of transfer latencies in 5xUS B6N mice suggests impairment only in a subpopulation (n=5). In contrast, the remaining B6N mice (n=5) did not re-enter the TC in the retention test indicating persistent avoidance as index of hypermnesic (flashbulb) memory as implicated in PTSD (Diamond et al., 2007; Kaouane et al., 2012).

The impaired performance at high arousal in complex tasks may be attributed to enhanced glucocorticoid release on long-term potentiation and memory consolidation in the hippocampus, the amygdala and prefrontal cortex (Joëls et al., 2006; Karst et al., 2005; Metcalfe and Jacobs, 1998; Rocher et al., 2004). Elevated glucocorticoid levels have also been shown to impair working memory and memory recall but only when elevated by aversive stimuli (Woodson et al., 2003) arguing against an exclusive glucocorticoid-mediated effect. Spatial convergence was proposed, i.e., memory facilitation may occur only when stress hormones (corticosteroids, noradrenaline, CRH) exert their actions in the same areas as those activated by the particular stressful situation (Joëls et al., 2006). Elevated glucocorticoids can impair memory consolidation and retrieval (Joëls et al., 2006) and cause hypermnesia of traumatic events as implicated in PTSD (Kaouane et al., 2012) and exhibited by persistent avoiders to re-enter the TC (B6J: 1xUS; B6N: 1xUS, 3xUS and 5xUS; Fig. 5.4).

Increased variation in fear expression across individuals at maximum learning performance reflects the display of individuality (Freund et al., 2013) in both substrains as recently described in the DualCage (Hager et al., 2014a). Overall, this highlights the importance of both genetic (the substrain difference) and environmental/epigenetic factors (the within substrain variation) and its interaction underlying the observed avoidance responses.

Despite significant differences in transfer latencies of different shock groups (1x/3x/5xUS), the progression of re-exploration of the TC did not differ in
these groups except for in the 5xUS group of B6N mice. This suggests that within-session extinction was largely unaltered in both substrains despite different reinforcement levels. This finding expands our previous results on similar within-session extinction of B6J and B6N mice irrespective of the stronger fear response in B6N mice (Hager et al., 2014a) and our own report on delayed extinction of B6N mice in classical fear conditioning (Stiedl et al., 1999). On the basis of these results we conclude that the primary emotional challenge of mice is to overcome the fear memory as prime inhibition to re-enter the TC. This critical decision step parallels that observed in the exploration pattern of fruit flies in a novel situation before entering an adjacent space (Gakamsky et al., 2013) as also reported in mice in refined open field experiments (Fonio et al., 2009).

**The diversity and relevance of various fear measures**

Our results underscore the importance to exploit unambiguous specific measures of fear in our refined fear learning assay.

**Freezing:** We conclude that the deliberate choice to enter the TC as a consequence of avoidance and risk assessment serves as more specific (animal centered) index of fear than freezing as more unspecific fear response without clear discrimination between specific and generalized fear, consciously or unconsciously expressed (LeDoux, 2014).

**Avoidance:** This measure is the core symptom according to the DSM-5 (American Psychiatric Association, 2013) and offers to explore an instrumental response with a choice directed to a target (here the door to the TC). It is independent of, e.g., the coping style, and thus the general activity of the mouse unlike freezing.

**Stretch-attend posture:** The filtering for movement with directionality of movement towards the TC drastically reduced the number of data points suitable for stretch-attend posture analysis (see Fig. 5.3b) compared to our previous study (Hager et al., 2014a). However, this filtering allowed for discrimination from other movements such as escape from the TC, and thereby, it increased the relevance of our interpretation of the stretch-attend posture as an index of learned contextual fear during approach towards the threat.
Perspective

To further increase the resolution of differential memory performance between B6J and B6N mice, low shock intensities (e.g., 0.3 - 0.5 mA) need to be tested in the DualCage as previously used in classical fear learning tests (Milanovic et al., 1998; Ögren and Stiedl, 2010). Thereby a faster facilitation effect on the rising side of the arousal-performance function may be identifiable in B6N mice. The specificity of the PTSD-like hypermnesia in the 5xUS group of B6N mice currently is unclear. It would need an altered DualCage approach, e.g., with two different test compartments, to determine whether this hypermnesia represents a generalized or a specific avoidance response mediated by unique stimuli of the TC such as the shock grid. The slower progression of TC re-exploration of the residual 5xUS mice (Fig. 5.5d) despite their short transfer latencies supports the assumption of increased generalized fear in this experimental group.

To further determine the influence of genetic factors on the reinforcement-dependent learning performance other mouse strains need to be tested in this approach. We would expect DBA/2J mice, based on their susceptibility to negative reinforcement in spatial learning tests (Youn et al., 2012), to show a better performance in the DualCage when unspecific stressors are removed. Similarly, unspecific stressors in classical fear learning are expected to worsen the performance of mouse strains with increased anxiety-like behavior as observed in hippocampus-dependent fear learning in DBA/2J mice and as also reported in munc18-1 heterozygous mice (Hager et al., 2014b). This underscores the high influence of aversive emotional states on cognitive performance in mice as revealed by the prominent impact of anxiety on spatial learning in the water maze (Wolfer et al., 1998).

Final conclusions

This study demonstrates the impairing effect of strong reinforcement on contextual fear memory under semi-naturalistic conditions based on avoidance responses with differential onset in two closely related C57BL/6 mouse sub-
strains. These findings indicate that this passive avoidance task in the DualCage classifies as a complex form of learning. Consequently, performance comparison between mouse strains in complex tasks based on a single reinforcement may fail to recognize that observed differences are attributable to the emotional impact on cognition, particularly when using classical behavior tests that provide for unspecific stress (e.g., handling, novelty) and when anxiety differences are observed in genetic mouse models. Thus, the DualCage approach appears highly suitable to address the consequences of therapeutic interventions on specific emotional responses in the absence of unspecific stressors. DualCage experiments may serve as improved anxiety model with high clinical relevance since avoidance behavior is exploited as important symptom of the DSM-V (American Psychiatric Association, 2013) and endophenotype to assess emotional dysfunctions. Overall the experiments show the modulatory effect of various reinforcement levels for amnestic and hypermnestic fear memories. The approach shows high translational value and is reminiscent of the modulation of memory formation under severe stress (e.g., crime exposure) as implicated in human eyewitness memory with amnestic-like effects by high reinforcement (Deffenbacher et al., 2004). Our data suggest higher genetic susceptibility to PTSD-like flashbulb memory in the B6N than in the B6J substrain.

Acknowledgements

We thank Drs. Sven Ove Ögren and Ilan Golani for helpful comments on the manuscript. We are grateful to Anton W. Pieneman for initial experimental support. We thank Dr. Christian Gutzen and Agniezka Piotrowski from Biobserve for essential data analysis support. Torben Hager and Agniezka Piotrowski were supported as early stage researchers by the European Union Seventh Framework Programs under grant agreements no. PEOPLE-ITN-2008-238055 (BrainTrain) provided to Oliver Stiedl and Christian Gutzen, respectively. August B. Smit and Matthijs Verhage were supported by a consortium grant from Agentschap NL (NeuroBasicPharmaPhenomics, FES- 0908).
6

General discussion
General discussion

The aim of this thesis was to characterize emotional memory and anxiety in mice using classical but also novel behavioral approaches for the characterization of (sub-)strain-specific and individual differences and effects of genetic interventions. In this Chapter, the studies included in this thesis are recapitulated and the findings on advanced autonomic phenotyping of emotional behavior are summarized and discussed. Additionally, the limitations of the current studies and further perspectives of advanced and integrative phenotyping are considered.

6.1 *Munc18-1* haploinsufficiency results in enhanced anxiety-like behavior (Chapter 2)

Behavioral phenotyping experiments of *munc18-1* heterozygous mice showed increased anxiety and impaired emotional learning (Maroteaux et al., 2014). The previously observed increase in anxiety might be (partially) caused by altered autonomic nervous system (ANS) function due to the haploinsufficiency. Consequently, we expected external factors like unspecific stress to have an increased influence on the emotional responsiveness of heterozygous mice. Despite observed changes in neurotransmitter release in *munc18-1* heterozygous mice (Verhage et al., 2000), no changes in heart rate dynamics, a prominent readout of ANS, were observed in freely moving heterozygous mice. We reasoned, that the most likely explanation for the observed increase in anxiety in *munc18-1* heterozygous mice is an altered coping style (expression) rather than cognitive impairment (encoding, consolidation and retrieval).

6.2 The functional and translational relevance of autonomic readouts (Chapter 3)

In Chapter 3 we characterized heart rate dynamics by nonlinear analysis based on detrended fluctuation analysis (Peng et al., 1995) during different behavioral and pharmacological interventions (in mice) and during different physiological
6.3 Recapitulation of Chapter 4

or pathological states (in humans) altering autonomic nervous system control. We hypothesized that non-linear analysis of heart rate in mice facilitates unambiguous conclusions about autonomic effects and potential pathological consequences. Furthermore, we expected to describe similar functional properties of the ANS in mice after behavioral or pharmacological intervention as in humans under pathological conditions. Using scale-invariant measures of heart rate variability, we described that neural control can be decoupled from autonomous cardiac control of heart rate in both mice and men, after treatment or due to pathological states. Linear measures of heart rate did not allow for this distinction. Overall, the findings of this Chapter show that, irrespective of differences in species-specific parameters, heart rate dynamics in mice and humans are determined by autonomic control under physiological baseline condition and depend on tonic parasympathetic control. Hence, this underlines the importance of translational relevant measures (here, measures of ANS activity) and models of increased construct validity (here, pharmacological intervention in mice to mimic human disorders) to understand the underlying mechanisms of affective disorders.

6.3 Inter-individual differences in genetically identical mice (Chapter 4)

In the DualCage, the advantage of uninterrupted long-term monitoring without human intervention in the home cage setup (De Visser et al., 2006; Maroteaux et al., 2012) was combined with the assets of deliberate exploration of an attached environment (Fonio et al., 2009; Kas et al., 2011). We expected approaches of increased etho- and etiological validity like the DualCage to increase the translational relevance of findings, e.g., avoidance behavior as translational measure found in mice and humans (American Psychiatric Association, 2013) as compared to freezing behavior, which cannot be translated to human behavior. We describe significant differences in fear responsiveness between two genetically closely related C57BL/6 substrains, similar to classical behavior tests (Bryant et al., 2008; Radulovic et al., 1998). However, in contrast to
classical behavior tests, the extinction of conditioned contextual fear in these two substrains does not differ under semi-naturalistic conditions (Hager et al., 2014a). The comparison of the behavioral performance in classical test with the results of this study supports the claim that the timescale of the test duration has to match the timescale of the examined physiological processes (Fonio et al., 2012a) to conclude valid interpretations. Furthermore, in the DualCage the observed inter-individual variation in fear expression in isogenic mice suggests individual differences in coping style. Based on the human etiopathology we suggest an increased construct validity of high avoiders in the DualCage as model of post-traumatic stress disorder (Daskalakis et al., 2013).

6.4 The relevance of arousal (or “stress”) on emotional learning (Chapter 5)

The exposure to reinforcing stimuli increases the state of arousal (“stress”), probably resulting in differential hypothalamic-pituitary-adrenal cortex axis activation in individual organisms. We hypothesized a different susceptibility to “stress” in two genetically closely related substrains of C57BL/6 mice. The results demonstrated a biphasic relation of reinforcement strength and contextual fear memory performance, i.e., increased arousal during training resulted in higher expression of conditioned contextual fear, while severe arousal resulted in decreased fear expression. The comparison of the two closely related substrains revealed a higher susceptibility of B6N mice to increased “stress” resulting in memory impairment by lower reinforcement strength than in B6J mice. Furthermore, severe reinforcement resulted in two performance subgroups of B6N mice with either low or maximum avoidance indicating weakened or PTSD-like memory, respectively. These results highlight the importance to recognize that differences in cognitive tests can emerge due to the differential impact of ”stress” on the emotional states of individual organisms.

6.5 General conclusion and perspective

The benefits of holistic phenotyping

Urbach et al. (2014) hypothesize that the simultaneous detection of multiple behavioral and physiological parameters in parallel might improve throughput, validity, and reliability in the behavioral characterization of rodents. The findings of Chapter 2 indicate that the correct interpretation of behavioral performance and the assessments of validities of animal models (e.g., models of neurodegenerative diseases or affective disorders) can be limited by the extent of tests and concomitantly by the reduced validity of interpretations. More precisely, despite observed changes in neurotransmitter release in munc18-1 heterozygous mice, no dysfunction of the ANS was found. This indicates that the altered behavioral performance is based on differences in the susceptibility to “stress”, most likely resulting in increased anxiety in heterozygous mice. A conclusion that could not have been drawn exclusively based on findings of classical behavior tests.

Advantages of novel behavioral approaches like the DualCage

In addition to the necessity of refined animal models (Borsini, 2012), the importance of refined behavioral approaches became increasingly acknowledged in scientific literature during the last years, while many methods are considered to be somewhat “frozen in time” (Spruijt et al., 2014). In Chapter 4 and Chapter 5 we emphasize the importance of refined behavioral approaches to strengthen the validity of findings. In order to minimize uncontrollable effects of unspecific “stress” on behavioral performance, and thereby eventually control the influence of emotionality, we developed a novel behavioral approach, the DualCage. We conducted studies in the DualCage with mice from two C57BL/6 substrains that have been extensively studied in literature and in our own lab in classical behavior tests. This increased the comparability with previous findings and facilitated the validation of our novel behavioral approach. The results of our studies in the DualCage demonstrate the importance to elicit and observe
General discussion

ethological relevant behavior under semi-naturalistic and controlled conditions in order to unambiguously interpret specific performances in animal models.

**Limitations of novel behavioral approaches like the DualCage**

Nonetheless, the development of novel behavioral models and approaches is commonly thwarted by several practical reasons (Fonio et al., 2012b). For example, classical behavioral tests of short test duration offer the convenience of high throughput experiments, often sequentially aligned in standardised test batteries (Rogers et al., 1997). These are considered the ‘gold standard’ and are commonly used in the majority of laboratories. Consequently, the use of non-standard approaches makes it harder to have results recognized by the scientific community. Additionally, the development and validation of novel approaches is considerably time- and cost-intensive. As an animal-centered approach the assay has to be tuned to the actual performance range of the animal model. Furthermore, this includes thorough quality control of the data, and extended revision of the many novel measures to focus on most informative readouts. This process is very laborious and requires the development of novel strategies for the analysis of raw data to handle large amount of information acquired over extended timescales. This has become obvious from customized software developments (AHCODA) in the PhenoTyper to extend or substitute the possibilities provided by the vendors (Maroteaux et al., 2012). In conclusion, the costs of new hard- and software development and the time to validate new experimental procedures may lead to relatively expensive systems that may not be easily adopted by the scientific community. The economic aspect of this dilemma was summarized already 10 years ago by Crabbe and Morris (2004) in their essay about high-throughput phenotyping by stating that “the average neuroscientist actually has greater access to enthusiastic students than to a large equipment budget".
The importance of translational measures for the validation of animal models

Furthermore, the translational validity of animal models depends on the translational relevance of measures. In Chapter 3 the comparative study of the effects of different treatments in mice and man on autonomic nervous function expressed on the basis of heart rate variability measures, endorsed the importance of scale-invariant measures of increased translational validity. Animal models of increased translational validity may provide an improved mechanistic understanding of affective disorders, such as post-traumatic stress disorder, depression and schizophrenia. Affective disorders show a high comorbidity with cardiovascular disorders, therefore altered heart rate dynamics may constitute a valuable endophenotype and are considered a valid (additional but not exclusive) biomarker in clinical research (Olshansky et al., 2008). Increased heart rate due to enhanced sympathetic drive and/or attenuated parasympathetic drive occurs in affective disorders under basal conditions. Alterations may be observed particularly in the diurnal/circadian cycle (Agorastos et al., 2013). Current hypotheses implicate reduced prefrontal cortex function (Thayer et al., 2009) in the rational control of emotional centers. However, the functional contribution of forebrain areas in the brain-heart interaction, that is anatomically well described (Ter Horst et al., 1996), has not yet been determined and depends on refined animal models.

Replicability of behavioral experiments

Increased variation of observations potentially increases the risk of type 1 errors (false positives) and type 2 errors (false negatives) facilitating the common replicability problem in behavioral neuroscience (Benjamini et al., 2010). As our studies in the DualCage indicated, variation of behavioral performance increases on extended timescales that correspond with underlying physiological processes. This entails a reduced statistical power in detecting significant strain- or treatment-dependent differences. Therefore, besides a potentially increased relevance of interpretations, larger group sizes are necessary to reach statisti-
General discussion

cal significance. This dilemma was recently described by Button et al. (2013), who states that in this context studies in neuroscience commonly appear to be underpowered.

In order to avoid underpowered studies and increase replicability across laboratories, it became common practice to increase the level of standardization of behavior assays and environmental factors involved. This includes amongst others rearing and housing conditions as well as the “quality” of human intervention such as handling procedures (Baker, 2011; Williams, 2011). However, it was recently suggested to accept the fact that laboratories are different in an unpredictable manner. Consequently, no level of standardization can entirely avoid unspecific variation since it is impossible to standardize unknown external stimuli such as differences between experimenters (Kafkafi et al., 2005). This is in agreement with the finding that despite rigorous standardization across several laboratories diverging behavioral performances could be observed (Crabbe et al., 1999; Lewejohann et al., 2006). Thus, as Kafkafi et al. (2005) further suggest, instead of “out-of-the-ordinary standardization” of experiments across laboratories, statistical estimates for particular measures of the variability of the genotype x environment interaction should be obtained.

The emergence of individuality

In addition to external and unspecific factors, strain-specific and individual-specific characteristics potentially influence the behavioral performance. Namely, besides the genetic identity of mice across laboratories, they will probably feature deviant epigenetic states already upon arrival in the testing facility (Figure 1). Furthermore, each member of a new group will further individualize while being exposed to deviant environmental factors of the particular facility. Hence, the magnitude of individual differences observed in replications of an experiment is likely to vary across studies, irrespective of the level of “external” standardization.

Neurobiological theories on the development of organisms suggest that small perturbations presumably related to a diversity of factors (e.g., intrauterine position, nutrition and interaction, imprinting errors, maternal stress and
disease, early postnatal interactions) may lead to initial individual differences already during ontogenesis (Raijmakers and Molenaar, 2004). These initial differences may trigger alterations in experience during further developmental progress that accumulate over time and may result amongst others in differential plasticity on a cellular level. While individual epigenetic states will further result in deviant developmental trajectories (Champagne, 2013).

Consequently, besides the introduction of robust measures, the appreciation of processes that effect deviant developmental trajectories might lead to increased reproducibility and validity of experiments. Therefore, Wolfer et al. (2004) found that increasing the amount of external stimuli provided by environmental enrichment does not seem to generally increase variability. Although some controversy exists with regard to parameters that cannot be linked to behavioral performance directly, such as body weight. Most studies in this context have been conducted using smaller cohorts of animals and behavioral tests of shorter timescales compared to the studies in the DualCage. Thus, whether long-term enrichment under semi-naturalistic conditions has an increasing effect on inter-individual differences remains to be determined. A recent study (Freund et al., 2013) describes a significant increase in inter-individual differences of explorative behavior among genetically identical individuals housed for three months in a complex environment. The authors hypothesize that the effect of the enriched environment differentially affects individuals over time, thereby rendering the observed emergence of “life space” (Lewin, 1935) and personality-like differences. However, the emergence of individuality as described here in mice, parallels the development of personality traits in humans (Jang et al., 1998). This highlights the importance of translational relevant models in biomedical research, e.g., for the understanding of underlying pathological mechanism in humans based on findings in animal models.

In summary, caused by ontogenic individualization, subpopulations of isogenic mice may emerge that show considerable variation in their epigenetic states. This in turn may create subpopulations that feature personality-like characteristics (Fig. 6.1), which can be exploited to investigate traits or disorder that reflect these characteristics.
Figure 6.1: Schematic diagram of the emergence of individuality during ontogeny in mice. Beginning at E0 (conception) small perturbations like imprinting errors and/or hormone exposure due to intrauterine position may lead to initial individual developmental differences of physiological and neurological features of the embryo *in utero*. Factors like nutrition and interaction, maternal stress and disease, and/or early postnatal interactions and experiences may cause further individual differences pre- (E0–P0) and post-partum (>P0). These differences may trigger variations that accumulate over time during the whole ontogenetic development and result in different epigenetic states leading to different developmental trajectories. Subpopulations of mice may emerge, that feature particular characteristics (exemplified here by a subpopulation of high avoiders or very low avoiders as extremes within a population that may characterize as phenotypes with increased affective disorder susceptibility).
For example, mice that show increased avoidance during the contextual fear retention test in the DualCage (see Chapter 5: maximum avoidance in a subpopulation of C57BL/6N mice after strong reinforcement during training) might serve as a model for post-traumatic stress disorder. We expect similar underlying hypermnesic memory processes (or ‘flashbulb’ memories; see Brown and Kulik (1977)) in this subpopulation of high-avoiders, as they are described in the human disorder (Kaouane et al., 2012). Furthermore, selection of individuals based on their responsiveness to specific stimuli or treatments, might also increase the predictive validity of animal models in pharmaceutical research. To increase the effectiveness of drugs globally (i.e., irrespective of personality-like differences), it is imperative to reduce the number of non-responders. In the case of the development of antidepressant drugs for instance, the assessment of drug-effectiveness should be based on the responsiveness of (pre-selected) antidepressant insensitive animals (Borsini, 2012).

**Perspective**

In conclusion, we expect the DualCage to foster the translational relevance of behavioral research. However, since behavioral performance of mice depends amongst others on rearing conditions, the cumulative effect of ontogenetic individualization in mice of advanced age needs to be evaluated. Also, further validation based on the performance of common reference mouse strains (e.g., C57BL/6J) in the DualCage is necessary to complement our current findings. This has to include variations of experimental protocols, like increased test-training intervals to assess mid- to long-term learning performance under semi-naturalistic conditions. Additionally, the behavioral performance of models of human disorders in the DualCage under conditions of minimized unspecific stress, like munc18-1 heterozygous mice, might confirm our findings by means of autonomic phenotyping regarding the increased susceptibility to external stimuli. Finally, a methodological integration of autonomic and electrophysiological readouts (EEG, multiple unit recordings) during longitudinal behavioral performance, possibly in combination with brain area-specific (e.g., optogenetic interventions) or pharmacological interventions will provide con-
comitant measures within one experiment instead of correlated findings across different experiments. Especially regarding the above mentioned emergence of individuality during development (i.e., the ‘self-enhancing’ differential effects of experience in individuals), the parallel extraction of concomitant measures instead of temporally separated measures extracted during several episodes of testing, gains importance. Consequently, the extraction of concomitant measures in longitudinal, ethological relevant approaches might additionally increase translational and construct validity of models by the increased analogy to the etiopathology of human disorders (e.g., PTSD).


References


Blank, T., Nijholt, I., Eckart, K., and Spiess, J. (2002). Priming of long-term potentiation in mouse hippocampus by corticotropin-releasing factor and
References


References


References


References


References


Liu, L., Wei, W., Kuang, H., Tsien, J., and Zhao, F. (2014). Heart rate and heart rate variability assessment identifies individual differences in fear response magnitudes to earthquake, free fall, and air puff in mice. PLOS One, 9:e93270.


cortex stimulation produces lethal cardiac arrhythmias – a mechanism of

Pamplona, F., Henes, K., Micale, V., Mauch, C., Takahashi, R., and Wotjak, C.
(2011). Prolonged fear incubation leads to generalized avoidance behavior

Paulus, M. (2007). Decision-making dysfunctions in psychiatry – altered home-

Peng, C., Buldyrev, S., Hausdorff, J., Havlin, S., Mietus, J., Simons, M., Stan-
ley, H., and Goldberger, A. (1994). Nonequilibrium dynamics as an indis-
ensable characteristic of a healthy biological system. *Integr Physiol Behav
Sci*, 29:283–293.

scaling exponents and crossover phenomena in nonstationary heartbeat time

parison of detrended fluctuation analysis and spectral analysis for heart rate

Petkov, P., Ding, Y., Cassell, M., Zhang, W., Wagner, G., Sargent, E., Asquith,
An efficient snp system for mouse genome scanning and elucidating strain


R Development Core Team (2008). *A language and environment for statistical
computing*. Foundation for Statistical Computing, Vienna, Austria. URL:

responses in C57BL/6N mice subjected to one-trial foreground contextual

References


Summary

Advanced autonomic and behavioral phenotyping of emotional behavior of mice

Behavioral neuroscience investigates the relation between behavioral functions and its underlying biological processes. In other words, the composite of an organism’s characteristics (the phenotype) is observed and described through behavioral characterization (phenotyping). The phenotype is determined by the interaction of the genetic makeup of an organism (the genotype, nature) with environmental factors (the environment, nurture) during the development of an organism. In order to investigate these processes in living organisms, different animal models are utilized and rodent models are advantageous for several reasons. Mice are the best mammalian model with respect to fast reproduction and genetic interventions. The performance of animal models in behavioral tests is observed and quantified in a particular behavior test or tasks. But importantly, the interpretation of particular performances and measures needs to be done carefully and fulfill certain criteria and validities. Furthermore, the behavioral test as well as the animal model itself should fulfill these requirements. When the behavioral performance is interpreted incorrectly or certain requirements and validities are not met, not only the investigation of principles of basic neuroscience might be negatively affected, but this might also affect and mislead the field of drug discovery, which has a worldwide economic cost of over $40 billion per year. Therefore, the aim of this thesis was to characterize emotional states such as anxiety and fear memory of mice using classical but also novel and advanced behavioral tests with refined behavioral and autonomic measures.
Chapter 1 generally introduces the topic of advanced phenotyping and summarizes the findings of existing literature. In the study underlying Chapter 2 we monitored heart rate responses of genetically modified mice with the haploinsufficiency of munc18-1 in different behavioral tasks and analyzed the time series of heartbeats by linear and a non-linear methods. By recording ECG by means of implanted radio-transmitters, we monitored the performance and heart rate of freely moving mice, predominantly in their home cage, thereby avoiding the influence of unspecific and uncontrollable stress. We reasoned, that the most likely explanation for the previously observed increase in anxiety in these mice is an altered coping style (emotional expression) rather than cognitive impairment (encoding, consolidation and retrieval of emotional information). This conclusion could not have been drawn based on the behavioral performance of mice alone in classical tests based on sometimes ambiguous measures such as freezing.

In Chapter 3 we characterized heart rate dynamics by nonlinear analysis during different behavioral and pharmacological interventions in mice and compared these data with different physiological or pathological states in humans. Using scale-invariant measures of heart rate variability, we described that heart rate dynamics in mice as well as in humans are determined by autonomic control and depend on tonic parasympathetic control under physiological baseline condition. These findings highlight the importance of translational relevant measures from mouse to humans (here, indirect measures of autonomous nervous system activity) and models of increased construct validity (here, pharmacological intervention in mice to mimic human autonomic dysfunction) to provide new approaches to better identify and understand the underlying mechanisms of affective disorders that show high comorbidity with cardiovascular risk probably though autonomic dysregulation.

In Chapter 4 we investigated the long-term responses to emotional fear learning of two closely related and most widely used inbred strains of laboratory mice C57BL/6J and C57BL/6N in the DualCage. We developed the DualCage as novel behavioral approach to automatically assess long-term behavioral performance of mice under semi-naturalistic conditions. Automated
analysis of particularly avoidance behavior in a home cage design does not suffer from many of the limitations described in classical behavioral tests. In our approach, the advantage of uninterrupted long-term monitoring without human intervention in the home cage setup was combined with the assets of deliberate exploration of an attached environment. We initially identified profound activity differences that were not identified in classical tests. In Chapter 4 we investigated the long-term responses to emotional fear learning of two substrains of the most widely used inbred strain of laboratory mice in the DualCage. We described significant differences in fear responsiveness, similar to classical behavior tests and showed considerable inter-individual performance differences. However, in contrast to classical behavior tests, the extinction of conditioned contextual fear in these two substrains did not differ in the DualCage. Based on the human etiopathology we suggested increased construct and translational validity of fear of mice conditioned to contextual cues in the DualCage as a model of post-traumatic stress disorder.

The exposure to reinforcing stimuli increases the state of arousal (“stress”), probably resulting in differential hypothalamic-pituitary-adrenal cortex axis activation in individual organisms. Therefore, in Chapter 5 we investigated the effect of increased arousal on emotional learning in the DualCage. We hypothesized a different susceptibility to “stress” in the two genetically closely related substrains introduced in Chapter 4. The comparison of the two closely related substrains revealed a higher susceptibility of C57BL/6N mice to increased “stress” resulting in memory impairment by lower reinforcement strength than in C57BL/6J mice. These results highlight the importance to recognize that differences in the behavioral performance can emerge due to the differential impact of ”stress” on the emotional state of individual organisms.

Chapter 6 summarizes the findings of the studies underlying this thesis. This summary led us to the conclusion that we expect the DualCage to foster the translational relevance of behavioral research, i.e., provide a better translation of findings from animal model to human. Furthermore, a methodological integration of autonomic and electrophysiological readouts (EEG, multiple unit recordings) during longitudinal behavioral performance, possibly in com-
Summary

Combination with brain area-specific (e.g., optogenetic interventions) or pharmacological long-term interventions, will provide concomitant measures within one experiment instead of correlated findings across different experiments. The extraction of concomitant measures in longitudinal, ethologically relevant approaches is expected to additionally increase translational relevance and construct validity of models by the increased analogy to the etiopathology of human disorders such as post-traumatic stress disorder.
Nederlandse samenvatting

Geavanceerde autonome en gedragsmatige fenotypering van emotioneel gedrag van muizen.

Gedragsneurowetenschappen onderzoekt de relatie tussen gedrag en onderliggende biologische processen. Met andere woorden, de samenstelling van de kenmerken van een organisme (fenotype) wordt geobserveerd en beschreven aan de hand van gedragskenmerken (fenotypering). Het fenotype wordt bepaald door de interactie van de genetische samenstelling van een organisme (het genotype, nature) met de omgevingsfactoren (milieu, nurture) tijdens de ontwikkeling van een organisme. Om deze processen in levende organismen te onderzoeken wordt gebruik gemaakt van verschillende diermodellen. Het gebruik van knaagdiermodellen heeft hierbij verschillende voordelen. Muizen zijn het beste zoogdiermodel ten aanzien van snelle voortplanting en genetische ingrepen. De prestaties van diermodellen in gedragstesten worden geobserveerd en gekwantificeerd in een bepaalde gedragstest of taak. Het is hierbij belangrijk dat de interpretatie van bepaalde prestaties en uitkomsten zorgvuldig gedaan wordt en voldoet aan bepaalde criteria en de eisen van validiteit. Daarnaast moet de zowel de gedragstest als het diermodel voldoen aan deze voorwaarden. Wanneer de gedragsprestaties verkeerd worden geïnterpreteerd of er niet aan de vastgestelde eisen en validiteit wordt voldaan, kan niet alleen het onderzoek naar fundamentele principes van de neurowetenschappen negatief worden beïnvloed, maar kan dit ook misleidend zijn voor het onderzoek naar geneesmiddelen, wat wereldwijd per jaar economische kosten van meer dan $ 40 miljard maakt. Het doel van dit proefschrift was daarom emoties zoals anx-
Nederlandse samenvatting

ijety en angstgeheugen van muizen met behulp van klassieke, maar ook nieuwe en geavanceerde gedragstesten, met verfijnde gedrags- en autonome metingen, te karakteriseren.

In hoofdstuk 1 wordt het onderwerp van geavanceerde fenotypering geïntroduceerd en een samenvatting gegeven van de bevindingen uit de bestaande literatuur. In hoofdstuk 2 monitorden we de hartslag reactie van genetisch gemodificeerde muizen met haploïsufficientie van munc18-1 in verschillende gedragstaken en analyseerden we de hartslag tijdreeksen met lineaire en niet-lineaire methoden. Door het opnemen van ECG met behulp van geimplanteerde radio-zenders, monitorden we prestaties en hartslag van muizen. Deze konden zich vrij bewegen, voornamelijk in hun kooi, om zo de invloed van niet-specifieke en oncontroleerbare stress te vermijden. We kwamen tot de conclusie dat de meest waarschijnlijke verklaring voor de eerder waargenomen toeneming van angst bij deze muizen een veranderde coping-stijl (emotionele expressie) is en niet een cognitieve stoornis (codering, handhaven en het ophalen van emotionele informatie). Deze conclusie had niet getrokken kunnen worden op basis van klassieke muizen gedragstesten, omdat bij deze soms meerduidig gedrag zoals freezing optreedt.

In hoofdstuk 3 karakteriseerden we hartslag dynamiek door niet-lineaire analyses uit te voeren tijdens verschillende gedrags-en farmacologische interventies in muizen en vergeleken we deze gegevens met diverse fysiologische of pathologische staten bij de mens. Door gebruik te maken van scale-invariant analyses van hartslag variatie, beschreven we dat hartslag dynamiek zo wel in muizen als in mensen door autonome controle bepaald wordt en afhankelijk is van tonische parasympathetische controle onder fysiologische basislijn condities. Deze bevindingen benadrukken het belang van translationeel relevant maatstaven van muis naar mens (hier, indirecte metingen van de activiteit van het autonome zenuwstelsel) en modellen van verhoogde constructvaliditeit (hier, farmacologische interventie bij muizen om de menselijke autonome disfunctie te reproduceren) om nieuwe benaderingen voor betere identificatie en begrip van onderliggende mechanismen van affectieve stoornissen, die een
hoge comorbiditeit met cardiovasculair risico, waarschijnlijk door autonome onregeling, laten zien, te leveren.

In hoofdstuk 4 onderzochten we de lange termijn respons op het emotionele angstgeheugen van twee nauw verwante en meest gebruikte ingeteelde stammen van laboratoriummuizen, C57BL/6J en C57BL/6N, in de DualCage. We ontwikkelden de DualCage als nieuwe aanpak om lange termijn gedragsprestaties van muizen onder semenaturalistische omstandigheden automatisch te beoordelen. Geautomatiseerde analyse van bijzonder vermijdingsgedrag in een thuis kooi ontwerp wordt niet onderworpen aan de vele beperkingen, zoals beschreven in klassieke gedragstesten. In onze aanpak wordt het voordeel van ononderbroken lange termijn monitoring zonder menselijke tussenkomst in de thuis kooi setup, gecombineerd met de mogelijkheid van het vrijwillig verkennen van een aangesloten omgeving. Aanvankelijk identificeerden we sterke verschillen in activiteit, die niet zijn geïdentificeerd in klassieke testen. In hoofdstuk 4 onderzochten we de lange termijn respons op het emotionele angstgeheugen van twee substammen van de meest gebruikte ingeteelde stam van laboratoriummuizen in de DualCage. We beschreven significante verschillen in angst responsiviteit, vergelijkbaar met klassieke gedragstesten en toonden aanzienlijke inter-individuele prestatie verschillen. In tegenstelling tot klassieke gedragstesten, verschilde het verdwijnen van geconditioneerd contextuele angst tussen de beide substammen in de DualCage niet. Op basis van de menselijke etiopathology suggereren we verbeterde construct- en translationele validiteit van angst, van muizen, die geconditioneerd zijn door contextuele signalen in de DualCage, als model voor posttraumatische stressstoornis.

De blootstelling aan de versterking van stimuli verhoogt de staat van opwinding (‘stress’). Dit resultert waarschijnlijk in verschillende activering van de hypothalamic-pituitary-adrenal cortex as in individuele organismen. Daarom hebben we in hoofdstuk 5 het effect van verhoogde opwinding op het emotionele leren in de DualCage onderzocht. We stelden de hypothese dat de twee substammen, die in hoofdstuk 4 voorgesteld werden, een verschillende gevoeligheid voor ‘stress’ hebben. De vergelijking van de twee nauw verwante substammen onthulde een hogere gevoeligheid van C57BL/6N muizen voor ver-
hoogde stress, wat resulteerde in geheugenstoornissen door mindere versterking dan in C57BL/6J-muizen. Deze resultaten benadrukken het belang om te erkennen dat verschillen in gedragsprestaties kunnen ontstaan als gevolg van de verschillende impacten van ’stress’ op de emotionele staat van individuele organismen.

Hoofdstuk 6 geeft een overzicht van de resultaten van de studies die ten grondslag liggen aan dit proefschrift. Deze samenvatting leidde ons tot de conclusie dat we verwachten dat de DualCage de translationele relevantie van gedragsonderzoek bevordert, dat wil zeggen, een betere vertaling van bevindingen van dierenmodellen naar de mens. Bovendien zal een methodologische integratie van autonome en elektrofysiologische read-outs (EEG, multiple unit recordings) in longitudinal gedragsprestaties, eventueel in combinatie met hersengebieds specifieke (bijvoorbeeld optogenetische interventies) of met farmacologische langdurige interventies, gelijktijdige resultaten binnen één experiment opleveren in plaats van gecorreleerde bevindingen in verschillende experimenten. We verwachten dat het verkrijgen van gelijktijdige resultaten in longitudinal, ethologisch belangrijke benaderingen de translationele relevantie en constructvaliditeit van modellen verhoogt, door een verbeterde analogie met de etiopathologie van stoornissen bij mensen, zoals posttraumatische stressstoornis.
Additional scientific contributions of Torben Hager

Besides the published chapters and the chapters prepared for publication additional work has contributed to the coauthorship of Torben Hager to the following publications:

Peer-reviewed publications


Contributions at Meetings and Symposia


home cage (DualCage) environment. Endo-Neuro-Psycho Meeting, Lunteren, The Netherlands (Oral presentation, Parallel Sessions E).


Torben Hager was born on the 14th October 1980 in Schwerte (Germany). He obtained the diploma from German secondary school qualifying for university admission or matriculation from the Friedrich-Bährens Gymnasium in Schwerte. Afterwards he studied Zoology and Neurobiology at the Ruhr-University Bochum and acquired his Master degree in Zoology and Neurobiology. His PhD project focused on advanced autonomic and behavioral phenotyping of emotional behavior of mice under the supervision of associate professor Dr. Stiedl and professor Dr. Verhage. Currently he works a Chief Scientific Officer at Biobserve in Bonn (Germany).
Acknowledgments

I would like to express my special appreciation and thanks to my promotor and head of the FGA department **Prof. Dr. Matthijs Verhage**. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. I greatly appreciate the countless efforts you invested in a plethora of BrainTrain-related symposia, meetings and workshops, Phenotyper-meetings, personal one-on-one ROLLS-ROYCE-Steering-Wheel moments, and much more, which made it possible for me to become the fortunate scientist I am today.

My deepest gratitude is to my co-promotor and head of the Behavioral Neuoscience Research Group **Dr. Oliver Stiedl**. You have been and you still are a tremendous mentor for me. Your advice on both research as well as on my career have been priceless. You gave me the freedom to explore on my own, and at the same time the guidance to recover when my steps faltered. Additionally, amazingly fortunate as I have been, I am more than glad that I can refer to you also as my friend. Besides teaching me how to think beyond the ivory tower of science, how to question thoughts, and express ideas, you also introduced me to the convenience of an ice-cold VELTINS while watching the SPORTSCHAU after a long day of hard work. In this context, I would also like to express my gratitude to your wife, **Brigitte Stield**, amongst others for ever unsurpassed hospitality and amazing gastronomical skills.

I would also like to thank my committee members, **Prof. Dr. Ilan Golani**, **Dr. Tommy Pattij**, **Dr. Martien Kas**, **Prof. Dr. Guus Smit**, and **Dr. Maarten Los** for serving as my committee members. I also want to thank you for let-
Acknowledgments

ting my defense be an enjoyable moment, and for your brilliant comments and suggestions, thanks to you.

I would especially like to thank Anton Pieneman for all the countless moments I could rely on your help and support, inside and outside of the VU. I am absolutely proud that you allowed me to make friends with you and Tee. I will benefit for ever from the exclusive wisdom you taught me, inside and outside of the VU.

I hope my expression of gratitude also reaches the far shores of Wellington, New Zealand. Over there, my amigo Dr. Ji Un Youn merits my gratitude for countless moments of scientific brilliancy and amusement. If you read this, please address my sincerest regards to Prof. Dr. Bart Ellenborek.

I would like to thank my friend and fellow BrainTrain-ESR Dr. Julia Kurps for her steady companionship during my years at the VU and during numerous BrainTrain-related odysseys all around the world. Without several strigiform-enabled coffee breaks provided by you I doubt I would have been able to conserve my mental sanity during frequent times of frustrated desperation. I also would like to thank Vera van der Niet, first of course for being a friend, but amongst others also for eloquently translating the summary of this thesis.

In this BrainTrain-related context, I would also like to express my gratitude to all my fellow ESRs of the ITN BrainTrain. Namely, Dr. Ashutosh Dhingra, Dr. Dave Tang, Dr. Cornelis Blauwendraat, Dr. Nikhil Pandya, Dr. Juan Carlos Valenzuela, Dr. Christian Seeger, Dr. Laszlo Bicskei, Dr. Min Seol, Dr. Ioannis Kramivs, Dr. Julia Dawitz, Dr. Nicholas Rajan, Dr. Giorgio La Fata, Dr. Sylvia Lombardo, and Dr. Tiberiu Stan. Thank you all for the fantastic times we had. Of course, Evelyn van Royen, the beloved mother of all BrainTrain ESRs, needs to be mentioned here too. Thank you Evelyn for everything you have done for us.

I would like to express my appreciation and thanks to Prof. Dr. Tobias Kalenscher for introducing me to the (scientific) beauty of Amsterdam. Without you my first months after immigration to the Netherlands would have been at least less enjoyable, and some special moments for sure even unbearable. In
this regard, I am still deeply concerned about the fact that I finally missed the opportunity to become the captain of the beautiful M.S. Antje.

For the scientific ingenuity and entertainment during the ongoing struggle on the improvement of Behavioral Neuroscience I would like to express my special gratitude to my fellow campaigner Dr. Gregoire Maroteaux, Esther Remmelink, Dr. Emmeke Aarts, Dr. Sophie van der Sluis, and Bastijn Koopmans. I really enjoyed working with all of you.

I would like to thank SYLICS B.V. and especially Annemieke Steenbergen for all you have done for me and for the wonderful times I had working with you.

I also would like to thank all the students who worked in our lab during my time in Amsterdam. Your work significantly contributed to the publication of this thesis, and I am grateful for the company of every one of you in the lab, in the lunch room, in the pub, or in my garden next to my beloved BBQ. I will try to mention all of you, but please forgive me if I fail. Namely, Paula, Annemarie, Inti, Vincent, Joris, Judith, Steven, Paul, Noor, Rick, Ayla, Roelof, Nikki, Henk, Josse, and Bart.

During my time working as a scientist in Amsterdam I was very fortunate to meet so many fantastic people, that unfortunately I will not be able to mention all of them in this acknowledgment. Therefore, I would like to address my gratitude and thanks to all people of the FGA and the CNCR, especially to Els Borghols, whose support and reliability is simply unmatched.

A special thanks to my parents Axel Hager und Sabine Hager. Obviously, without you, none of all this would have been possible for me to achieve. But beyond this, beyond the maternal and paternal care, even beyond any kind of support that anyone could ever possibly expect, it is merely beyond the scope of my literacy to sufficiently express my thanks for all that you have done for me during the last decades.

Unfortunately, there are also no words to express my interminable gratitude to my best friends Andreas Kolodinski, Dennis Staks, and Marcel Merling for literally always standing at my side, rain or shine. Please accept this humble
text as a proportionate token of my appreciation. May there be countless more
Jungs-WE to come. In this context, I also have to thank the corresponded wives
and girlfriends, Sandra Dieckerhoff, Melanie Fischer, and Katrin Krüger,
for dismissing the boys when I am in need.

Furthermore, I would like to thank Dr. Christian Gutzen for being not
only an employer, but also a friend, sports-companion and guide to my new
home in Bonn. In this regard, I would also like to express my gratitude to Dr.
Jan Nagy for providing me with the opportunity to work with Christian.

Finally, I mourn the loss of my friend and colleague Dr. Rene F. Jansen.