NCS-1 dependent learning bonus and behavior outputs of self-directed exploration

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Animals explore a new environment and learn about their surroundings. “Exploration” refers to all activities that increase the information obtained by an animal. For this study, I determined a molecule that mediates self-directed exploration, with a particular focus on rearing behavior and vocalization. Rearing can be either self-directed exploration or escape-oriented exploration. Self-directed exploration can be driven by the desire to gather information about environments, while escape-oriented exploration can be driven by fear or anxiety. To differentiate between these two concepts, I compared rearing and other behaviors in three different conditions 1) novel dim (safe environment), which induces exploration based rearing; 2) novel bright (fearful environment), which elicits fear driven rearing; and 3) familiar environment as a control. First, I characterized the effects on two distinct types of environment in exploratory behavior, and its effect on learning. From this, I determined that self-directed exploration enhances spatial learning, while escape-oriented exploration does not produce a learning bonus. Second, I found that NCS-1 is involved in exploration, as well as learning and memory, by testing mice with reduced levels of Ncs-1 by point mutation and also siRNA injection. Finally, I elucidated other behavior outputs and neural substrate activities, which co-occurred during either self-directed or escape-oriented exploration. I found that high-frequency ultrasonic vocalizations occurred during self-directed
exploration, while low-frequency calls were emitted during escape-oriented exploration. Also, with immediate early gene imaging techniques, I found hippocampus and nucleus accumbens activation in self-directed exploration. This study is the first comprehensive molecular analysis of learning bonus in self-directed exploration. These results may be beneficial for studying underlying mechanisms of neuropsychiatric disease, and also reveal therapeutic targets for them.
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Abbreviation

5-HTT  Serotonin transporter
ACh   Acetylcholine
AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPARs AMPA receptors
Amyg  Amygdala
ANOVA Analysis of variance
AP1, 2 Activator protein 1, 2
ARF1   ADP-ribosylation factor-1
ASD   Autism spectrum disorder
BLA Basolateral amygdala
BoNT Botulinum neurotoxin A or incobotulinumtoxin A
BSA   Bovine serum albumin
CA1,3 Carno Ammon 1,3
Ca2+  Calcium
CaMKII, IV Ca2+/calmodulin-dependent protein kinase II, IV
CAPS  Ca2+-dependent activator protein for secretion
Cav 2.1 Ionotropic P/Q-type Ca2+ channel
Cbl-b  Cbl proto-oncogene B, E3 ubiquitin protein ligase
CFI   Comparative fit index
CgC   Cingulate cortex
CI    Confidence interval
Ct    Threshold cycle
CTN   Interfering NCS-1 C-terminal peptide
CREB  cAMP Response Element-Binding
CRH   Corticotropin-releasing hormone
CRHR2 Corticotropin-releasing hormone receptor 2
CRH-BP Corticotropin-releasing hormone binding protein
CR    Conditioned response
CS    Conditioned stimuli
D2R (Drd2) Dopamine type 2 receptor
D3R (Drd3) Dopamine type 3 receptor
D4R (Drd4) Dopamine type 4 receptor
DA Dopamine
DG Dentate gyrus
DO Displaced objects
DLS Dorsolateral striatum
DMS Dorsomedial striatum
DNA Deoxyribonucleic acid
eIF2α Eukaryotic translation initiation factor 2A
EntC Entohinal cortex
ENU N-ethyl-N-nitrosourea
EPM Elevated plus-maze
EPSP Excitatory post synaptic potential
FB Familiar bright
FD Familiar dim
FFT Fast Fourier transforms
fMRI Functional magnetic resonance imaging
FM Frequency modulated
FO Familiar object
GAD Glutamate decarboxylase
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GCAP Guanylate cyclase-activating protein
GDNF Glial cell line-derived neurotrophic factor
GRK2 G protein-coupled receptor kinase 2
HC Hydrophobic crevice
HF High-frequency
H.M. Henry Gustav Molaison
   (February 26, 1926 – December 2, 2008)
HPC Hippocampus
H-ras Harvey rat sarcoma viral oncogene homolog,
   Transforming protein p21
IEG Immediate early gene
IL1RAPL1 Interleukin receptor accessory protein like-1
Ins(1,4,5)P3R  Inositol 1,4,5-trisphosphate receptor
KChIPs  K+ channel interacting proteins
KO  Knockout
Kv4.2  Voltage-gated Potassium Channel 4.2
LM  Learning and memory
LF  Low-frequency
LTD  Long-term depression
LTP  Long-term potentiation
MAOA  Monoamine oxidase A
MAOB  Monoamine oxidase B
MEG  Magnetoencephalography
mGluR  Metabotropic glutamate receptor
mPFC  Medial prefrontal cortex
mRNA  Messenger ribonucleic acid
NAc  Nucleus accumbens
NacC  Nucleus accumbens core
NacS  Nucleus accumbens shell
NB  Novel bright
ND  Novel dim
NMDA  N-Methyl-D-aspartate
NMR  Nuclear magnetic resonance
NO  Novel objects
NR1  N-methyl-D-aspartate receptor subunit 1
NR2  N-methyl-D-aspartate receptor subunit 2
NR2B  N-methyl D-aspartate receptor subtype 2B
ORL1  Opioid receptor-like 1
PAG  Periaqueductal gray
PBS  Phosphate buffered saline
PBS-BT  PBS containing 5 % BSA and 0.1 % Triton X-100
PC12 cells  Pheochromocytoma cells
PDE  Phosphodiesterase
PeriC  Perihinal cortex
P14K  Phosphatidylinositol-4-kinase
<table>
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<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>RMSEA</td>
<td>Root mean square error of approximation</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SNS</td>
<td>Safe, novel space</td>
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<tr>
<td>SO</td>
<td>Stationary objects</td>
</tr>
<tr>
<td>StEM</td>
<td>Structural equation model</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
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<tr>
<td>TBS-T</td>
<td>Tris buffered saline with 0.1 % Tween-20</td>
</tr>
<tr>
<td>TGFβR1</td>
<td>Transforming growth factor beta receptor 1</td>
</tr>
<tr>
<td>TRPC5</td>
<td>Transient receptor potential channel 5</td>
</tr>
<tr>
<td>UR</td>
<td>Unconditioned response</td>
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<tr>
<td>US</td>
<td>Unconditioned stimuli</td>
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<td>USV</td>
<td>Ultrasonic vocalization</td>
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<td>X-ray- induced T (X;Y) V7 rearrangement</td>
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Chapter 1

1 Introduction

Although learning and memory (LM) have been extensively studied and highly debated for a long time, it remains a critical subject requiring more scholarly attention, as it has immensely broad implications for cognitive sciences and other related areas. Current and, for that matter, future researchers should recognize the importance of dealing, in their research, with the fundamental mechanisms and neural basis of LM.

The clinical research about memory impairment shows why researchers should address this topic. As many researchers suggest, memory impairment is a feature of a number of mental disorders, including mental retardation (Vaillend et al., 2008), Alzheimer’s disease (Dubois et al., 2013; Selkoe, 2001), Parkinson’s disease (Barch and Ceaser, 2012; Foster et al., 2013), anxiety disorder (Zlomuzica et al., 2014), and schizophrenia (Matza et al., 2006). Although neuropsychiatric disorders are highly prevalent, and increasingly recognized as challenges by healthcare systems worldwide (Bebbington, 2001), it is well understood that developing targeted therapies have been hampered for various (and unknown) reasons, leading to lack of mechanism-based treatments. The causes of these disorders are multiple, including genetic, environmental, and developmental. And also, often no unifying molecular or cellular abnormalities in these human diseases can be used to validate animal models. This situation suggests that researchers need to direct their attention to developing models that allow them to examine the problem of memory impairments from the more comprehensive understanding of human cognition and learning processes. Dosing so will allow research to develop therapies with promising outcomes.

Memory formation depends strongly on learning strategies - that is, ways that individuals explore or process information. “Exploration” is a widely employed concept in behavioral research, and refers to all activities directed at increasing information intake from the learning environments (Archer et al., 1983; Berlyne, 1966). Different types of exploration can be used by subjects, depending upon their respective environments, including novelty, degree of stress, threat, volition, and/or affection (Kormi-Nouri et al., 2005; Schwabe and Wolf, 2010; Voss et al.,
These factors might influence the efficiency of LM. For example, volitional control during exploration benefits memory performances in cats (Held and Hein, 1963) and humans (Voss et al., 2011). In addition, learning or exploration in a safe, non-threatening environment helps students improve their performance (Flum et al., 2006), while learning under mild stress tends to impair memory formation in humans (Schwabe and Wolf, 2010). Therefore, certain environments and/or exploration strategies may promote learning and improve memory performances.

This study aims to determine types of exploration that facilitate LM, and to find a target molecule and neural circuits to provide additional behavior readouts involved in this process. Discovering this information is beneficial not only in order to find therapeutic targets for memory impairments, but also useful to more effectively clarify behavioral phenotypes in the genetically engineered mice used to study different mental disorders, including neurological disease related to memory impairment and anxiety disorder.

1.1 Introduction to Learning and Memory (LM)

Although innate human behaviors and reflexes exist, our brain heavily relies on LM and experience for many purposes; therefore, investigating the principles of learning will be beneficial for understanding why, whether in a normal or diseased states, we behave the way we do.

Memories can be relived, and they represent a “storehouse” of accumulated knowledge about the world, which contributes to a multitude of acts of perception, coordination, and emotion. Memories accrue by learning from experiences, and they shape our individual mental and emotional characteristics. Thus, investigating the principles of learning will help us to understand normal and adaptive behaviors, and may also help develop more effective therapies for diseases related to memory impairment. While individuals differ in their behaviors, these differences may be explained by differing learning experiences, and/or genetic effects. Undoubtedly, many human attributes are molded in a similar way by the interaction of genes and the environment with the learning process. It may be possible that a person can create an environment to encourage certain traits he or she wishes to have; at the same time, it may also be possible that certain types of exploration will facilitate better learning compared to others.
Due to its significance in shaping human life, LM has long been studied and discussed. For thousands of years, philosophers have pondered and scholars have written about the quality of memory. In the last 100 years, LM has continued to generate tremendous research interest, and remains a current research topic of interest; psychologists have focused on examining the nature of learning and its consequences on memory, while neuroscientists have focused on exploring the basis of LM in terms of structure and function of our nervous system.

1.1.1 Modern memory research

The learning process allows an organism to survive in changing environments by providing stored information about sources of satisfaction or danger with experience. The theory of learning is about adaptation to the environment, and extends from Charles Darwin’s theory (1872) of evolution. At the turn of 20th century, research on LM gained greater traction with notable scholars developing experimental methods to study LM, including Hermann Ebbinghaus, who used himself as his sole subject, Ivan Pavlov who conducted studies on conditioning in dogs, and Edward Thorndike who studied trial-and-error learning in cats.

Hermann Ebbinghaus (1850-1909) performed the first human memory studies and established experimental design to study LM, such as the retention curve and the learning curve. He taught himself nonsense syllables consisting of consonant-vowel-consonant trigrams for which he had no prior experience. Ebbinghaus learned lists of 13 syllables to the point where he was able to repeat the lists twice in order without error. He then examined at his ability to recall these lists with various delays. He measured the amount of time needed to relearn the lists in order to pass the same criterion, specifically two perfect recitations. Here, Ebbinghaus was particularly interested in determining how much easier it became to relearn the lists. He expressed these savings concerning a percentage of the early learning, which he referred to as the retention curve, where the initial forgetting is rapid while the rate of forgetting slows down dramatically over time. The negative acceleration in the retention curve is a consequence of the rapid decrease in the rate of forgetting. In another experiment, Ebbinghaus relearned the lists of nonsense syllables each day for six days, showing the number of trials needed to relearn the lists each day. The number of trials decreased over time, reflecting improved learning of the lists. As such, the learning curve consisted of an adverse acceleration with smaller gains each day. Immediate after these findings, memory research has been spurred.
Ivan Petrovich Pavlov (1849-1936) discovered the conditioned reflex in his experiments. The basic methodology starts with a biologically significant unconditioned stimulus (US), which reflexively evokes some unconditioned response (UR). For example, when food is used as US, the salivation after look at US will result in an UR. Subsequently, the food would be paired with a neutral conditioned stimulus (CS), such as a bell. After such pairings, the bell, CS, was able to evoke the response, conditioned response (CR), itself. The classical conditioning paradigm has received considerable research focus in the last few decades and has been applied to measure many types of LM.

Edward L. Thorndike’s (1874-1949) behavioral studies were differed from those of Pavlov in letting the animals to learn by trial and error rather than conditioning. Thorndike placed a cat in a puzzle box with food outside. If the cat hit a device or lever, the door would open, and the cat was able to escape and eat the food. With the repeated trials of this task, Thorndike was able to measure how quickly a cat learned to escape from puzzle box. Once the cat hit the device randomly, over trials, the randomness of this behavior gradually diminished as the cats learned the task. This became known as instrumental learning, which is a volitional type of learning, and differs from classical learning. Pairing of stimuli and the consequences therein, is required for certain forms of learning (Held and Hein, 1963). This trial-error type of operant conditioning has since been extended to behavior modification as a treatment approach.

With the development of experimental methodologies in LM to study learning processes, human patients with amnesia extended the LM research further, allowing researchers to study how and where memory is stored. The concept of retrograde amnesia was first posited by Ribot (1882), and Ribot’s law explains temporally graded retrograde amnesia that spares more distant memories than recent ones. Korsakoff (1887) studied retrograde amnesia patients who had impaired recent memory without intelligent or judgment loss. These studies have led to an influential model of human LM (Schacter and Tulving, 1994; Fig. 1-1).
1.1.2 Types of memory

Psychologists have developed a conceptualization of memory in terms of stages, types, and processes. This chapter will review three major memory store stages, including sensory, short-term, and long-term store/memory, and then two main categories of long-term memory, explicit and implicit (Atkinson and Shiffrin, 1968). Subsequently, the outline of long-term memory, with a particular emphasis on the distinct brain regions involved in each type of memory process, is followed. And finally, the three processes of episodic long-term memory, encoding, storage, and retrieval will be introduced.

As shown in Figure 1-1, memory can be characterized by stages with different holding time and capacity. After our sensory organs acquire data from environments, the first stage, called 'sensory memory,' holds the incoming sensory/perceptual information for a very brief period; the sensory information system transiently retains the 'copies' of what sensory organs sense. From all of the sensory information, only salient information undergoes to the short-term/working memory. Ignored information is lost, and new perceptual data replaces old information (Ericsson and Chase, 1982). Information stored for short-term moves on to long-term storage through repetition and rehearsal; some information is stored for a life-time, while a person will lose some information over time. Often, it is this long-term memory that people consider memory, with large.

Long-term memory includes two major subdivisions: explicit/declarative memory and implicit/procedural memory. Explicit/declarative memory consists of the memory of facts and events, which can be explicitly stored, and requires conscious and explicit thought in order to be recalled (or "declared"). This type of memory can be further categorized into episodic and semantic memory. Implicit/procedural memory ("knowing how") is the memory of skills and performances without conscious awareness, which is acquired through repetition and practice, and further allows automatic sensory behavior, such as playing a piano or riding a bike.

Each type of long-term memory is stored in particular regions of the brain through different processes. For example, the hippocampus (HPC) is involved in declarative memories, but not in procedure memories. Thus, as shown in a patient "H.M." (Scoville & Milner, 1957), who had bilateral medial temporal lobe resection, this resulted in removal of parts of the HPC.
and amygdala (Amyg). Researchers found that H.M. had retrograde amnesia as well as anterograde amnesia. In testing H.M’s performance with the repeated trials, the patient could not remember completing the task; however, he improved in the performance itself. This example not only proves the existence of different types of memory, but also demonstrates that procedure memory and declarative memories are distinct. Although particular brain regions are involved in different types of long-term memory, the cellular and molecular processes shown in 1.1.4 are not different between different types of long-term memory (Bailey et al., 1996). However, different types of molecules may be involved in different memory processing stages of episodic memory, encoding, consolidation, storage and information recall. The encoding stage is where learning takes place, and without learning there is no memory. As such, the factors that affect learning/encoding processes and the molecules involved in encoding stages will be further discussed.
Figure 1-1. Types of memory (Based on Atkinson and Shiffrin (1968) and Schacter and Tulving (1994)).

The top level shows Atkinson and Shiffrin Multi-store Model (1968) of memory as consisting of three distinct components called the sensory register, the short-term store, and the long-term store. The long-term memory divides into two broad categories, as defined by experimental evidence that suggests each is a separate phenomenon. Anatomical regions are distinct to types of memory. For example, the hippocampus is not required to encode long-term unmoral associated memory.
1.1.3 Exploration as a learning process

The encoding parts of LM are the processes by which we explore, gather, and organize information on sensory inputs from our environment, and provide the raw information in which associations are built. Exploration or learning is essential aspects of behavior. In a laboratory mouse, for example, we observe “exploration” by behavioral outputs, such as running around, sniffing, rearing upright using one’s hind legs, making contact with stimuli by means of vibrissae or paws, and turning the head and body towards particular stimuli (Archer et al., 1983). However what motivates exploration is less clear.

In the field of exploratory behaviors, both Harlow (1950) and Montgomery (1952) stressed the importance of considering all motivational processes of behavior. Different types of motivation for exploration occur depending on the environmental factors, such as novelty, as well as the degree of stress, threat, or volition, all of which can also affect LM performance (Kormi-Nouri et al., 2005; Schwabe and Wolf, 2010; Voss et al., 2011). Studies about exploration drive began in the 1960s, and this section reviews different types of exploration by providing their definitions, motivations, and factors in relation to their effects on LM.

Many scholars have attempted to define exploration. For example, Berlyne (1960) categorized the types of exploration into extrinsic vs. intrinsic, inspective vs. inquisitive, and specific vs. diversive exploration. Extrinsic responses are defined as direction at obtaining information about a conventional reinforce, or other biologically significant event, such as food; in contrast, intrinsic exploration is defined as exploration directed at stimuli of little apparent biological consequences. Inspective exploration refers to responding to environmental change, while inquisitive exploration refers to responding to change. Finally, specific exploration is defined as behavior directed at obtaining information about a particular changed object or event, while diversive exploration is behavior directed at obtaining stimulus change and information from any environmental source. With the Berlyne's categorization of exploration, it is important to investigate how intrinsic exploration occurs.

I have focused on two types of motivation that drive exploration, and have implications for survival: 1) instrumental motivation, such as food, safety, and reproduction (Montgomery and Monkman, 1955; Zimbardo and Montgomery, 1957); and 2) intrinsic motivation to
investigate novel stimuli (curiosity) (Bunzeck et al., 2011; Guitart-Masip et al., 2010). The attempts to account for the motivation of intrinsic exploration include the postulation of exploratory-investigatory drives, appeals to concepts of optimal arousal, and explanations based on the energizing effects of fear widely applied to the intrinsic exploration of nonhuman animals. This motivation of intrinsic exploration may be advantageous or disadvantageous for learning. Other factors, such as novelty of the items of learning and how volitionally the organism engaged in learning, also influence the learning efficacy.

**Optimal stimulation and arousal theories**

According to the optimal stimulation and arousal theory postulated by Berlyne (1960), organisms behave in order to maintain an optimum level of stimulation or arousal due to environmental changes (novelty). Therefore, environmental change may increase arousal above the optimum; in such an event, arousal can be reduced in two ways: through inspective exploration or through withdrawal and avoidance. However, this theory is limited in several ways. First, the theory lacks the ability to explain the motivation for exploration. As Fowler (1965) commented, arousal theory tends to explain everything, but predicts nothing. A second basic problem with this theory is its inability to determine what is optimal, and gauge whether an organism is above or below that threshold. Finally, this theory does not account for the fact that animals can be aroused not only by environmental changes (novelty) but also by changes in internal states (instrumental motives).

**Fear and exploration**

Environmental changes can elicit one of either exploration or withdrawal, based on a biphasic or a monophasic system. Biphasic theories postulate two separate but interacting motivational systems (Montgomery and Monkman, 1955; Schneirla, 1959; Valle, 1972). According to Montgomery, the behavior elicited by environmental change is the net outcome of the competition between the tendency to explore (motivated by curiosity or basic drives) and the tendency to withdraw (motivated by fear). Monophasic theories, on the other hand, postulate a single motivational process operating with a threshold, below which exploration is more likely to occur and above which withdrawal is more likely to occur. The fear generated by environmental change usually motivates exploration (Halliday, 1966; Lester, 1967; Mowrer, 1960), assuming
an inverted U-shape relationship between the notion that high levels of fear elicit withdrawal and lower levels of fear elicit approach and investigation (Lester, 1967). This theory has sparse evidence for support. Although exploration reduces fear, it is unlikely that fear alone motivates the behavior (Russell, 1973).

**Novelty**

Novelty not only triggers exploration and learning, but also has potential benefits over familiarity for new memory formation, with the effects of priming and/or distinctiveness. The novelty effects arise primarily from a line of cognitive research that examine the detrimental effect that pre-study repetition of study materials has on participants’ ability to study the repeated materials later. In such cases, research suggests that participants are less able to successfully identify the repeated materials. This effect was first observed by Kinsbourne and George (1974). Subsequently, Tulving and Kroll (1994 and 1995) conceptualized the novelty effect as priming effects; the researchers concluded that novel stimuli are encoded better than familiar stimuli, and also revealed a significant difference in the HPC by positron emission tomography (PET). This basic finding has been replicated many times (Åberg and Nilsson, 2001; Kormi-Nouri et al., 2005). More recently, some have suggested that novelty effects may also be linked to distinctiveness (Tulving and Rosenbaum, 2006).

**Volition**

In humans, self-directed ‘volitional’ exploration of an object, and its location, results in improved recognition performance, with the benefit being linked to a brain network centered on the HPC (Voss et al., 2011). This is consistent with the observation in rats, showing that the experimentally manipulated motivation to explore objects differentially recruits specific sub-regions of the HPC (Albasser et al., 2010). Previous research revealed the importance of the dentate gyrus (DG) in driving the exploratory behavior of safe, novel space (Saab et al., 2009).

Although researches have determined the benefits of environmental change (novelty) over familiarity and volitional over non-volitional exploration in learning, the fear factor related to novelty has not been considered.
1.2 Characterizing Behavior Phenotypes

The use of genetically modified mice in neuropsychiatric research has grown exponentially in the last decade with the development of methods that allow researchers to target precise genetic alterations and because of the practical advantage of using mice for research. Genetically modified mice are used to study the neural basis of behavior, to discover new molecular mechanisms of illnesses, to identify new drug targets for the generation of novel efficient treatments, and to further validate gene candidates of diseases identified in clinical samples. However, studies can produce conflicting results due to difficulties in phenotyping transgenic mice, such as lack of standardization, background differences of transgenic mice and confounding factors that affect the behavior test results. Here, I would like to focus on studies of LM and anxiety.
Figure 1-2. How to test for hypothesis on causes of a disease in gene-targeted mutant mice. (Adapted from Crawly, 2007)

A line of mice with the mutated gene is generated. The characteristics of the mutant mice are identified relative to wild-type mice. Salient characteristics relevant to the human disease are quantified. These disease-like traits are then used as surrogate markers to evaluate the effectiveness of treatments. Putative therapies are administered to the mutant mice. A treatment that prevents or reverses the disease traits in the mutant mice is taken forward for further testing as a potential therapeutic treatment for the human genetic disease.
1.2.1 Pre-assessment

Crawly (2005: Table 1-1) suggests a three-tiered strategy for comprehensive behavioral phenotyping. The three tiers include: 1) general health and neurological reflexes (to perform most behavioral assays requires mice not be physically compromised); 2) sensory and motor abilities (mice must be able to move, see, hear and smell); and 3) at least three behavior tests for each behavioral domain of interest.

The first and second tiers to measure the gross general health should be done before measuring the hypothesized domain of interest. For example, the most common behavioral assays, the open field activity test and the Morris water maze, are useful in measuring anxiety and LM, respectively. However, because both of these tests depend on successful ambulation of the mouse, without knowing motor and/or swimming function, we cannot conclude whether behavior test results are anxiety or LM related, respectively. Also, for the Morris water maze, if mice vision is impaired, mice will perform badly because of blindness, not because of a decreased LM ability. By knowing physical ability, mice with a mutation that reduces locomotor activity can be tested accurately for an anxiety-like phenotype using the Vogel task, which requires minimal locomotion. Similarly, the light↔dark task will be inappropriate for a blind background strain of mice or a mutation that causes blindness. Thus, knowledge about general health, neurological reflexes, and sensory and motor abilities is essential to allow investigators to design behavioral tasks appropriate for the domain of interest.
Table 1-1. Preliminary observations for general health (Modified from Crawly 2007).

The main behavior tests that are suitable to determine general health, neurological reflexes, motor skills, sensory skills are introduced. These tests are important to remove confounds and to design further specific domains of analysis.
1.2.2 Domain of interests: exploration based-tasks

The third step of phenotyping is performing at least three different behavior tests in the domain of interest. Distinct domains of interest may include LM, anxiety, depression, social behavior, and reward. The success of this approach is largely dependent on the usefulness of available behavioral tests for each domain of interest in mice. The optimal behavior tests are quantitative, reproducible, and can be conducted in controlled laboratory environments. Exploratory behaviors are often used to measure responses in mice, such as in the object recognition memory test, most anxiety-related tasks, and in the three-chamber social behavior test.

Here, I focus on the effects of exploration in LM. The learning process (or exploration) cannot be studied directly; instead, it must be inferred from changes in behavior. Learning is something that occurs as a result of certain experiences and process changes in behavior. The inaccessibility of learning is one reason many approaches are used to study it. Exploratory behaviors, such as direct investigation and locomotor changes, have been measured as an indicator of LM. Therefore, I introduce hippocampal-dependent LM behavior tests that use exploration as an indicator of LM, namely the object recognition memory test.

Exploration based tasks are used to understand some LM tasks. However, I cannot exclude the fact that exploration-based tasks also exploit the conflicting tendencies to approach versus avoidance of a potentially dangerous area. Therefore, approach–avoidance conflict tests are available for evaluating anxiety-like behaviors in mice, such as open field, elevated plus-maze (EPM) and light/dark box. The avoidance of the aversive area can be used to indicate the level of innate anxiety in transgenic/mutant mice.

Here, I describe these techniques and postulate on the contribution of genetic modeling in animals to our understanding of the underlying neurobiology of anxiety disorders.

1.2.2.1 The measurement of exploration for object recognition tasks

The object recognition task assesses cognition, specifically spatial memory and discrimination. Rodents spontaneously tend to spend more time exploring novel objects or areas than familiar
objects or areas. This tendency allows the researcher to discriminate between objects a mouse considers novel versus familiar based on the time the mouse spends exploring the objects. Displaced (novel) versus stationary (familiar) objects can similarly be tested. The choice of a behavior output to measure as an indication of direct investigation or exploration requires careful consideration. Berlyne (1950) used the duration of sniffing at objects as a measure of exploration in rats. Latency to approach an object has also been used as indicator of direct investigation. In addition, locomotion in a novel environment is commonly assumed to represent exploratory behavior; however, animals may also be moving around while attempting to search for an escape. Furthermore, it is difficult to determine by simple observation whether a rodent that moves about more is exploring more to acquire information. For example, a fast-moving rat may be relatively inattentive to environmental cues whereas one that remains stationary may be attentively watching and sniffing the air.

Dissociation of exploration from locomotion is another factor to consider. Several studies suggest that locomotor activity can be distinguished from other investigatory responses. Leyland and colleagues (1976) reported a dissociation between locomotion and direct investigation of novel stimuli (contacting the stimuli) in a modified Berlyne’s box. In this box, novel stimulation increased exploration but not locomotion. Injection of amphetamine had the reverse effect. Activity and exploratory investigation have been separated by physiological manipulations, including injection of dopaminergic agonists (Isaacson et al., 1978), lesions of septal nuclei (Kelsey et al., 1989) and hippocampectomy (Morris et al., 1982). The use of locomotion as a measure of exploration has limitations. Animals in novel environments move more, but the assumption that the level of activity provides an accurate measure of exploratory tendency should be avoided, although locomotory increase is usually accompanied by increased environmental sampling (Prescott, 1970).

I propose that rearing should be one of the several ethological measures that are more commonly employed in assessing hippocampal LM. A four-legged mammal is said to rear when it stands only on its hind legs, raising its forelimbs off the ground, usually momentarily. The number of rearings and/or the rearing duration have been measured and suggested to represent exploration (Bindra and Spinner, 1958), ‘excitability level’ in relation to internal factors (Lat, 1965), and an environmental novelty marker that can be profitably used to assess hippocampal
LM (Lever and Burton, 2006). One mechanism for HPC-dependent LM is the hippocampal spatial mapping program in which environment specific maps are constructed and updated by spatial exploration; during the mapping process rearing occurs in a distributed fashion throughout the environment. Rearing may function as an environment information gathering process, since rearing does not change upon novel objects and object position changes (Maier et al., 1988; Moses et al., 2002; Wesierska et al., 2003), but rearing movements are elicited by distal environmental changes (Lever et al., 2006). In novel situations, rearing as well as locomotor changes occur. However, rearing might be more sensible and efficient compared to locomotion in determining and updating spatial information. For example, in the Morris water maze test, when mice first find the hidden platform, they rear a lot, and the number of rearing movements is reduced as training proceeds (Sutherland et al., 1982). On the test day, when the hidden platform is moved to another quadrant, mice once again rear a lot on finding the platform. In the Morris water maze test, mice use distal cues to identify the location of the hidden platform (Sutherland et al., 1982), and thus rearing may serve as an environmental novelty marker, or it may contribute to information gathering.

Whether rearing is done as a means of information gathering remains questionable. The assumption made here is that rearing can potentially be used to examine memory encoding, and long-term plasticity models associated with spatial/contextual learning functions of the HPC. It is also possible that rearing is functional, but not essential, for normal place navigation. Rearing may also be avoidance and escape reaction. In the open field test, mice rear at the boundaries of an enclosure, bringing their forelegs onto the walls. Moreover, electrical stimulation of the periaqueductal grey matter (PAG) and the medial hypothalamus enhances the positive relationship between rearing or wall-supported rearing and a threat factor.

1.2.3 Molecular basis of learning and memory

In the process of learning, cascades of molecular and cellular events occur. Depending on the biochemical changes, synaptic connections decrease or increase, as championed by Hebb (1949). The two main molecular mechanisms of LM at a synapse are long-term potentiation (LTP) and long-term depression (LTD). The main molecules involved in this process are NMDA receptors, AMPA receptors, and protein kinases triggered by intracellular calcium (Ca\(^{2+}\)), such as
Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII), and protein kinase C (PKC).

As LTP and LTD have emerged as strong candidates for some of the neurophysiological mechanisms underlying the cellular basis of synaptic strengthening (Bliss, 1996; Lynch, 1986), researchers have started investigating whether the molecules involved in the process of LTP and LTD are also required or necessary for LM behaviorally by using transgenic and knockout (KO) mice of gene of interests (Thomas and Huganir, 2004). For example, kinases such as CaMKII, PKC, PKA, and several tyrosine kinases or phosphatases deleted mutant mice showed deficits in hippocampal LTP, and in hippocampal dependent memory tasks (Silva et al., 1992a,b; Abeliovich et al., 1993a,b; Bourtchuladze et al., 1994; Sakimura et al., 1995). In comparison, a transgenic ‘Doogie’ mice overexpressing the NR2B subunit of the NMDA receptor in adult forebrain showed more robust levels of LTP, as well as enhanced performance in hippocampal dependent LM tasks, such as the novel-object recognition test, Morris water maze, and contextual and cued fear memory (Tang et al., 1999). To date, more than 200 gene-targeted mutant mice are created and tested for LM in physiology and behavioral studies (Lee and Silva, 2009).

With the plethora of genetically modified mice, careful observation of behaviors and phenotypes are more than necessary to uncover the neural substrates of memory, and not to exclude possibilities involving other domains of behaviors (Fossella and Casey, 2006). For example, mice with targeted disruption of the gene subunit NR1 of NMDARs (known to play an important role in hippocampal LTP (Collingridge et al., 1983)) in the CA1 region of the HPC show severe impairment in spatial LM (Cui et al., 2004). Mice expressing 5% of normal levels of the NR1 subunit also produce a schizophrenia-like phenotype (Mohn et al., 1999). Therefore, this example reveals the importance of measuring more complete behavior phenotypes of transgenic mice. This is also true for other domains of behaviors, such as anxiety-related behaviors; systematic phenotyping of all behaviors is rarely done.

1.2.3.1 Measuring anxiety in laboratory animals

A variety of tests have been employed in animal modeling of anxiety disorders as listed in Table 1-2. The tests can be categorized based on whether they involve in conditioning, social or non-social behavior, single responses or behavior patterns, and response elicitation or suppression.
(Handley, 1991). Among these tests, open field, elevated plus maze (EPM) and dark/light transitions are the most frequently used to determine anxiety-related phenotypes in transgenic mice (Fig. 1-3). These tests are based on the assumption that mice are a naturally exploratory species, and that exploration-based tasks exploit the conflicting tendencies to approach versus avoid a potentially dangerous area. The aversive area takes different forms in the various tests: open, elevated arms (EPM), a light compartment/arena (light/dark exploration test, dark/light emergence test), or the central area of a brightly lit open field (open field test) (Belzung and Griebel, 2001; Holmes, 2001). The avoidance behavior of the aversive area can be measured to indicate the level of innate anxiety in transgenic/mutant mice. The neural systems involved in detecting danger and producing defensive responses are highly conserved in evolution, i.e. they are remarkably similar in all vertebrates (LeDoux, 1995). In addition, the neurochemical changes involved in normal/adaptive and pathological/maladaptive forms of anxiety may differ only in quantitative terms (File, 1992).

Approach-avoidance tests are critical in the assessment of anxiety-related behavior in mice and are popularly used among researchers (Table 1-3). Approximately half of experiments shown in Table 1-3 draw their conclusions using only one behavioral test, most commonly the percent of open arm entries in the EPM test or of central entries in the open field test. This raises the questions of whether one or two variables can adequately represent a complex psychological trait (such as learning). Additionally, the interpretation of animal models of anxiety-induced inhibition of exploration should be interpreted with caution since increased time spent in an aversive area can be interpreted in several ways: as decreased anxiety, or increased motivation, arousal or general locomotion (Wahlsten, 2001; Wahlsten et al., 2003; Würbel, 2001). For example, in the case of serotonin transporter (5-HTT) KO mice that show a general reduction in exploratory locomotion in a brightly lit open field arena, changes in exploration-based tests for anxiety behaviors difficulty distinguishing between a strong anxiety-like phenotype and an impairment in exploratory locomotion (File, 2001; McNaughton and Gray, 2000). In addition, these tests can also be influenced by differences in basal cognition (Refojo and Deussing, 2011). For example, animals may use different strategies, which can easily be misinterpreted as changes in anxiety like behavior. Therefore, mice with targeted genetic mutations that do not alter exploratory motivation, motor ability, or recognition memory are also suitable test subjects for
these paradigms

The use of multiple different testing strategies may minimize the influence of confounding factors in behavioral assays. In addition, since each test for anxiety-like behavior comes with limitations and no single test provides the ideal measure of anxiety, the importance of using several different types of anxiety tests cannot be overstated. In addition, internal controls such as behavior comparisons between dark and bright zones should be done, and behavior assessments should include not only the main parameter of interest but also other behavioral phenotypes, especially rearings and ultrasonic vocalizations (as discussed in chapter 1.4) Limitations of exploration-based tests highlight the need for new tests, including non-anxiety based tests.
Table 1-2. A list of behavior tasks for testing anxiety phenotype. (Adapted from Holmes 2001)

<table>
<thead>
<tr>
<th>Unconditioned responses</th>
<th>Refs</th>
<th>Conditioned responses</th>
<th>Refs</th>
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</thead>
<tbody>
<tr>
<td><strong>Exploratory behavior</strong></td>
<td></td>
<td><strong>Conflict tests</strong></td>
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<tr>
<td>Open field(s)</td>
<td>(Prut et al., 2003)</td>
<td>Geller-Seifter</td>
<td>(Geller et al., 1962)</td>
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<tr>
<td>Elevated Plus-Maze</td>
<td>(Rodgers et al., 1997)</td>
<td>Vogel</td>
<td>(Vogel et al., 1971)</td>
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<tr>
<td>Elevated T-Maze</td>
<td>(Viana et al., 1994)</td>
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<tr>
<td>Elevated Zero-Maze</td>
<td>(Shepherd et al., 1994)</td>
<td></td>
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<tr>
<td>Holeboard(s)</td>
<td>(File et al., 2001)</td>
<td></td>
<td></td>
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<tr>
<td>Free exploration paradigm</td>
<td>(Griebel et al., 1993)</td>
<td></td>
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<tr>
<td>Dark/light transition</td>
<td>(Bourin et al., 2003)</td>
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<tr>
<td><strong>Social behavior</strong></td>
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<tr>
<td>Separation-induced US</td>
<td>(Gardner et al., 1985)</td>
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<tr>
<td>vocalization (pups)</td>
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<tr>
<td>Stress-induced</td>
<td>(Sanchez et al., 2003)</td>
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<tr>
<td>vocalization (adults)</td>
<td></td>
<td></td>
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<tr>
<td>Social interaction</td>
<td>(File et al., 1980)</td>
<td><strong>Avoidance tests</strong></td>
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<td></td>
<td></td>
<td>Passive avoidance</td>
<td>(Sahgal et al., 1993)</td>
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<td></td>
<td></td>
<td>(Two-way) Active</td>
<td>(Fernandez-Teruel et al., 1991)</td>
</tr>
<tr>
<td>Anxiety test battery</td>
<td>(van Gaalen et al., 2000)</td>
<td>avoidance</td>
<td>(Graeff et al., 1998)</td>
</tr>
<tr>
<td>Mouse defense test battery</td>
<td>(Blanchard et al., 2003)</td>
<td>Inhibitory avoidance</td>
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<tr>
<td></td>
<td></td>
<td>(Elevated T-Maze)</td>
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<tr>
<td>Predator odor avoidance</td>
<td>(Staples et al., 2010)</td>
<td>Fear-potentiated</td>
<td>(Korte et al., 2003)</td>
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<tr>
<td></td>
<td></td>
<td>behavior in the EPM</td>
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<tr>
<td><strong>Others</strong></td>
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</tr>
<tr>
<td>Hyponeophagia</td>
<td>(Britton et al., 1981)</td>
<td>Conditioned</td>
<td>(Sanger et al., 1991)</td>
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<td></td>
<td></td>
<td>suppression</td>
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<tr>
<td>Open field drink test</td>
<td>(Stout et al., 1994)</td>
<td>Fear-potentiated</td>
<td>(Sanger et al., 1991)</td>
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<td></td>
<td></td>
<td>startle</td>
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<tr>
<td>Startle response (baseline)</td>
<td>(Schwegler et al., 1997)</td>
<td>Conditioned</td>
<td>(Sanger et al., 1991)</td>
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<td></td>
<td></td>
<td>emotional response</td>
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</tbody>
</table>
Table 1-3. Representative behavior test results for genetically engineered mice.

(Adapted from Holmes 2001) 5-HT=5-hydroxytryptamine/serotonin; CRH=Corticotropin-releasing hormone, CRH R1,2= CRH receptor 1,2; DRD3,4=Dopamine type 3,4 receptor; EPM=elevated plus-maze; GAD 65=Glutamate decarboxylase 65; KO= Knockout; MAO=Monoamine oxidase; NO=Novel object, OE=Overexpression

<table>
<thead>
<tr>
<th>Genetic Mutation</th>
<th>Anxiety-related phenotype</th>
<th>Behavioral test (Ref)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GABA(_A) (\alpha)(_1) subunit mutant</td>
<td>No anxiety-related phenotype</td>
<td>EPM (Rudolph et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light/dark transition (Rudolph et al., 1999)</td>
</tr>
<tr>
<td>GABA(_A) (\delta) subunit KO</td>
<td>No anxiety-related phenotype</td>
<td>EPM (Mihalek et al., 1999)</td>
</tr>
<tr>
<td>GABA(_A) (\gamma)(_2) subunit heterozygous KO</td>
<td>Increased anxiety-like behavior</td>
<td>EPM (Crestani et al., 1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light/dark transition (Crestani et al., 1999)</td>
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<tr>
<td></td>
<td></td>
<td>Free exploratory paradigm (Crestani et al., 1999)</td>
</tr>
<tr>
<td>GABA(_A) (\gamma)(_2) subunit (long variant) KO</td>
<td>Increased anxiety-like behavior</td>
<td>EPM (Homanics et al., 1999)</td>
</tr>
<tr>
<td>GAD 65 KO</td>
<td>Increased anxiety-like behavior</td>
<td>Elevated zero maze (Kash et al., 1999)</td>
</tr>
<tr>
<td>MAOA KO</td>
<td>Decreased anxiety-like behavior</td>
<td>Open field center time (Kash et al., 1999)</td>
</tr>
<tr>
<td>MAOB KO</td>
<td>No anxiety-related phenotype</td>
<td>EPM (Grimsby et al., 1997)</td>
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<tr>
<td></td>
<td></td>
<td>Open field center time (Grimsby et al., 1997)</td>
</tr>
<tr>
<td>Catechol-O-Methyltransferase KO</td>
<td>Increased anxiety-like behavior</td>
<td>Light/dark transition (Grimsby et al., 1997)</td>
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<tr>
<td>DRD3 KO</td>
<td>Decreased anxiety-like behavior</td>
<td>EPM (Steiner et al., 1997)</td>
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<td></td>
<td></td>
<td>EPM (Xu et al., 1997)</td>
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<tr>
<td>DRD4 KO</td>
<td>Increased anxiety-like behavior</td>
<td>Open field center time (Dulawa et al., 1999)</td>
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<td></td>
<td></td>
<td>Emergence test (Dulawa et al., 1999)</td>
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<td></td>
<td></td>
<td>NO exploration (Dulawa et al., 1999)</td>
</tr>
<tr>
<td>5-HT1(_A) receptor KO</td>
<td>Increased anxiety-like behavior</td>
<td>EPM (Ramboz et al., 1998)</td>
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<tr>
<td></td>
<td></td>
<td>Open field center time (Heisler et al., 1998; Ramboz et al., 1998)</td>
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<tr>
<td></td>
<td></td>
<td>Elevated zero maze (Heisler et al., 1998)</td>
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<tr>
<td></td>
<td></td>
<td>NO exploration (Heisler et al., 1998)</td>
</tr>
<tr>
<td>5-HT1(_B) receptor KO</td>
<td>Decreased anxiety-like behavior</td>
<td>EPM (Brunner et al., 1999)</td>
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<td></td>
<td></td>
<td>Open field center time (Zhuang et al., 1999; Malleret et al., 1999)</td>
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<td>Ultrasound vocalization in pups (Brunner et al., 1999)</td>
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<td>EPM (Malleret et al., 1999)</td>
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<td>Light/dark transition (Ramboz et al., 1995)</td>
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<td>Open field center time (Ramboz et al., 1995)</td>
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<td>Emergence test (Tecott et al., 1998)</td>
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<tr>
<td>Genotype</td>
<td>Behavior Description</td>
<td>EPM Studies</td>
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1.3 Introduction to Neuronal Calcium Sensor-1 (NCS-1)

As previously described, “volitional exploration” might be an effective learning strategy to have better memory performance. A plethora of molecules are known to be involved in LM processes on normal, pathological or enhanced cognitive states in animals. However, the molecular understanding in “volitional exploration” is unknown. I suggest neuronal calcium sensor-1 (NCS-1) to be a candidate gene for “volitional exploration”, due to its known role in exploration and LM.

The Roder lab has previously shown that over-expression of NCS-1 in the murine DG selectively increased exploration as well as spatial LM (Saab et al., 2009). Several lines of evidence suggest that NCS-1 plays a role in LM, and may be involved in the motivation to learn. In addition to its established role in LM, NCS-1 is likely involved in the degree of intrinsic reward associated with exploration and by consequence, exploratory drive. This may provide a unique advantage to learning, by eliciting unique sensory features from the environment in a self-directed manner. One hypothesis I will explore is that NCS-1 is involved in the relationship between intrinsically motivated exploration and memory performance. Here I will overview the biochemical characteristics of NCS-1 and also its possible involvement in “active exploration” by reviewing the functions of NCS-1 in depth.

1.3.1 Neuronal calcium sensor family

Ca$^{2+}$ signaling with extensive versatility is one of the central processes required for a wide repertoire of biological components comprising the Ca$^{2+}$ modulated molecules (Berridge et al., 2000). Belonging to the Ca$^{2+}$ sensitive molecules, neuronal calcium sensor (NCS) proteins are the subfamily of the calmodulin super family that and trigger different biological processes to regulate signal transduction in neurons and photoreceptor cells (Bourne et al., 2001; Burgoyne and Haynes, 2012; Burgoyne and Weiss, 2001; McFerran et al., 1999; Weiss et al., 2010). Amongst the family members, NCS-1, neurocalcins/VILIPs (visinin-like proteins), and KChIPs (K+ channel interacting proteins) are expressed in various neurons of the nervous system whereas recoverin and GCAPs (guanylate cyclase-activating proteins) are predominantly or solely expressed in the retina (Polans et al., 1996). Whilst being similar to Calmodulin, they are distinguished usually by having a higher affinity for Ca$^{2+}$ binding. And certain family members
are membrane targeted through N-terminal myristoylation (NCS-1, VILIPs, recoverin, and KChIP1) or palmitoylation (certain KChIPs). The N-terminal glycine is myristoylated in vivo through the action of N-myristoyltransferase. It is widely accepted that the myristoyl chain confers the capability to link the NCS family proteins to the membrane through the “myristoyl switching” mechanism (Ames et al., 1997; Burgoyne and Weiss, 2001; McFerran et al., 1999; O'Callaghan et al., 2002), which, eventually, can be modulated by changing Ca$^{2+}$ concentration (O'Callaghan et al., 2002). Some NCS proteins display reversible membrane association through a unique Ca$^{2+}$/myristoyl switch mechanism (Ames et al., 1997; O'Callaghan, 2003; O'Callaghan et al., 2002; Spilker et al., 1997). Despite having high levels of sequence and structural similarity between NCS proteins, NCS-1 (also called frequenin homolog, frq) has a number of known non-redundant functions (Burgoyne, 2007; Burgoyne and Haynes, 2012).

1.3.2 NCS-1 conserved calcium sensor: structure and its interactors.

_Ncs1_ was first discovered (as the _frequenin_ homolog) a decade ago in a _Drosophila melanogaster_ mutant and assigned a role in synaptic transmission (Pongs et al., 1993). The X-ray- induced T (X;Y) V7 rearrangement, close to the _shaker_ gene, of mutants were found to shake their legs vigorously, thus due to genetic and behavioral evidence the mutants were originally thought to be a Shaker-like mutant (Tanouye et al., 1981). Subsequently, the frequency-dependent facilitation of neurotransmitter release was observed in V7 mutants dna also the upregulation of a novel calcium- binding protein was found named the gene as _frequenin_ (Pongs et al., 1993). This is how _Ncs1_ was first identified.

NCS-1 is a conserved protein and has orthologues from _Saccharomyces cerevisiae_ (Frq1) (Hendricks et al., 1999) to man (McFerran et al., 1998; Pongs et al., 1993). The protein sequence of NCS-1 across species is highly preserved with 100 % among mammals and 60 % between yeast and humans (Bourne et al., 2001). The structure of NCS-1 is characterized by EF-hand motifs, which can bind Ca$^{2+}$ ions. There are 4 EF-hand motifs (Fig. 1-3), namely EF1, EF2, EF3 and EF4, but EF-1 is not able to bind Ca$^{2+}$ ions (Bourne et al., 2001; Burgoyne and Weiss, 2001). NCS-1 is N-terminally myristoylated, a modification that allows its association with the plasma membrane and the trans-Golgi network (O'Callaghan et al., 2002). Through the myristoylation site, NCS-1 cycles between membrane and cytosolic pools (Handley et al., 2010). Opposing to the EF- motifs is a central and wide hydrophobic crevice (HC) (Ames et al., 2000; Amici et al.,
A recent study of the nuclear magnetic resonance (NMR) solution structure of human NCS-1 showed that the C-terminal tail of NCS-1 occludes the C-terminal part of the groove, acting as an auto-inhibitor to block substrate access to the HC and confer stability to the protein by shielding the HC from the environment (Martin et al., 2013) that can interact with various physiological targets activating specific biological processes (Ames et al., 2000; Burgoyne, 2007; Lim et al., 2011; Weiss et al., 2010). The known interactors of NCS-1 are following proteins (Haynes et al., 2006; McCue et al., 2010; Fig. 1-4): PI4K IIIβ (phosphatidylinositol-4-kinase IIIβ) (de Barry et al., 2006; Haynes et al., 2005) and its orthologue Pik1 in yeast (Hendricks et al., 1999), ARF1 (ADP-ribosylation factor-1) (Haynes et al., 2005; 2007), IL1RAPL1 (interleukin receptor accessory protein like-1) (Bahi et al., 2003), TRPC5 (transient receptor potential channel 5) (Hui et al., 2006), Ins(1,4,5)P$_3$R (inositol 1,4,5-trisphosphate receptor) (Boehmerle et al., 2006; Schlecker et al., 2006), Cav 2.1 (ionotropic P/Q-type calcium channel) (Tsujimoto, 2002), dopamine type-2 receptor (D2R) and dopamine type-3 receptor (D3R) (Kabbani et al., 2002). Biological functions of NCS-1 vary among organisms due to the particular interaction partners that are expressed.

To figure out the function of NCS-1 and its interacting partners, an interfering NCS-1 C-terminal peptide (CTN), consisting of the last 33 amino acids of NCS-1, has been used to disrupt binding of target proteins to this region (Dason et al., 2009; Hui and Feng, 2008; Hui et al., 2007; Romero-Pozuelo et al., 2007; Tsujimoto, 2002). This peptide consists of part of the fourth EF hand, but does not bind Ca$^{2+}$ (Saab et al., 2010). It is unknown which proteins or how many bind to this region. The CTN affects Ca$^{2+}$ currents and signals; thus, it is possible that one of the target proteins is a Ca$^{2+}$ channel or a protein that regulates Ca$^{2+}$ channels.
Figure 1-3. A diagram showing NCS-1 structure.

(A) Diagram showing NCS-1 protein with its structural domains including EF hands (orange=functional, green=nonfunctional) and the position of the P144S mutation. Surface representation of the most representative structures. (B) The most representative structure for the MD-NMR trajectory, showing the surface representation highlights the shape of the HC and the allocation of the L3 into the crevice. Taken from Belluci et al., 2013.
Figure 1-4. A protein-protein interaction map showing the known interactions of NCS-1.

The figure summarizes data on the known protein interactions made by NCS-1 protein and indicates whether these are Ca\(^{2+}\)-dependent or -independent. The solid lines linking the proteins indicate that direct protein–protein interactions have been identified. Taken from Burgoyne, 2007. AP1,2=Activator protein 1,2; ARF1=ADP-ribosylation factor-1; CAPS=Ca\(^{2+}\)-dependent activator protein for secretion; Cav 2.1=ionotropic P/Q- type Ca\(^{2+}\) channel; D2R=dopamine type-2 receptor; GRK2=G protein-coupled receptor kinase 2; IL1RAPL1=interleukin receptor accessory protein like-1; Ins(1,4,5)P\(_3\)R=inositol 1,4,5-trisphosphate receptor; Kv4.2=Voltage-gated Potassium Channel 4.2; PDE=Phosphodiesterase; PI4K IIIβ=phosphatidylinositol-4-kinase IIIβ; TRPC5=transient receptor potential channel 5
1.3.3 NCS-1 expression patterns

Ncs1 mRNA and protein expression is widely distributed in the nervous system (both CNS and peripheral nervous system (PNS)) in adult mammals. NCS-1 expression in the CNS of flies (Pongs et al., 1993), rats (Martone et al., 1999), mice (Jinno et al., 2002), and humans (Chen et al., 2002) has been detected by western blot and immunohistochemistry. It is also expressed in the PNS: Presynaptic terminals in neuromuscular junctions of Drosophila larvae (Pongs et al., 1993), crayfish (Jeromin et al., 1999), frogs (Jeromin et al., 1999), and adult and developing rats (Garcia et al., 2005) are all labeled with anti-NCS-1 antibodies. NCS-1 is expressed essentially in most neuronal cell types in all brain regions (Gierke et al., 2004). NCS-1 is particularly highly expressed in several neural networks commonly known to play an essential role in associative LM, including the HPC (Génin et al., 2001; Gomez et al., 2001; Martone et al., 1999; Olafsson et al., 1997). In the HPC, Ncs1 is highly expressed in the DG, CA1, and CA3 stratum oriens and stratum radiatum where Ncs1 mRNA is upregulated after LTP (Génin et al., 2001). Additionally, NCS-1 co-localizes with syntaxin at axon terminals of the DG/CA3 synapse (Jinno et al., 2002). It is expressed primarily in axons and dendrites (Olafsson et al., 1997). Expression levels of NCS-1 vary in different neurons. For instance, it is expressed in the Purkinje cell layer of the mouse cerebellum but compared to that in the granule cell layer (Olafsson et al., 1997). NCS-1 is also present in some glial cells such as astrocytes, but it is absent in Schwann cells and oligodendrocytes (Olafsson et al., 1997). Higher levels of NCS-1 are found in presynaptic terminals of crayfish and frog phasic motor neurons than in their tonic counterparts (Bélair et al., 2005; Olafsson et al., 1997), raising the possibility that the differential expression of NCS-1 may contribute to the known functional differences at these synapses.

NCS-1 was originally thought to be exclusively expressed in the nervous system, but has since been detected in non-neuronal tissue (Gierke et al., 2004), including the heart (Guo et al., 2002; Nakamura et al., 2003). By far, the strongest expression in mammalian species occurs in the CNS. Localization studies have been conducted only in flies, mice and rats. Future localization studies on NCS-1 should use existing KOs to confirm specificity of NCS-1 antibodies.
1.3.4 NCS-1 functions

NCS-1 is the most extensively studied and founding member of the NCS family. Genetic manipulation at an organism level indicates many different roles for NCS-1, for reviews see (Amici et al., 2009; Burgoyne, 2007; Burgoyne and Haynes, 2010; Burgoyne and Weiss, 2001; Burgoyne et al., 2004; McFerran et al., 1999; Weiss, 2001). NCS-1 has been implicated in a wide range of important functions. It is a regulator of ion channels and postsynaptic receptors, with consequent effects on neurite outgrowth and synaptic transmission (Dason et al., 2009; Hui et al., 2007; Jo et al., 2008; Pongs et al., 1993; Romero-Pozuelo et al., 2007; Saab et al., 2009). Through these effects, NCS-1 affects higher functions, such as LM (Gomez et al., 2001; Saab et al., 2009). NCS-1 has also been implicated in neuroprotection (Nakamura, 2006) and axonal regeneration (Yip et al., 2010) and may thus have potential as a therapeutic agent. Here I will review NCS-1 function in LM related function and in relation to mental illnesses.

1.3.4.1 Survival

NCS-1 is essential for survival in yeast (Hendricks et al., 1999), but not in worms (Gomez et al., 2001), flies (Dason et al., 2009), and mice (Nakamura et al., 2011), although Ncs1 KO mice have a 30% reduction in survival compared to controls (Nakamura et al., 2011). A question that remains unanswered is why the absence of NCS-1 is lethal in yeast (Hendricks et al., 1999), but not in worms (Gomez et al., 2001), flies (Dason et al., 2009), and mice (Nakamura et al., 2011). One possibility is functional complementation between NCS-1 and other NCS proteins (neurocalcin, VILIP, hippocalcin, recoverin, GCAP, and KChIP). NCS-1 is the only NCS protein found in yeast, whereas additional NCS proteins are found in worms, flies, and mice. This hypothesis could be tested in genetically tractable and modifiable organisms such as flies and mice.

1.3.4.2 Neuronal development

Due to its spatial and temporal expression patterns, NCS-1 has been implicated in neuronal outgrowth, synaptogenesis in specific organ systems, and finally the formation of organs. Neuronal morphology was shown to be affected by NCS-1 expression levels in Drosophila, Lymnaea and rats. In Drosophila, Frq overexpressers and null mutants had motor terminals with
reduced number and length of branches, and fewer synaptic boutons (Angaut-Petit et al., 1998; Dason et al., 2009). In cultured *Lymnaea* primary neurons, blocking NCS-1 function by application of CTN and RNAi enhanced neurite outgrowth. PC12 cells (pheochromocytoma cells) expressing a dominant negative form of NCS-1 displayed enhanced neurite outgrowth (Hui et al., 2006). Similarly, the number of neurites per cell, branches per neurite, and length of neurites were reduced in NG-108-15 cells (Mouse neuroblastoma x Rat glioma hybrid) transfected with NCS-1 (Chen et al., 2001). NCS-1 has also been implicated in synaptogenesis in the rat retina (Reynolds et al., 2001) and axon outgrowth in the chick retina (Bergmann et al., 2002), in the developing mouse olfactory pathway (Treloar et al., 2004), and in the developing rat spinal cord (Kawasaki et al., 2003). Finally, in zebrafish, NCS-1 knockdown abolished formation of the semicircular canals of the inner ear (Blasiole et al., 2005). All of these effects are consistent with a role for NCS-1 as a regulator of neurite outgrowth, synaptogenesis and neuronal development.

### 1.3.4.3 Regulation of neurotransmission and short-term plasticity

NCS-1 has a modulatory role on exocytosis. Overexpression of NCS-1 in PC12 cells was found to enhance growth hormone release in intact cells, demonstrating that NCS-1 is a positive regulator of evoked dense-core granule exocytosis (McFerran et al., 1998). However, overexpression had no effect on Ca\(^2+\)-induced exocytosis in permeabilized PC12 cells, suggesting that NCS-1 has a modulatory role on exocytosis instead of a direct action on the exocytotic machinery.

NCS-1 is involved in regulating basal levels of neurotransmitter release, with effects on short-term synaptic plasticity consistent with alterations in initial release probability. The overexpression of NCS-1 in *Drosophila* and *Xenopus* is known to enhance neurotransmitter release at the neuromuscular junction (Olafsson et al., 1995; Pongs et al., 1993; Rivosecchi et al., 1994). Overexpression of NCS-1 in *Drosophila* was reported to enhance paired-pulse facilitation and transmission during high- frequency trains, but had no effect on spontaneous transmitter release or on neurotransmission evoked by low- frequency stimulation (Mallart et al., 1991; Pongs et al., 1993; Rivosecchi et al., 1994). In addition, injection of NCS-1 into *Xenopus* embryonic spinal neurons caused an increase in both spontaneous and evoked neurotransmitter release (Olafsson et al., 1995). Glial cell line-derived neurotrophic factor (GDNF), which
upregulates NCS-1, potentiates paired-pulse facilitation in *Xenopus* nerve-muscle cocultures by enhancing the amplitude of the first evoked synaptic response (Wang et al., 2001). Further, application of an CTN to mouse hippocampal slices impaired basal levels of synaptic transmission and enhanced paired-pulse facilitation (Saab, 2010), as in the *Drosophila* studies (Dason et al., 2009; Romero-Pozuelo et al., 2007).

### 1.3.4.4 Learning and memory

NCS-1 may also modulate higher cognitive functions such as LM by regulating long-term synaptic plasticity (both LTP and LTD) (Jo et al., 2008; Saab et al., 2009). LTP and LTD of synaptic responses are widely considered to be a cellular model for LM. A role for NCS-1 in LM was first demonstrated in a study on *C. elegans*, in which NCS-1 is expressed in sensory neurons and NCS-1 KO worms showed defects in associative long term memory for thermosensation, while overexpression of NCS-1 resulted in accelerated learning and prolonged memory (Gomez et al., 2001). Induction of LTP in rats increased postsynaptic *Ncs1* mRNA levels in the DG (Génin et al., 2001). This transcriptional upregulation was dependent on NMDA receptor activation. Application of a metabotropic glutamate receptor (mGluR) agonist, (R, S)-3,5-dihydroxyphenylglycine, induces a form of LTP with slow onset and enhances the level of NCS-1 protein (Brackmann et al., 2004). *Ncs1* KO mice are viable, they present impaired exploratory behavior, anxiety- and depressive-like phenotypes together with deficits in non-aversive long-term memory (de Rezende et al., 2014). Conversely, overexpression of NCS-1 in the hippocampal DG has been implicated in exploratory behavior and in the acquisition of spatial memory, by regulating the surface expression of D2R, while having no effect on NO recognition tasks (Saab et al., 2009).

### 1.3.4.5 Neuroprotection and axonal regeneration

A trauma or injury to the brain can often result in neuronal apoptosis. NCS-1 appears to promote neuronal survival since NCS-1 expression in cultured cortical neurons increased after the application of GDNF and overexpression of NCS-1 in the absence of GDNF mimicked the survival-promoting effects of GDNF (Nakamura et al., 2001). In addition, overexpression of NCS-1 in PC12 cells and cultured cortical neurons has neuroprotective effects under apoptotic conditions. Similarly, lentiviral overexpression of NCS-1 in axotomized corticospinal neurons in
living rats reduced cell atrophy (Yip et al., 2010). NCS-1 is upregulated in injured neurons and can activate anti-apoptotic pathways that may prevent neuronal loss after a trauma by activating PI3K/Akt pathway (Nakamura et al., 2001). NCS-1 has also been implicated in regeneration. Lentiviral overexpression of NCS-1 in the rat corticospinal tract induced axonal sprouting and regeneration from the lesioned cortico- spinal tract and collateral sprouting from the intact cortico- spinal tract (Yip et al., 2010). Both behavioral and electrophysiological tests indicated improved forelimb function in injured rats that were overexpressing NCS-1 in the intact corticospinal tract.

1.3.4.6 NCS-1 in cognitive and psychiatric disorder

NCS-1 is implicated in neuronal regulation, cognitive and psychiatric conditions, such as bipolar disorder, schizophrenia and autism spectrum disorder (ASD) (Amici et al., 2009; Braunewell, 2005; Handley et al., 2010; Koh et al., 2003; Torres et al., 2009). Postmortem samples of the dorsolateral prefrontal cortex of schizophrenia and bipolar patients showed increased expression of NCS-1 (Koh et al., 2003). It is thought that dysregulation of dopaminergic neurotransmission in schizophrenia may be due to altered levels of dopamine receptor interacting proteins (Bai et al., 2004; Braunewell, 2005). NCS-1 interacts with D2R (Kabbani et al., 2002; Lian et al., 2011) and attenuates D2R internalization in a Ca\(^{2+}\)-dependent manner by interacting with GRK2 (G protein-coupled receptor kinase 2) (Kabbani et al., 2002). Therefore, NCS-1 may play a role in dysregulating the dopaminergic neurotransmission leading to neuropathology of mental disorders (Kabbani et al., 2002). Another interactor of NCS-1, IL1RAPL, may be involved in X-linked mental retardation via regulation of exocytosis and neurite outgrowth through their interaction (Bahi et al., 2003). Mutations in IL1RAPL are linked to ASD and also a rare missense mutation at the NCS-1 gene was suggested to be linked to one autistic patient (Piton et al., 2008). This mutation appears to impair NCS-1 cycling between the plasma membrane and cytoplasm, resulting in a signaling defect (Handley et al., 2010). Given these links to disease, several studies have attempted to find compounds that bind to or alter NCS-1 levels. The neuroleptic drug chlorpromazine binds to NCS-1 (Muralidhar et al., 2004), while several antipsychotic drugs (haloperidol, clozapine, olanzapine, and aripiprazole) do not alter NCS-1 levels (Souza et al., 2007). Interestingly, Paclitaxel (also called Taxol: used in cancer treatment), a chemotherapeutic drug, reduces NCS-1 levels by activating calpain (Boehmerle et al., 2007).
1.3.5 Conclusions

Over the last two decades, we have come a long way in our understanding of NCS-1's role in synaptic function and development. Early evidence suggested a presynaptic role for NCS-1 as a Ca\(^{2+}\) sensor affecting short-term synaptic plasticity, but recent studies favor a role for NCS-1 in regulating basal levels of synaptic transmission, with effects on short-term synaptic plasticity being a consequence of altered initial release probability. Several studies have disputed whether the presynaptic effects of NCS-1 were due to Ca\(^{2+}\) channels or an interaction with PI4Kβ.

NCS-1 also has a postsynaptic role in regulating both LTP and LTD in the mammalian CNS. The different stimulation protocols used to induce LTP and LTD result in different sources of Ca\(^{2+}\) activating NCS-1, which leads to NCS-1 binding to different target proteins (Fig. 1-4). NCS-1 may also have differential effects in different neurons due to the expression of different target proteins. These effects on long-term synaptic plasticity likely contribute to the LM phenotypes reported in worms and mice.
1.4 Introduction to Ultrasonic Vocalizations (USVs): association with approach and avoidance behavior

Characterizing ultrasonic vocalizations (USVs) may help us to distinguish the level of threat or reward that the subject perceived while exploring novel environments, since studies in vocal communication of rodents have shown that they communicate with USVs during or in anticipation of threats or rewards (Wöhr and Schwarting, 2013).

Vocalization can categorize behavior and general state of the organisms. The vocal communication of animals has been extensively studied for many species, including songbirds, whales, and humans (Au et al., 2006; Wilbrecht and Nottebohm, 2003). Rodents communicate with USVs and emit 22 kHz calls during or in anticipation of threats or 50 kHz calls during or in anticipation of rewards (Knutson et al., 2002; Wöhr and Schwarting, 2013). Threat cues include exposure to predators (Blanchard et al., 1991), defensive displays during aggressions (Kaltwasser, 1990), and other stressors such as foot shock (Tonoue et al., 1986) or being touched by an experimenter (Brudzynski et al., 1993). Behaviorally, these 22kHz calls have been associated with freezing and strong escape movements (Wöhr and Schwarting, 2008; 2013). Conversely, rewarding situations (Knutson et al., 2002; Wöhr and Schwarting, 2013) encompass juvenile play (Knutson et al., 1998) and socio-sexual interactions (Blanchard et al., 1993; McIntosh and Barfield, 1980). In such situations, rats emit 50 kHz USV in parallel with approach behaviors, including locomotor activity and rearing on their hind limbs (Fu and Brudzynski, 1994). While rat calls are distinctive in the 22 kHz and 50 kHz range and their behavioral associations have been extensively documented, the mouse vocal repertoire has only recently begun to be examined (Chaboud et al., 2012; Scattoni et al., 2008). Moreover, the functions of their vocal expressions, in terms of behaviors, have rarely been studied, especially in a non-social context.

A few studies in mice have found the communicative and affective function of USVs in situations with each developmental stage, such as pup isolations, adolescent play and adults in social contexts (Scattoni et al., 2009; Wöhr and Schwarting, 2013). Mouse pups emit USVs when taken away from their nest and isolated (Zippelius and Schleidt, 1956). Mothers respond to the sound and move closer (Zippelius and Schleidt, 1956) as confirmed by a later playback study.
(Sewell, 1970). Additionally, some temporal analysis of behavior association with USVs found these cries from pups happen prior to or during locomotion and head raising, a feature, which appear to encourage approach behaviors (Branchi et al., 2004). HF calling in adolescent mice is positively correlated with social investigation (Panksepp et al., 2007). Studies on USVs of adult mice were not comprehensively conducted in any context other than reproduction related contexts (Kerchner, 2004). Recently, female-induced vocalization has been used to phenotype cholinergic and dopaminergic KO adult mice (Wang et al., 2008). However, a few papers found that adult male mice emit USVs in non-social contexts including exploration of novel environments and various stressors (Chabout et al., 2012; Ko et al., 2005). Mice may emit low frequency 30 kHz (26 to 36 kHz) calls with the existence of stressors (Ko et al., 2005) and emit high frequency – 40kHz calls with novelty exploration (Chabout et al., 2012). Surprisingly, the analysis of behavior in association with USVs is rarely measured as an important factor in interpreting the functions of USVs. Some have tried to score a selection of behaviors together with USV emissions, but behavioral analyses, which also encompass USVs emissions, are rarely performed. This current study is an attempt to remedy this shortcoming, and is founded on the premise that consistent recording of USVs coupled with distinct behavioral expressions will allow us to accumulate behavioral correlates of a wide range of USVs and ultimately build the library of the “mouse language”. While much of the literature has focused on USV emissions in social contexts or threat-reward related contexts, the behavior of adult male mice emitting USVs during exploration of novelty has not been studied extensively. What is generally and curiously missing is a simultaneous analysis between USVs and detailed mouse’ behavioral repertoire during exploration of novelty, an account that will help “decode” mouse’ USVs and provide new insight into mouse language.

During novelty exploration, the approach and avoidance conflict occurs, according to subject’s affective states as explained in Chapter 1.2. I propose that the distinctive contexts that alter the affective states of subjects would also trigger different levels of exploration. To determine this hypothesis, we first need to prove whether animals in different contexts feel differentially by using a scientific mean. Since mice’s emotion cannot be directly measured, unlike humans, we can measure a factor that is influenced mainly by emotion then predict animal’s affective states in different contexts. Vocalization is such a factor that can be measured and characterized to reveal their affective states of subjects, because the communicative and
affective functions of USVs have been extensively studied in rodents (Knutson et al., 2002; Wöhr and Schwarting, 2013).

High rates of USVs occur throughout the life span of rodents in a variety of relevant situations. Vocalization happens not only in social contexts such as isolated pups from their mothers, juveniles in social play, adult females during social investigation, and adult males when exposed to females or during aggression, but also in non-social contexts including exposure to stressors. Most studies on USVs of rodents mainly focused on pups or adults in social contexts and revealed emotional relations and behavior correlation with USVs. In rats, it is well documented that, in such situations, the acoustic features are divided into HF (50 kHz) and low frequency (22 kHz) USV calls. HF calls tend to happen during anticipation of reward or approach behavior, and low frequency calls tend to occur during anticipation of punishment or avoidance behavior (Knutson et al., 2002; Fig. 1-5). However in mice, only recently, the vocalizations have been classified into syllable types based on spectrotemporal characteristics (Fig. 1-6), and still have a room for more investigation in terms of behaviors (Chabout et al., 2012; Scattoni et al., 2008; Wiaderkiewicz et al., 2013). Moreover knowledge of USVs emissions in non-social contexts is limited. The affective states of animals are largely dependent on different situations where approach and avoidance conflict would occur on different levels.

In conclusion, by measuring USVs during novelty exploration, I will be able to predict the affective states of mice in non-social contexts, in particular, with a special attention to approach and avoidance behaviors. The outcomes of this study have important implications for preclinical research, ranging from practical considerations, such as the effect of ambient ultrasonic noise on the well-being of the animal, to new approaches for the assessment of psychological states and functions in rodents that are not readily accessible with the standard paradigms in use today. For example, USVs could be utilized to measure communication deficits or baseline USVs during exploration in rodent model of autism.

Here, I will review the USV patterns in mice and rats observed at their developmental stages from pups to adults in the settings, which trigger approach or avoidance behavior including socially relevant contexts and non-social contexts.
**22 kHz vocalizations (Aversive vocalizations)**

1. Short 22 kHz
2. Long 22 kHz

**50 kHz vocalizations (Appetitive vocalizations)**

3. Flat 50 kHz
4. Step FM 50 kHz
5. Trill FM 50 kHz
6. Step-Trill FM 50 kHz
7. Other FM 50 kHz

**Figure 1-5. The classification of adult rat vocalization**

The exemplary sonograms show the acoustic structure of vocalizations over time. (1) Short 22 kHz call: duration 99.6 ms, peak frequency 28.7 kHz; (2) Long 22 kHz call: duration 1005.5 ms, peak frequency 22.7 kHz; (3) Flat 50 kHz call: duration 81.6 ms, peak frequency 49.7 kHz; (4) Step FM 50 kHz call: duration 85 ms, peak frequency 82.5 kHz; (5) Trill FM 50 kHz call: duration 111.1 ms, peak frequency 88.6 kHz; (6) Step-trill FM 50 kHz call: duration 83 ms, peak frequencies at 60.0 kHz; (7) Example of other type of FM 50 kHz call: duration 22 ms, peak frequency 55.5 kHz. Modified from Brudzynski et al., 2013.
Figure 1-6. The classification of adult mice vocalization.

(1) Spectrogram of a typical mouse calls in response to acute pain. First, the scissors made a sound as they closed, removing part of the mouse’s tail. Then, approximately 0.3 s later, the mouse made a broadband call with audible (below the dashed line at 20 kHz) and ultrasonic (above the dashed line) components. Obtained from Grimsley et al., 2013. (2-11) Typical sonograms of ultrasonic vocalizations, classified into ten distinct categories of calls emitted by c57/B6 mice. Descriptive statistics (mean ± SEM) are given for the duration of each call type, as well as the beginning and ending dominant frequency. Obtained from Scattoni et al., 2008.
1.4.1 Socially relevant contexts

Social interactions in rodents can be specific to a broad range of contexts throughout their lives from maternal care, courtship, to territorial defense. The level of sociability is contingent on social contexts, individual status and age. In general, social interactions among adults are closely connected to procreative opportunities (Crews, 1997). However, juvenile animals with lack of reproductive competence (Cameron, 2004; Laviola et al., 2003; Sisk and Foster, 2004) may usually have more playful quality (Spinka et al., 2001). Therefore, USVs might also be different to the social contexts depending on their ages.

**Pups:** The communicative function of USVs between mother and infant rodents was well established in isolation and payback experiments. The first production of pure ultrasound by small mammals other than bats was reported from laboratory rodents, as the USVs (23 to 28 kHz) were detected when they were isolated in their cages (Anderson, 1954). Mice isolated pups (from birth to 13 days) emitted audible sounds with soft clicks and loud pain squeaks and USVs in isolations (Zippelius and Schleidt, 1956). The USVs in mice pups had positive effect on maternal orienting, searching, and retrieving, while mothers did not respond to dead or anesthetized pups unable to vocalize (Zippelius and Schleidt, 1956). The maternal care response to pups’ USVs was confirmed in the following studies which showed rodent pups in distress emitting USVs and their adults apparently receiving these calls and reacting to them by displaying various types of maternal behaviors (Noirot, 1972). Later, it was confirmed again by means of playback experiment, in which mothers searched the places from which isolation-induced USVs sound originated (Sewell, 1970). In mice, a temporal simultaneous analysis revealed that a significant increase of head rising behavior occurs immediately prior to or during the USVs emissions, suggesting that pups behave in a way of favoring interactions with their mothers (Branchi et al., 2004).

**Juvenile:** In rodent juvenile, sociality is engaged with play in general. Play in rats produce 50 kHz, but not 20 kHz USVs. During or in anticipation of play and playful tickling by an experimenter, rats elicited HF USVs (Knutson et al., 1998; Panksepp and Burgdorf, 2003). Behaviorally, rats preferred or approached playful contexts and individual rats would subsequently run into the tickling hand (Panksepp and Burgdorf, 2000). During encounter experiments, rats emitted both 20 kHz for submissive behavior and 50 kHz for aggressive
fighting (Sales, 1972). Similar to rats, as juvenile mice consistently emitted HF calls during and anticipation of play, HF calls are positively correlated with social investigation (Panksepp et al., 2007).

**Adults:** In adult rats, social stressors, such as predators and fighting, are suggested to evoke 22 kHz USVs as well as avoidance behavior including freezing and flight and they both are found to be associated. Predators and associated stimuli such as presence of cats or cat odor could potentiate 22 kHz vocalizations and also inhibit emission of 50 kHz (Blanchard et al., 1991; Panksepp and Burgdorf, 2003). 22 kHz USVs emissions by experimental touch were contended to be behaviorally associated with tense, immobile crouching behaviors and strong expiratory movements (Brudzynski et al., 1993). Rats emitted 22 kHz with defensive and submissive displays during inter-male fighting (Thomas et al., 1983) and the refractory period following copulation during sexual interactions (Barfield and Geyer, 1975). This USV therefore has been thought to be a message indicating that the sender is in a socially withdrawn state. Conversely, 50 kHz vocalizations during socio-sexual interactions were emitted in adult rats (Thomas and Barfield, 1985; White and Barfield, 1987), including male approach and ejaculation during copulation (McIntosh and Barfield, 1980), male and female social exploration (Blanchard et al., 1993; Brudzynski et al., 2002). In one study, presentation of anesthetized (and thus silent) mice evoked primarily 50 kHz USVs in individual adult rats during social investigation, with females emitting more of these vocalizations than males (Blanchard et al., 1993).

1.4.2 Non-social contexts

**Non-social aversive situations induce USVs emissions in rodents.** Mice pups or adults emitted audible sounds with soft clicks and loud pain squeaks and pure ultrasound vocalizations in response to stressors including cold temperature (Zippelius and Schleidt, 1956) and restraint stress (Chabout et al., 2012). Conversely, rats emitted 22 kHz USVs when stressed (Brudzynski and Ociepa, 1992; De Vry et al., 1993), with aversive stimuli including bright light (Knutson et al., 2002; Panksepp and Burgdorf, 2003). The presence or anticipation of foot shock cues decreased levels of 50 kHz calls (Burgdorf et al., 2000; Tonoue et al., 1986). Opiate antagonist drugs (e.g., peripheral naloxone, central carbachol) with aversive behavioral properties such as freezing or escape elicited 22 kHz USVs while decreasing 50 kHz USVs. Conversely,
physiological withdrawal from drugs (e.g., peripheral morphine) that acutely elicited conditioned approach increased startle response with association of 22 kHz USVs elicited (Burgdorf et al., 2001; Knutson et al., 1999). Finally, electrical stimulation of brain areas implicated in pain perception and also resulted in 22 kHz USVs (Yajima et al., 1976).

**Non-social rewarding situations induce USVs emissions in rodents.** Rats emitted USVs with approaching food (Burgdorf et al., 2000), electrical self-stimulation of the brain (Burgdorf et al., 2000; 2007), and addictive drugs (Burgdorf et al., 2001; 2008; Knutson et al., 1999). Behaviorally, the administration of amphetamine and glutamate into medial preoptic area of the hypothalamus triggered the emission of 50 kHz USVs and the HF calls were associated with locomotor activity, rearing, and exploration (Fu and Brudzynski, 1994). With the conditioned approach, the drugs (e.g., peripheral morphine) associated with rewarding and approach properties conditionally elicited 50 kHz USVs (Burgdorf et al., 2001; Knutson et al., 1999).

USV emissions may have associations with exploratory behaviors such as locomotor activity, rearings in hind limb and sniffing (Sirotin et al., 2014). In rats a high vocalization rate is paralleled by high levels of locomotor activity (Hofer, 1987b). Mice emitted USVs during exploration in the novel environments (Chabout et al., 2012). The function of USVs emissions during exploratory behaviors in novel environments is still unknown, whether it is due to rewarding or anxiety or even calling for distance cage mate.

22 kHz USV emission causes freezing behavior in experience partner rats, rather than sensitization. An aversive stimulation prior to 22 kHz USV exposure is necessary for inducing freezing behavior through 22 kHz USV (Kim et al., 2010). Therefore suggest that auto conditioning is sufficiently rapid, reliable, and stimulus-specific to serve an adaptive defensive function in rats (Parsana et al., 2012).
1.5 Brain Activity Mapping

Exploratory behavior in novel environments and spatial learning involves the HPC system (Lever et al., 2006). O’keefe & Nadel claim that the HPC is the critical structure for a spatial mapping theory (O’Keefe and Nadel, 1978). This claim is later supported by two main pieces of evidence. First, in the HPC there are cells, which respond only in specific places in a familiar environment, which are later described as “place” cells. Ranck (1982), who originally proposed a different classification of receptive field types, now accepts the notion of a place field. Olton (1978), using semi-automated analysis techniques, has also observed place fields in rats searching for food in a radial maze. The second evidence is that the DG, a subregion of the HPC, is important for novelty detection (Kesner, 2007). It should be noted that the idea of the HPC as novelty detector and comparator is based on various clinical, behavioral, and neurophysiological data (Miller and Matzel, 1988; Squire and Shimamura, 1989). This idea has fundamental significance for understanding the double interconnected function of the HPC: selective attention with inhibitory control protects the processing of information from interference, and the global transfer function of information (e.g., relational, temporal, and spatial) into cortical memory storage (Eichenbaum, 1997; Squire and Shimamura, 1989). The presentation of a novel stimulus producing a robust increase in hippocampal activity is found at single-unit recordings (Fyhn et al., 2002; Vinogradova, 2001) and imaging studies using PET (Tulving et al., 1996), fMRI (Strange and Dolan, 2001; Yamaguchi, 2004), and c-fos expression (Jenkins et al., 2004). Therefore, the HPC is one of the prime candidate regions involved in safe novelty exploration although there are likely more brain regions involved. These will be formally identified in the future.

The studies relating specific brain function with specific behaviors were conducted in various ways including lesion studies in animals and human patients: in vivo PET and functional magnetic resonance imaging (fMRI) in humans; and in vitro IEG staining methods in animals. Neuroanatomical lesion studies in animals were initiated by Lashley (1929). More selective lesioning techniques employ a stereotaxic apparatus to locate small brain regions from a three-dimensional brain atlas (e.g., Paxinos and Franklin, 2004). Focused electrolytic lesions revealed a major role of the HPC in spatial learning (Cohen and Eichenbaum, 1994). Postmortem correlations from patients with lesions in discrete regions of the HPC and cerebral cortex revealed selective neuronal loss that correlated with specific types of memory (Damasio et al.,
1985). The most famous case is H.M., a man who lost all ability to retain new information (anterograde amnesia) after surgical bilateral hippocampal lesions for treatment of his intractable epileptic seizures (Scoville and Milner, 1957).

In vivo positron emission tomography and fMRI techniques now allow investigators to identify specific human brain regions that mediate performance on specific types of memory tasks (Posner and Raichle, 1998). Elegant fMRI studies map site–selective activation patterns in brain pathways and structures including the cerebral cortex, HPC, or cerebellum of normal human volunteers, while they are engaged in diverse memory and cognitive tasks such as picture recognition (Robbins et al., 1996; Squire et al., 1992; Ungerleider, 1995), maze learning (Van Horn et al., 1998), error detection (Carter et al., 1998), pain processing (Derbyshire et al., 1997), facial emotion recognition (Morris et al., 1998), retrieval of names for faces (Zeineh et al., 2003), and fear acquisition and extinction (LaBar et al., 1998). Imaging techniques reveal a characteristic pattern of hypometabolism and reduced receptor occupancy by neurotransmitters in many brain regions of patients suffering from Alzheimer’s disease (Meltzer et al., 1998; Minoshima et al., 1997).

The c-fos mapping studies provide viable evidence supporting the existence of a brain-wide network, which is involved in processing of information (or even underlying widespread plastic changes) and in learning. However, at the same time, careful behavioral analyses into correlations between various animal reactions and c-fos expression patterns reveal that specific aspects of information processing (plastic changes) are encoded by well-determined brain structures (such as emotions in the Amyg).

The immediate early gene (IEG), of which c-fos is a proto-oncogene, has become a popular neurobiological tool for mapping functional activity as a specific marker for neuronal activity at the single cell level (Chaudhuri, 1997; Hughes and Dragunow, 1995; Kaczmarek and Chaudhuri, 1997). C-fos with other IEGs are activated and expressed within neurons shortly after cell stimulation following voltage-gated Ca\(^{2+}\) entry into the cell (Morgan and Curran, 1986) without the requirement for de novo protein synthesis, which provides ‘immediate’ reaction. The protein product can be detected within neurons by immunohistochemical techniques 20-90 minutes after neuronal excitation, and disappears 4-16 hours later (Menétrey et al., 1989; Morgan
et al., 1987; Mugnaini et al., 1989). Electron microscopy has shown that neuronal excitation induces c-fos-like immunoreactivity within the nuclei of neurons, but not within glial, ependymal, or endothelial cells (Mugnaini et al., 1989). This gene is widely distributed throughout the brain, and a variety of events that bring about increased neuronal activity will produce an up-regulation of c-fos in numerous (but not all) brain sites (Chaudhuri et al., 2000; Herrera and Robertson, 1996; Kovács, 2008). In the brain, once expressed, c-fos protein enters the cell nucleus and participates in protein complexes that interact with DNA (Sambucetti and Curran, 1986) and serve as transcription factors.

These complementary results for IEG imaging and electrophysiology lead to the prediction that, compared to exposure to novel stimuli, exposure to familiar stimuli should be associated with overall decreases of perirhinal cortex (PeriC) activity. This prediction is supported by fMRI studies of humans where, for fMRI, the typical finding is a reduction of the parahippocampal BOLD signal when familiar stimuli are compared with novel stimuli (Fernández and Tendolkar, 2006; Henson et al., 2003; Montaldi et al., 2006). Magnetoencephalography (MEG) studies again suggest that familiar stimuli lead to a reduction in temporal lobe activity (Bunzeck et al., 2009).

Novelty detection is considered a prerequisite for learning and almost simultaneously exploration occurs during learning (Tischmeyer and Grimm, 1999; Tulving et al., 1996), which has an association with c-fos (Fleischmann et al., 2003; Herdegen and Leah, 1998; Kasahara et al., 2001; Nikolaev et al., 1992; Tischmeyer and Grimm, 1999). Several brain regions have been implicated in novelty detection and novelty induced exploration (Ranganath and Rainer, 2003; Yamaguchi, 2004), but the HPC (Brown and Aggleton, 2001; Jenkins et al., 2004; Knight, 1996; Nyberg, 2005; Yamaguchi, 2004), and the prefrontal cortical areas (Daffner et al., 2000; De Leonibus et al., 2006; Dias and Honey, 2002; Matsumoto et al., 2007; Xiang and Brown, 2004) are considered the most essential components in the processing of novelty. Perirhinal c-fos activity may be a critical requirement for effective, stable object (novel element) recognition memory in rats (Brown and Aggleton, 2001; Seoane and Brown, 2007; Zhu et al., 1995). Additionally, lesions of the nucleus accumbens core (NacC) cause a decline in novelty-induced exploration (Fink and Smith, 2002; Kelly and Iversen, 1976; Schwarting and Huston, 1996) and the nature of its functional links with the HPC remains highly contentious (Shohamy and
Adcock, 2010; Lisman, 2011).

In previous studies, animals were exposed to either novel or familiar stimuli (Aggleton and Mishkin, 1985; Mishkin and Delacour, 1975; Mumby et al., 1990) with spontaneous exploration (Ennaceur and Delacour, 1988) without consideration of levels of threat or stress that mice encounter when they are exposed to novelty. One of the shortcomings of these studies is the difficulty to determine whether an increased IEG expression was a consequence of exposure to stress, novelty, or the effect of an enhanced motor activity (Montag-Sallaz et al., 1999). The association between IEG expression and specific neural coding has remained largely unclear. Therefore, having controls for stress, novelty, and motor activity will be necessary.
Chapter 2

2 Materials and Methods

2.1 Discovery of the \textit{NcsI}^{P144S/P144S} Mouse Line

In collaboration with RIKEN Bioresources Center in Japan, one mutation in \textit{NcsI} was found after screening over 2,000 mouse genomic DNA samples of DBA/2 × C57BL/6F1 males (= G1 males) derived from ENU-treated C57BL/6 males (=G0 males). The C to T mutation in \textit{NcsI} resides within exon 6 at position 2:31,284,698 (ENSMUSG00000062661) and changes the proline at position 144 to a serine (P144S) in a region between the third and fourth EF-hand (Fig. 2-1). There are no known SNPs between C57BL/6J and DBA/2 at or near the mutation site. Once the P144S mutation in NCS-1 was identified, the sperm was used to rederive mutant mice (\textit{NcsI}^{P144S/+}) by \textit{in vitro} fertilization and implantation into pseudopregnant C57BL/6J females. Thus, the mice obtained from this process were only 2\textsuperscript{nd} generation C57BL/6J and contained several mutations (as many as 30 non-silent point mutations in coding regions of genes) in addition to contaminating donor DBA/2 alleles. To limit the chances that resultant phenotypes were due to unknown point mutations or DBA/2 genes, we followed the standard backcrossing procedure for 10 generations.

Genotyping of mice was performed by PCR using DNA from tail clips and the following primers to amplify \textit{NcsI}: Forward primer, 5’-GCC TAA GGC TGC ACT GAC TC-3’; Reverse primer, 5’-TGG GAA ACT CAC CAA AGA CC-3’, which yielded a PCR product of 245 bp. The PCR product of the P144S mutant allele was cut by \textit{Hpy188I} (New England Biolabs, Beverly, MA, USA), such that the wild-type allele ran as a 245 bp product while amplification and digestion of \textit{NcsI}^{P144S/+} DNA resulted in 245, 168 and 84 bp fragments, and that of \textit{NcsI}^{P144S/P144S} DNA produced 168 and 84 bp fragments.
Figure 2-1. Discovery of P144S mutant in *Ncs1*.

(A) A diagram showing NCS-1 protein with its structural domains including EF hands (orange=functional, green=nonfunctional) and the position of the P144S mutation. (B) In the left panel, the 78 bases shown in uppercase letters reflect exon 6 of *Ncs1* and the corresponding amino acid coding is shown underneath. The C to T transition causes a P144S missense mutation (shown in red). In the right panel, sample sequence chromatograms are shown for a wild-type showing as C and littermate heterozygous mutant showing as N, which signifies mix of C/T. Figures taken from Saab, 2009.
2.1.1 Animal surgery and infusion of small interfering RNA (siRNA)

Male C57BL/6J (10–12 weeks old, TCP) were handled while housed three-five per cage for at least 1 week before surgery, and subsequent to surgery for hippocampal cannula insertion were housed one per cage. I delivered in vivo either Ncs1 siRNA (Mm Freq 3 HP siRNA; QIAGEN, Valencia, CA, USA) (Fig. 3-12) or negative control siRNA (Shanghai GenePharma Ltd., Shanghai, China) as per previous method (Saab et al., 2009). For the injection of siRNA (fear memory test), mice were anesthetized with isoflurane and Stainless steel guide cannulae (custom built by Small Parts, Inc., Logansport, IN, USA) were cemented onto the skull after positioning the tip at coordinates -2.0 mm from bregma, +/- 1.5 mm from midline and -1.2 mm from the dura. Seven days after recovery from surgery, animals were injected with siRNA. Each siRNA was diluted to 0.5 µg/µL in 5% glucose and mixed with six equivalents of a 22 kDa linear polyethyleneimine (PEI; Fermentas Inc., Glen Burnie, MD, USA). After 10 min of incubation at room temperature, 2 µL were injected into each HPC through an infusion cannula protruding 0.5 mm below the termination of the guide cannulae (to -1.7 mm from the dura) with an infusion syringe pump (Model 22; Harvard Apparatus, Natick, MA, USA) at 0.5 µL/min for 2 min, for a total of 1µL per hemisphere. Animals were handled gently to minimize stress. A total of three infusions of siRNA were given over a period of 3 days (1 µg siRNA per HPC per day). For the exploratory experiments, mice were tested 3 days after the last siRNA injection. For the fear memory test, mice were trained 3 days after the last siRNA injection and tested 24 hours later.

2.1.2 Botulinum neurotoxin A (BoNT) injections

C57BL/6J (8-10 weeks old, TCP, Canada) mice were randomly divided into two groups of 20 mice. Botulinum neurotoxin A or incobotulinumtoxin A (BoNT, Xeomin; Merz Pharma, Canada Ltd., Burlington, ON, Canada) was reconstituted and diluted in PBS. Two dilutions were made, 0.2 LD50 mouse units (MU, or U) and 0.1 U of BoNT per 20 µL. Injections were performed with a Hamilton microsyringe (33G; Hamilton, Reno, NV, USA) with a volume of 20 µL in one or both hindlimb muscles, similar to described work (Kutschenko et al., 2011). Following BoNT or saline injection (control group), the mice were observed daily and behavior tests were performed 3 days after the injections.
2.2 Exploratory Experiments

Daily animal handling was performed starting from 3 days prior to the behavioral tests. Experiments were performed during the light cycle, from 8:00 AM to 11:00 AM. Experimenters were blind to the treatments of the subjects. Handled C57BL/6J adult male subjects were placed in an empty clear Plexiglas chamber (42 x 42 x 42 cm; AccuScan Instruments, Columbus, OH, USA). The chamber was lit overhead at the following intensities: Dim at 20-40 lux and Bright at 400-500 lux. The behaviors were scored by an automated VersaMax animal activity monitoring system (AccuScan Instruments). The positions of the nose, tail and center of mass of each mouse were tracked using EthoVision 7.0 software (Noldus Information Technology, Leesburg, VA, USA) and analyzed total distance traveled. In addition, time spent on attending to objects and the number of rearings were observed and scored manually using Observer 5.0 software (Noldus information Technology). The following criteria were used for scoring the behaviors:

- Still: Animal is stationary and not performing any of the other scored behaviors (including “in-between” behaviors).
- Walking: Animal is walking.
- Grooming: Animal is grooming their paws, head and body.
- Unsupported rearing: Animal is upright and supported exclusively on hind legs, potentially sniffing the environment.
- Rearing to the wall: Animal is standing and touching the wall with one or two paws.
- Rearing to the object: Animal is standing and touching the objects with their one or two paws.
- The total number of rearings: The sum of unsupported rearings, rearing to the walls and rearing to the objects.
- Attending to objects: Animal is actively investigating the objects, generally by sniffing within 1 cm.

2.2.1 Experiment 1 (exploration and learning)

Experiments were conducted in chambers. The test consisted of three phases (Fig. 2-2): 1) habituation/training (15 min) with either dim or bright lighting, with four identical objects
presented, 2) displaced object (DO) phase (5 min) under dim lighting with two objects displaced towards the arena center, and 3) NO discrimination phase (5 min) under dim lighting by presenting one FO from the training phase and one NO. Between phases, mice were returned to their home cages for 3 min. Total distance traveled, the number of rearings, and time spent on attending objects were measured and analyzed.

Figure 2-2. Schematic representation of experimental procedure

The test consisted of three phases: 1) habituation/training (15 min) with either dim (Blue: 20-40 lux) or bright lighting (Yellow: 400-500 lux), with four identical objects presented, 2) displaced object phase (5 min) under dim lighting with two objects displaced towards the arena center, and 3) novel object discrimination phase (5 min) under dim lighting by presenting one familiar object exposed during the training phase and one novel object.
2.2.2 Experiment 2 (environmental novelty)

Handled but naïve to any behavior experiments, C57BL/6J adult male subjects were used in five treatments. The first four represent a crossed design of the treatments novel vs. familiar with the treatments bright light vs. dim light. I expected exploratory behavior to decrease in these four treatments, ranging from highest to lowest in the following order; novel, dim light (ND); novel, bright light (NB); familiar, dim light (FD); familiar bright light (FB)/ familiar water (FW) treatments. Bright light and water are used as an aversive stimulus. As a second aversive stimulus, in a fifth treatment, mice were placed in a previously visited chamber (familiar) that was filled with shallow water (FW; see Figure 2-3 for an overview of treatment conditions). Twelve mice were used in each group. The experimental container was an empty clear Plexiglas chamber (42 × 42 × 42 cm) that was lit from overhead. Two light treatments were used: a dim (20–40 lux) and a bright (400–500 lux) light intensity. For the novel treatments, data were collected from mice exposed to the chamber for the first time, for 30 min in either the dim or bright light condition. For the familiar treatments (under dim or bright light conditions), mice used had been exposed on the previous day to the same chamber under dim lighting (20–40 lux). As a final treatment, water was used as a second stressor, with mice exposed to a novel dimly lit chamber filled with 5 cm of water; this water depth is sufficiently shallow that mice do not need to swim. USVs were recorded (see below) for the first 5 min during each 30 min trial. Mice were then sacrificed 30 min post-exposure to open field for real time PCR (RT-PCR) (Fig. 2-3). Total distance traveled and the number of rearings were measured and analyzed. Animals were returned to their home cages after trials and the entire apparatus was cleaned with 70% ethanol between trials to remove any scent.
On day 1, for the familiar groups, mice were pre-exposed to the open field under dim light for 30 min. On the test day, novel groups were exposed to the open field for the first time under two lighting conditions; dim (Blue: 20-40 lux) and bright (Yellow: 400-500 lux). USVs were recorded for the first 5 min. Mice were then sacrificed 30 min or 90 min post-exposure to open field for RT-PCR or c-fos detection, respectively. While boxes without line patterns represent novel conditions, boxes with the line patterns represent familiar conditions. Additionally a box with diamond symbols represents water conditions. Each black arrow bar represents 30 minutes. A red bar represents 5 min. RT-PCR: real time polymerase chain reaction, USV: ultrasonic vocalization.
2.2.3 Experiment 3 (spatial novelty)

To reduce anxiety levels, the habituation session was preceded the test session. All groups were habituated for 30 min/day for 5 days in the open field with different objects; one in the left front corner and the other in the right back corner under dim lighting. During the test sessions, one of the two objects was dislocated for the ND and NB groups, while both objects remained in the same locations for the FD and FB groups. The dim groups, both ND and FD groups, were tested under 20-40 lux, while the bright groups, both NB and FB groups were tested under 400-500 lux light as described in Figure 2-4. The final condition was the control group for motor function. For the first five days, mice were exposed with the not running wheel in the chamber, and on day 6, mice were exposed to running wheel. Mice were then sacrificed 90 min post-exposure to the open field for immunofluorescence detection of c-fos expression (Fig. 2-4). Total distance traveled, the number of rearings, and time spent on attending objects were measured and analyzed.
Figure 2-4. Schematic representation of experimental procedure.

On day 1-5, mice were habituated to the open field with two different objects under dim light for 30 min each day. On the test day, novel groups (boxes without line patterns) exposed to the open field with one of the object displaced in two lighting conditions; dim (Blue: 20-40 lux) and bright (Yellow: 400-500 lux) and familiar groups (boxes with line patterns) exposed to the open field without any changes in both dim and bright conditions. For the final condition, mice were
exposed to a non-running wheel, and then on day 6 mice were exposed with the running wheel. Followed by test session, brain was removed for c-fos imaging. Each arrow bar represents 30 minutes.

2.3 Ultrasonic Vocalization Recording

An UltraSoundGate Condenser Microphones (CM 16; Avisoft Bioacoustics, Berlin, Germany) was placed 15 cm above the experimental chamber; this was high enough so that the receiving angle of the microphone covered the whole area of the testing chamber. This microphone was sensitive to frequencies of 15-180 kHz with a flat frequency, and were connected via an Avisoft-UltraSoundGate 416 USB Audio device (Avisoft Bioacoustics) to a personal computer, where acoustic data were displayed in real time by Avisoft-RECODER USG (Avisoft Bioacoustics), and were recorded with a sampling frequency of 250 kHz with 1024 points of fast Fourier transform (FFT) length in 16 bit format. For all behavioral conditions, USVs were analyzed off-line with Avisoft-SASLab Pro (Avisoft Bioacoustics; 512 FFT-length, 100 % frame, Hamming window and 75 % time window overlap). Correspondingly, the spectrograms were produced at the resolution of 488 Hz and 0.512 ms. The number of HF (>35 kHz) and LF (20–35 kHz) calls was filtered out automatically using SASLab Pro. The following acoustic features were counted by Pulse train analysis: (1) call duration: mean duration of a single USV, and (2) mean peak frequency: expressed as peak frequency at maximal amplitude. To compare USVs with behavior, I synchronized audio and video files by performing a “clap” with fingers in the field of the camera to time-match video and audio files. In the audio files, we cut the information before this sound, and in the video files I selected the exact time frame of this event and started behavioral scorings at this time-point forward. This manual synchronization permitted to link those behaviors described above with USVs elicited at the time of the behavioral event.
2.4 Behavior Tests to Determine the Phenotype of Ncs1\textsuperscript{P144S/P144S} Mouse Line.

2.4.1 Hole-board test

Hole-board test were used to measure possible differences in exploratory behaviors using hole poking as a behavioral response. Behavioral observations were made for 5 min in a dimly-lit or a brightly-lit clear Plexiglas chamber of the same size as the one used in object recognition testing, but containing 8 circular holes (r = 1 cm) in the center, two holes at the corner (r = 2 cm) and 2 rectangular holes on the wall (width = 2 cm height = 1 cm) elevated 8 cm above a transparent surface. The behavioral activities such as time spent exploring the holes and number of rearings were scored. It should be noted that the holepoke response involves dislogging of up to the eyes as opposed to below eyes also known as superficial ‘mini-pokes’, as latter is more closely associated with stereotypical behaviors (Brosnan-Watters, 1997).

2.4.2 New Frontier exploration test

The New Frontier exploration test was conducted as described previously (Saab et al., 2009). Mice were allowed to climb from their home cage to any of four platforms; each elevated 15 cm above the floor. The platforms connected the home cage to novel environments (18 cm × 30 cm), that were also 15 cm above the floor. Placement of two or more paws in the novel environment was recorded as a crossing event. A visit to the same novel platform environment was only counted as a second crossing event if subjects subsequently returned to their home cage area. Mice were given 15 min to explore the platforms.

2.4.3 Contextual and tone fear memory

Mice were introduced to the fear conditioning and sound attenuating chamber (Med Associates., St. Albans, VT, USA) equipped with a computer-controlled fear conditioning system (Actimetrics, Wilmette, IL, USA). Each mouse was allowed to explore the chamber for 3 min before presentation of a CS, a tone (80 dB, 3600 Hz) which persisted for 30 s. The CS was followed immediately by the US, a mild foot shock (0.7 mA for 1 s). Three CS–US pairings were
presented with a 1 min interval between trials. The mice were then returned to their home cage. Freezing behavior, defined as the complete absence of any movement except for respiration and heartbeat, was measured during the context- and tone-conditioning tests at 0.25 second intervals by using FreezeFrame automated fear-conditioning software (Actimetrics). Memory for contextual learning was probed 1 hour and 24 hour after training by re-exposing the mouse for 3 min to the same fear-conditioning chamber. Memory for tone learning was also probed 30 min and 24 hour after training by allowing the mouse to explore a modified chamber, which had a plastic floor and cardboard walls. The chamber was scented with 5 % acetic acid. After 3 min, the audio tone was presented (2 Hz pulsating tone, 80 dB, 3600 Hz) for another 3 min.

2.4.4 Elevated plus maze (EPM)

Anxiety-like responses or exploratory tendency were measured in the EPM test, as previously described (Soleimani et al., 2008). The apparatus is a plus shaped acryl maze with two opposite open arms (25 × 5 cm; 70 lux) and two opposite closed arms (25 × 5 × 30 cm; 1.3 lux) extending from a central platform (5 × 5 cm) and elevated 50 cm from the ground. The floor of the arms was made of white Plexiglas and the walls of the closed arms were made of black Plexiglas. Similar arms were opposite to each other and at a 90° angle from dissimilar arms. The test mouse was placed in the central area facing an open arm and allowed to explore the open or closed arms of maze for 5 min. The maze was cleaned with 70 % ethanol after each trial to prevent influences of previously tested mice. The number of entries and head dips, and the time spent in the open arms, closed arms, and central platform were recorded. Two paws had to be inside the entrance line to each arm, which signaled the start of the time spent in the specific arm, and then the end time was recorded when all four paws were outside the line again. Generally, entries into open arms are considered to be stressful for animals, thus measures in the open arms provide indices of exploratory tendencies similar in nature to that of exploration of the open quadrants of the open field.

2.4.5 Accelerating rotarod

This experiment was performed as previously described (Soleimani et al., 2008) using an Economex Rotarod apparatus (Columbus Instruments, Columbus, OH, USA). The original 3 cm
ribbed plastic rotating axle was divided using four adjustable flanges, which enabled testing a maximum of four mice simultaneously. The rod is suspended at a height of 30 cm above the plastic surface. Mice are placed on top of the rod, facing away from the experimenter. In this orientation, forward locomotion opposite to rotation of the rod is necessary to avoid falling. During the stationary mode, each mouse was first observed for 10 seconds without any rotation. The axel was then adjusted for a constant motor speed of 5 rpm, and each mouse observed for a total of 10 seconds (fixed speed mode). Next, beginning at 5 rpm, the rotation gradually increased by increments of 0.1 rpm/s and the latency to fall off the axle was recorded in seconds for each mouse for the maximum period of 300 seconds (accelerating speed mode). On each day, 3 trials were completed with a 1 h intertrial interval. The mean latency was then calculated by averaging the latency of three consecutive trials. The stationary and fixed speed mode sessions were training periods and allowed the animals to become accustomed to the apparatus. Motor learning was determined by observing an improved latency on the last compared to the first day (motor learning ratio = latency day 3 / [latency day 1 + 3]). Impaired performance in these sessions served as early indicators of motor abnormality. The accelerated Rotarod procedure was repeated for 3 constitutive days by 3 trials per day to measure motor learning.

2.4.6 Forced swim test

The forced swim depression protocol was performed as described (Cryan et al., 2005). The mice were released individually into a transparent plastic cylinder (25 cm height, 18 cm diameter), which contained water at 25 °C to the depth of 18 cm. The experiment lasted 6 min, and an observer, blinded to the genotypes, scored the following parameters during the last 4 min of the trial: (1) active swimming (including crossing the quadrants of the container) and (2) floating (no limb movement and making only minimal movements to keep the head above the water). Each mouse was allowed to dry after the test, and the water was changed between subjects. I chose the forced swim test, as it is the most widely used paradigm for assessing depression- or antidepressant-related behavior in mice.

2.4.7 Sucrose preference test

A 5 day sucrose preference protocol was conducted as described (Roybal et al., 2007). Mice
were individually housed and were presented with two identical water bottles with balled stoppers. The positions of the bottles were alternated daily to avoid a side bias. The weight of each water bottle was recorded daily to assess the amount of solution consumed. On days 1 and 2, mice were presented with two identical bottles filled with water (water/water). On days 3 and 4, bottles contained 5 % sucrose solution dissolved in the drinking water (sucrose/sucrose). On day 5, one bottle was filled with water and the other was filled with 1 % sucrose solution. Preference for sucrose was calculated as $100 \times \frac{\text{sucrose}}{\text{sucrose} + \text{water}}$ consumption.

2.5 Biochemistry

2.5.1 Transfection of siRNA into N2A cells

The N2A (CCL 131; ATCC, Rockville, Maryland, USA) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen™ Life Technologies, Carlsbad, CA, USA) supplemented with 10 % cosmic calf serum (GE Healthcare HyClone™, Logan, UT, USA) and 2 mM L-glutamine. All of the cell cultures were maintained at 37 °C in 5 % CO$_2$. Double-stranded siRNA was transfected into N2A cells using RNAimax (Invitrogen™ Life Technologies) as the transfection reagent. Before transfection, the cells were washed and resuspended in 900 μl of RPMI culture medium. Cationic lipid complexes, prepared by incubating 2 μM siRNA duplexes with 3 μL of oligofectamine in 100 μL of RPMI medium, were added to the wells. After a 24 h transfection, the NCS-1 level was measured by western blot.

2.5.2 Western blotting

Mice were sacrificed by cervical dislocation, followed by immediate removal and freezing of the hippocampi on dry ice. The removed HPC was preserved at -80 °C. Each HPC was lysed in 300 mL radioimmunoprecipitation assay (RIPA) buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing protease inhibitor (Santa Cruz Biotechnology). Homogenates were centrifuged at 20,000 × g for 10 min at 4 °C, supernatants were collected, and protein concentrations were determined using Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturers’ instructions. 40 μg of lysate with loading buffer (Bio-Rad Laboratories) containing 2-mercaptoethanol (Sigma, Oakville, ON, Canada) was incubated at 95 °C for 5 min, and was separated (100 V, 2 h) by sodium dodecyl sulphate-polyacrylamide gel
electrophoresis (SDS-PAGE), using Bio-Rad Criterion electrophoresis system (Bio-Rad Laboratories), and blotted (50 V, 2 h) onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5 % nonfat dry milk (Bio-Rad Laboratories) in TBS-T solution (TBS with 0.1 % Tween-20 (Sigma)) for ≥1 h, and then incubated with one of the following primary and secondary antibodies: NCS-1 (1:10,000; ProteinTech Group, Chicago, IL, USA or Abcam, Cambridge, UK) and anti-rabbit IgG (1:10,000; HRP-conjugated; GE Healthcare), respectively. NCS-1 protein levels were normalized to β-tubulin on the same blot.

2.5.3 Quantitative RT-PCR

Upon completion of the novelty test sessions in the Experiment 2 (Fig. 2-3), mice were euthanized by cervical dislocation. HPC was dissected in ice-cold PBS for RNA extraction. RNA was isolated using Trizol (Invitrogen™ Life Technologies) according to the manufacturer’s specifications. Complementary DNA was generated using Reverse transcriptase III (Invitrogen™ Life Technologies). Complementary DNA was synthesized and RT-PCR performed using ABI prism and SDS 2.1 software. ABI assays on demand (AppliedBiosystems Inc., Foster City, CA, USA) were used for Ncs1, Drd2, Creb, c-fos, Gapdh and Actin. Quantitative PCR were run in triplicate and threshold cycle (Ct) values were averaged. Data were then normalized to Gapdh. A region of the Ncs1, Drd2, Creb, c-fos, Gapdh and Actin mRNA was amplified using primers shown; NCS1 2F (5’- CTG AAG TTG TGG AGG AGC TG-3’) and NCS1 2R (5’- CTT GTT CTC GTC GAA GAC G-3’), mGAPDH F (5’-GCA CAG TCA AGG  CCG AGA A-3’) and mGAPDH R (5’-GCC TTC TCC ATG GTG GTG AA-3’), DRD2 F (5’-TAT GCC CTG GGT CGT CTA TC-3’) and DRD2 R (5’-AGG ACA GGA CCC AGA C AA TG-3’), CFOS F (5’-CTC CCG TGG TCA CCT GTA CT-3’) and CFOS R (5’-TTG CCT TCT CTG ACT GCT CA-3’), CREB F (5’-CTT CCA CTT CTG CCC TCA AG-3’) and CREB R (5’-TCC CTA AGG CAA TCA TGG AG-3’), mActin F (5’-CGG TTC CGA TGC CC T GAG GCT CTT-3’) and mActin R (5’-CGT CAC ACT TCA TGA TGG AAT TGA-3’).

2.5.4 Immunofluorescence; c-fos staining

Sixty minutes after completing the novelty test session in the Experiment 2 and 3 (Fig. 2-3 and 2-
4), each animal was anaesthetized with isoflurane and transcardially perfused with 20 mL of saline solution followed by 20 mL of 4 % paraformaldehyde (PFA) in PBS. The brain was rapidly removed and post-fixed overnight in 4 % PFA in PBS. 50 µm coronal sections were obtained using a vibrating microtome (VT10005; Leica Microsystems, Weltzlar, Germany) and were stored at -4 ºC in PBS. Sections to be processed for c-fos-immunoreactivity were transferred to PBS (pH 7.4) and washed several times. After 1 h of incubation in PBS containing 5 % BSA and 0.1 % Triton X-100 (PBS-BT), sections were incubated overnight in anti-Fos rabbit polyclonal antibody (sc-52; Santa Cruz Biotechnology) diluted 1:500 in PBS-BT, at 4 ºC and with constant orbital rotation. Sections were washed three times in PBS and incubated in secondary antibody diluted 1:1000 in PBS-BT (goat anti-rabbit IgG; Cy5-conjugated; Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. After several PBS rinses, sections were mounted onto slides using Prolong Gold media (Life Technologies). Sections from groups to be directly compared were processed at the same time and using the same conditions and reagents in order to reduce variability.

2.5.5 Analysis of c-fos positive cells

The number of cells displaying c-fos immunoreactivity was measured in the following brain regions; In Experiment 2, the CA1, CA3 and DG sub-regions of the HPC, nucleus accumbens (NAc), and Amyg were assessed; In Experiment 3, dorsomedial striatum (DMS), dorsolateral striatum (DLS), NacC, nucleus accumbens shell (NacS), DG, medial prefrontal cortex (mPFC), CgC, EntC, PeriC were analyzed. The brain regions were defined by stereotaxic coordinates of mouse brain (Paxino and Franklin, 2004). At least two to three non-consecutive sections were stained and imaged bilaterally for each brain region, for each subject. Fluorescence images were acquired at 20 × (0.75 NA) magnification, using a laser-scanning confocal microscope Nikon C1si (Nikon Canada, Mississauga, Canada). After image acquisition, counting of the stained cells was carried out using NIS-Elements AR software (Nikon Canada). Briefly, for each region, stained cells were automatically detected based on their intensity of staining relative to background and their size. The experimenter was blind to experimental grouping throughout image acquisition and processing. Counts from both hemispheres and from all rostro-caudal levels were averaged in order to obtain a single value for each subregion for each subject. For each experiment, the count of c-fos-positive cells from each region was normalized by dividing it
by area, in order to allow comparison of the relative novelty-induced changes between the various brain regions and across different experiments.

2.6 Statistical Analyses

Behavioral data were analyzed by mixed factor analysis of variance (ANOVA) where the F-test rejected the null hypothesis of equal means, this was followed by Bonferroni’s post-hoc testing. Elsewhere, two-tailed t tests for statistical significance were employed. Values in figures are expressed as mean ± standard error of the mean (SEM). Correlation data were analyzed with a Spearman correlation test. Differences were considered statistically significant at p < 0.05. C-fos imaging data were analyzed by structural equation modeling (StEM) to assess inter-relationships among correlated variables comprising an underlying theoretical structure, as previously described (Albasser et al., 2010). Three measures of goodness of fit include a nonsignificant chi-square ($\chi^2$), the comparative fit index (CFI), and the root mean square error of approximation (RMSEA). In addition to a nonsignificant $\chi^2$ along with a ratio of the $\chi^2$ to the degrees of freedom of $< 2$, a good-fitting model that is a plausible representation of the underlying data structure was considered to have either a CFI = 0.90–0.95 or an RMSEA = 0.05 (good) or $< 0.08$ (acceptable) (Tabachnick et al., 2001).
Chapter 3

3 Exploration of safe and novel space invokes a learning bonus in mice that requires NCS-1

Contributions to project

H.S. Mun contributed to all experiments and the writing of the manuscript.

B.J. Saab, E. Ng, J. Georgiou helped review and edit the manuscript.

A. Mcgirr helped inject botulinum toxin A.

E. Ng performed forced swim test.

Contents of this chapter have been published in Scientific Reports: Mun HS et al.


A link to the published paper can be found at

http://www.nature.com/articles/srep17697
Summary

Understanding the mechanisms underlying memory formation is fundamental to establishing optimal educational practices and restoring cognitive functions arising from brain diseases. Here, I show for the first time in a non-primate species, that spatial learning receives a special bonus from self-directed exploration. In contrast, when exploration is escape-oriented, or when the full repertoire of exploratory behaviors is reduced, no learning bonus occurs. These findings permitted the first molecular and cellular examinations into the coupling of exploration to learning. I found elevated expressions of $\text{Ncs1}$ and $\text{Drd2}$ upon self-directed exploration, in concert with increased neuronal activities in the hippocampal DG and area CA3, as well as the NAc. I probed further into the learning bonus by developing a point mutant mouse ($\text{Ncs1}^{P144S/P144S}$) harboring a destabilized NCS-1 protein, and found this line lacked the equivalent self-directed exploration learning bonus. Acute knockdown of $\text{Ncs1}$ in the HPC also decoupled exploration from efficient learning. These results are potentially relevant for augmenting LM in health and disease, and provide the basis for further molecular and circuit analyses in this direction.

3.1 Introduction

LM have long been studied and understanding their underlying mechanisms and neural basis is of great importance for educational practices, and preserving or restoring cognitive functions in human aging and brain disorders. Upstream of LM is the processes by which we explore, gather, and organize the raw information from our surrounding on which memories are built. Here I investigated the molecular and behavioral mechanisms that mediate self-directed exploration and its associated benefits for spatial LM.

In behavioral research, “exploration” widely refers to all activities directed at increasing information intake from the environment (Archer, 1983; Berlyne, 1964). Exploration has important implications for survival, and can give rise to sources of food, safety, and reproduction (Montgomery and Monkman, 1955; Zimbardo and Montgomery, 1957), which may explain the
clear evolutionarily conserved intrinsic reward to investigation of novel stimuli (Bunzeck et al., 2011; Guitart-Masip et al., 2010).

In humans, self-directed ‘volitional’ exploration of an object and its location results in improved performance on recognition tasks and this benefit is linked to a brain network centered on the HPC (Voss et al., 2011). Similarly in rats, the experimentally manipulated motivation to explore objects differentially recruits specific subregions of the HPC (Albasser et al., 2010), and previous research in mice revealed the importance of the DG in driving exploratory behavior of safe, novel space (SNS) (Saab et al., 2009).

The type, extent and underlying motivation for exploration varies as function of environmental factors such as novelty and the degree of stress, threat or volition, all of which also affect LM performance (Barker et al., 2004; Held and Hein, 1963; Kormi-Nouri et al., 2005; Schwabe and Wolf, 2010; Voss et al., 2011). In particular, safe non-threatening environments improve academic performance (Flum et al., 2006), while mild stress can impair memory formation in humans (Schwabe and Wolf, 2010). Here, I experimentally manipulated the level of fear during self-directed exploration by employing different lighting and novelty conditions in order to examine the underlying basis for the effects that exploration has on LM.

The Roder lab has previously shown that over-expression of NCS-1 in the murine DG increases exploratory behavior and spatial LM (Saab et al., 2009). NCS-1 is a member of the neuronal calcium sensor protein superfamily (Burgoyne, 2007) that regulates presynaptic Ca\textsuperscript{2+} influx in flies (Dason et al., 2009) and long-term synaptic plasticity in rodents (Génin et al., 2001; Jo et al., 2008; Saab et al., 2009). NCS-1 plays also a key role for LM in C. elegans (Gomez et al., 2001).

Yet, NCS-1 and its binding partners may further facilitate LM in concert with motivation and reward circuitry to seek out additional unique environmental inputs, or to more efficiently encode information about the surrounding environment. Indeed, allelic variation in Ncs1 is implicated in addictive behaviors (Multani et al., 2012), characterized by pathological motivation and reward. Overexpression of NCS-1 in the hippocampal DG promotes exploratory behavior and the acquisition of spatial memory, likely through regulating surface expression of the D2R
(Saab et al., 2009), a binding partner of NCS-1 (Kabbani et al., 2002; Saab et al., 2009). Chronic cocaine exposure reduces D2R-modulated Ca\textsuperscript{2+} influx and activity of the adenylate cyclase-calcineurin pathway in the NAc (Hu, 2004; Perez et al., 2011). \textit{Ncs1} KO mice exhibit impaired exploratory behavior, together with deficits in non-aversive long-term memory (de Rezende et al., 2014). Thus, in addition to the established role of hippocampal NCS-1 in exploration and LM, extra-hippocampal NCS-1 may govern the degree to which intrinsic motivation associated with exploration and resultant exploratory drive.

I present data from mice showing that volitional vertical exploration, quantified by rearing, correlates with better spatial learning in an object discrimination task involving object displacement, and that experimentally enhancing and preventing this form of exploration, in turn, enhances and prevents spatial learning. Furthermore, I demonstrate, through multiple independent approaches, that NCS-1 in the brain, and specifically the DG, is a factor for intrinsically motivated exploratory behavior and the associated learning bonus. Our experiments show that exploration up-regulates the expression of unique genes including \textit{Ncs1} and \textit{Drd2}, and that knockdown of NCS-1 levels by siRNA or by point mutation reduces exploration, causing deficient spatial memory. Thus I demonstrate that NCS-1 is not only essential for cognitive capacity, but also gene regulation associated with intrinsically motivated exploration.

3.2 Result

To identify precise links between exploration and learning, and to study the potential roles of NCS-1 in these two processes, I first searched for a behavioral assay that met two specific criteria: 1) it must allow for the quantification of both exploration and learning and the correlation of the two measures, and 2) it must provide a way to manipulate the level of exploration. Given its satisfaction of the first criteria (Fig. 2-2), and its ability to modulate the level of perceived threat by adjusting the brightness of arena affecting the level of exploration (Fig. 3-1 A-B and 3-2 B-C), object recognition task was selected as primary measure of exploration/spatial learning.
3.2.1 High exploration and enhanced spatial learning in novel, dimly-lit environments.

I studied whether lighting conditions (Dim vs. Bright) alter various measures of exploratory behavior in an open field containing NOs (Fig. 2-2). Dimly-lit novel environments specifically increased two forms of self-directed exploration, namely vertical exploration in the form of rearing (on hind-limb) frequency and horizontal exploration in the form of path length travelled in the center of the arena (Fig. 3-1 A-B and 3-2 B-C). Dimly-lit novel environments, however, did not affect overall ambulation (Fig. 3-1 C-D and 3-2 D), grooming (Fig. 3-1 D) or object bias (Fig. 3-2A). Therefore, I interchangeably refer to the dimly-lit condition as a high-exploration environment and the brightly-lit condition as a low-exploration environment.

Rearing in SNS allows animals to make use of a superior vantage point (Lever, 2006) from which they can see farther, sniff alternative air currents and potentially obtain novel auditory information. If rearing is an exploratory strategy that improves the acquisition of spatial information, it may be associated with improved spatial learning. Alternatively, rearing could coincide with a brain state independently primed for efficient learning.

After training in either high- or low-exploration environments, I tested the performance of DO and NO recognition memory, the former being more difficult and hippocampal-dependent. Interestingly, only the group trained in the high-exploration environments showed preference for the DOs, implying a superior spatial map of the environment relative to the low-exploration group (Fig. 3-2 E). In contrast, both groups of mice demonstrated a preference for the NO in phase three of the task (Fig. 3-2 F). These results suggest a special role of the HPC in coupling exploration to efficient spatial learning.

In the event that mice trained under low-exploration condition performed poorly on the object displacement task because they misinterpreted the environment during the DO phase as novel, I compared the level of rearing in the first versus the second phase of the task (Fig. 2-2). Expectedly, no enhanced rearing was observed (Fig. 3-2 G) and no exploratory time for stationary objects (SO) was observed in the second phase (Fig. 3-2 H), as would be expected if the change in lighting conditions gave the impression of a novel environment.
Figure 3-1. Behavioral activity in the open field test of the novel dim, novel bright or familiar dim condition.

**Figure 3-1. Bayerical activity in the open field test of the novel dim, novel bright or familiar dim condition.**

*(A-D)* In the open field, *(A)* C57B6/J mice were tested in novel dim *(n=35)*, novel bright *(n=31)* or familiar dim *(n=29)* environments. Plots show vertical rearing *(B)*, horizontal movement in the center *(C)* or the entire arena (taken as a measure of overall ambulation) *(D)* and also walking and grooming behavior in the first five minutes of open field testing *(E)*. Data are expressed as mean ± SEM. **P = 0.01, ***P < 0.001. n.s.=not significant
Figure 3-2. Exploratory rearing promotes spatial learning. Mice were trained in an arena with four objects and under dim (n=14) or bright (n=15) lighting while object preference (A), rearing (B), rearing to wall, objects and unsupported rearing (C) and horizontal travel distance (D) were recorded. Later, preference towards two displaced objects (DO) over stationary objects (SO) was evaluated. Ability to recognize a novel object (NO) over a familiar object (FO) was evaluated. To confirm that mice trained under bright lighting did not interpret the environment during the training phase of the object recognition test, mice trained under dim or bright lighting were assessed for the number of rearings in each phase (H) and contact time with the two stationary objects during the habituation/training phases and the displaced objects preference phase which showed no differences. Data are expressed as mean ± SEM. * P = 0.05, ** P = 0.01, *** P < 0.001.
While a high-exploration environment favors rearing, a dimly-lit environment is intrinsically less threatening and therefore may improve DO memory in a manner not contingent on exploration. I thus sought to explore the contingent relationship between rearing and DO memory by selectively limiting rearing by way of focal partial hind-limb paresis induced via BoNT injection to the quadriceps (Kutschenko et al., 2011). I established an optimal dose of 0.1 unit that impaired rearing but not horizontal ambulation or other behaviors (Fig. 3-3 and 3-4 A-C). Despite being trained in a non-threatening dimly-lit high-exploration environment, and otherwise performing behaviorally the same as controls, BoNT-treated mice demonstrated impairment in memory for DO location (Fig. 3-4 D). NO recognition on the other hand was unaffected (Fig. 3-4 E), showing the general ability to learn remained intact. I thus conclude that the act of rearing, and the ability to explore at will, are essential to efficient learning.
Figure 3-3. Establishment of BoNT injection reducing rearing activity without affecting ambulation in the open field test.

(A-B) Comparison of unilateral (uni) and bilateral (bi) botulinum neurotoxin A (BoNT, XEOMIN) hindlimb injections on rearing (A) and ambulation (B) at 0.1 and 0.2 U delivery of BoNT injection and testing in the open field. Mice (n=10 per group) were assessed in the open field three days post-injection a dose of 0.1 U uni caused focal hindlimb paresis that impaired rearing without imposing any significant reduction in horizontal activity. (C) During the behavioral activity in the first five minutes of open field testing, no differences were found in walking and grooming between mice given hindlimb injections of BoNT or saline. Data are expressed as mean ± SEM. * P = 0.05, ** P = 0.01, *** P < 0.001.
Figure 3-4. Relationship between exploratory rearing and spatial memory in the object recognition test for mice injected with saline or botulinum toxin A. Object preference (A), rearings (B) and horizontal travel distance (C) were recorded during object habituation and subsequent testing for displaced objects (DO) preference over stationary objects (SO) (D) and novel object (NO) preference over familiar object (FO) (E). Data are expressed as mean ± SEM. **P = 0.01, ***P < 0.001.
To further evaluate the role of exploratory rearing in learning, I examined the correlation (Fig. 3-5A; Table 3-1) between rearing (Fig. 3-2 B and 3-4 B) during the training phase and DO preference (Fig. 3-2 E and 3-4 D). I found that subjects trained in dim lighting that had the most rearing events also showed the highest preference for DO (Fig. 3-5A; blue dots). The same relationship was not observed for mice trained in the bright environment (Fig. 3-5A; yellow dots). The observed difference indicates that the level of exploration *per se* may not be the critical factor eliciting learning bonus, but instead requires the synergy of rearing and appropriate motivation to explore. This is further supported by the fact that BoNT-treated mice (Fig. 3-5A; green dots), that were trained under dim lighting, showed a leftward shifted range of data but with slope similar to that of untreated explorers, and relatively high correlation coefficient value (not significant; $p = 0.09$). I found no correlation between horizontal movement and spatial memory in any of the three groups (Fig. 3-5B; Table 3-1).

![Figure 3-5](image)

**Figure 3-5. Exploratory rearing promotes spatial memory.**

(A) Plot of rearing data (Fig. 3-2 B and 3-4 B) against displaced objects preference (Fig. 3-2 E and 3-4 D) as a spatial memory index. (B) Plot of travel distance data (Fig. 3-2 D and Fig 3-4 C) against displaced object preference (Fig. 3-2 E and 3-4 D). Pearson correlation ($r^2$), slope (m), and p-value were determined.
Table 3-1. The statistical summary of correlation analysis.

The analyses of correlation between exploratory activity (Rearing or Horizontal activity) and preference to objects (Novel or Displaced objects, NO and DO respectively) were performed and slope, Pearson r, r², F-value and P-value are shown for all the conditions related to Figure 3-5, 3-11 I-J, and 3-13 J-K. * P = 0.05, ** P = 0.01, *** P < 0.001.

<table>
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<th></th>
<th>Comparison</th>
<th>Slope</th>
<th>Pearson r</th>
<th>r²</th>
<th>F-value</th>
<th>P-value</th>
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<tr>
<td>Novel Dim</td>
<td>Rearing-DO</td>
<td>0.72 ± 0.26</td>
<td>0.63</td>
<td>0.39</td>
<td>F(1,12)=7.8</td>
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<td>(n=14)</td>
<td>Rearing-NO</td>
<td>-0.21 ± 0.36</td>
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<td>Horizontal-DO</td>
<td>-0.22 ± 0.17</td>
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<td>F(1,12)=0.02</td>
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<td></td>
<td>Horizontal-NO</td>
<td>-0.11 ± 0.18</td>
<td>-0.18</td>
<td>F(1,12)=0.38</td>
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<tr>
<td>Novel Bright</td>
<td>Rearing-DO</td>
<td>0.29 ± 0.21</td>
<td>0.35</td>
<td>0.12</td>
<td>F(1,13)=1.8</td>
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<td>(n=15)</td>
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<td>0.45</td>
<td>0.20</td>
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<td></td>
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<td>F(1,13)=0.14</td>
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<td></td>
<td>Horizontal-NO</td>
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<td>F(1,13)=0.44</td>
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<td>Rearing-DO</td>
<td>0.49 ± 0.06</td>
<td>0.94</td>
<td>0.89</td>
<td>F(1,8)=62</td>
<td>***</td>
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<td>Saline injection</td>
<td>Rearing-NO</td>
<td>0.39 ± 0.33</td>
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<td>0.15</td>
<td>F(1,8)=1.4</td>
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<tr>
<td>(n=10)</td>
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<tr>
<td></td>
<td>Horizontal-NO</td>
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<td>Rearing-NO</td>
<td>0.05 ± 0.81</td>
<td>0.02</td>
<td>0.00</td>
<td>F(1,8)=0.00</td>
<td>0.95</td>
</tr>
<tr>
<td>(n=10)</td>
<td>Horizontal-DO</td>
<td>0.11 ± 0.18</td>
<td>0.20</td>
<td>F(1,8)=0.35</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Horizontal-NO</td>
<td>-0.46 ± 0.30</td>
<td>-0.48</td>
<td>F(1,8)=2.3</td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>Bi HPC injection</td>
<td>Rearing-DO</td>
<td>0.45 ± 0.11</td>
<td>0.74</td>
<td>0.55</td>
<td>F(1,14)=17</td>
<td>***</td>
</tr>
<tr>
<td>Negative siRNA</td>
<td>Rearing-NO</td>
<td>0.18 ± 0.13</td>
<td>0.34</td>
<td>0.11</td>
<td>F(1,14)=1.8</td>
<td>0.20</td>
</tr>
<tr>
<td>(n=16)</td>
<td>Horizontal-DO</td>
<td>-0.03 ± 0.18</td>
<td>-0.05</td>
<td>F(1,14)=0.03</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Horizontal-NO</td>
<td>0.06 ± 0.16</td>
<td>0.09</td>
<td>F(1,14)=0.13</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>Bi HPC injection</td>
<td>Rearing-DO</td>
<td>0.07 ± 0.19</td>
<td>0.10</td>
<td>0.01</td>
<td>F(1,14)=0.14</td>
<td>0.72</td>
</tr>
<tr>
<td>Ncs1 siRNA</td>
<td>Rearing-NO</td>
<td>-0.31 ± 0.22</td>
<td>-0.35</td>
<td>0.12</td>
<td>F(1,14)=1.9</td>
<td>0.19</td>
</tr>
<tr>
<td>(n=16)</td>
<td>Horizontal-DO</td>
<td>0.19 ± 0.14</td>
<td>0.33</td>
<td>0.11</td>
<td>F(1,14)=1.7</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Horizontal-NO</td>
<td>-0.23 ± 0.16</td>
<td>-0.35</td>
<td>F(1,14)=2.0</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>Ncs1 P144S/P144S</td>
<td>Rearing-DO</td>
<td>0.95 ± 0.30</td>
<td>0.64</td>
<td>0.41</td>
<td>F(1,14)=9.8</td>
<td>**</td>
</tr>
<tr>
<td>(n=16)</td>
<td>Rearing-NO</td>
<td>0.47 ± 0.28</td>
<td>0.41</td>
<td>0.17</td>
<td>F(1,14)=2.8</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Horizontal-DO</td>
<td>-0.15 ± 0.12</td>
<td>-0.31</td>
<td>F(1,14)=1.5</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Horizontal-NO</td>
<td>-0.05 ± 0.10</td>
<td>-0.15</td>
<td>F(1,14)=0.32</td>
<td>0.58</td>
<td></td>
</tr>
<tr>
<td>Ncs1 P144S/P144S</td>
<td>Rearing-DO</td>
<td>0.48 ± 0.44</td>
<td>0.29</td>
<td>0.08</td>
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<td>(n=15)</td>
<td>Rearing-NO</td>
<td>0.55 ± 0.33</td>
<td>0.42</td>
<td>0.17</td>
<td>F(1,13)=2.7</td>
<td>0.12</td>
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<tr>
<td></td>
<td>Horizontal-DO</td>
<td>0.03 ± 0.18</td>
<td>0.04</td>
<td>F(1,13)=0.02</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Horizontal-NO</td>
<td>-0.14 ± 0.13</td>
<td>-0.28</td>
<td>F(1,13)=1.1</td>
<td>0.31</td>
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3.2.2 Exploration of SNS activates the DG, CA3, NAc and expression of Ncs1 and Drd2.

To gain insight into the neural and molecular substrates responsible for the differences in exploratory behavior and the efficiency to learn in low- versus high-exploration environments, I examined the expression of the IEG, c-fos as a marker of neuronal activity (Hunt et al., 1987; Morgan et al., 1987) during the acquisition phase of the object recognition paradigm. First, I have confirmed the c-fos antibody specificity by using c-fos peptide (Fig. 3-6). Then, I focused on the HPC, due to its suggested role in spatial navigation, exploration and novelty detection (Saab et al., 2009; Vazdarjanova et al., 2002; Lisman et al., 2001); the NAc as it is involved in processing motivational relevance of environmental stimuli (Carlezon et al., 2009); and the basolateral amygdala (BLA) as a fear center (Davis et al., 1992). I found SNS, high-exploration environments, were associated with more c-fos-positive cells in the DG, hippocampal subregion CA3 and the NAc (Fig. 3-7). Novel bright environments, in contrast, only led to pronounced increases in amygdalar Fos-positive cells (Fig. 3-7).
Figure 3-6. c-fos antibody validation.

In the upper panel, c-fos positive cells are shown without peptide. In the lower panel, when the c-fos antibody was mixed with the peptide, the c-fos positive cells are not distinguished, with even increased exposure time.
A

CA1/DG  CA3  Amyg  NAc

ND  [Image]

NB  [Image]

FD  [Image]

B

![Bar chart showing Fos positive cells in various brain regions.](image)

- ** Novel Dim
- Novel Bright
- Familiar Dim

<table>
<thead>
<tr>
<th>Region</th>
<th>Fos positive cells</th>
</tr>
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<tbody>
<tr>
<td>CA1</td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td></td>
</tr>
<tr>
<td>Dentate Gyrus</td>
<td></td>
</tr>
<tr>
<td>Nucleus Accumbens</td>
<td></td>
</tr>
<tr>
<td>Amygdala</td>
<td></td>
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*Significance levels: ** p < 0.01, *** p < 0.001.
Figure 3-7. Novel dim environments that enhance rearing induce c-fos.

(A) Sample confocal images showing localization of c-fos positive cells 90 min post-exposure to novel dim (ND), novel bright (NB) or familiar dim (FD) environments. Brain regions examined include hippocampus CA1/dentate gyrus (DG), CA3, amygdala (Amyg) and nucleus accumbens (NAc) (n=3 for each condition). Scale bar = 100 microns. (B) Counts of c-fos-positive cells in brain regions at three sections per mouse, n= 3 per group. Data are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.
Given the prior work that links D2R and NCS-1 to novelty-driven exploration and spatial learning (Saab et al., 2009), I examined whether changes in Drd2 and Ncs1 expression are modulated by exposure to environments that vary in lighting and novelty. Quantitative PCR revealed Ncs1 and Drd2 are specifically upregulated following exploration in SNS, in comparison to exploration in either novel bright or familiar dim environments (Fig. 3-8).

Two additional transcripts were uniquely modified between conditions. The known memory regulator cAMP Response Element-Binding (Creb) and Fos both increased as a function of novelty, independent of the lighting or level of exploration (Fig. 3-8), confirming our immunohistofluorescence data (Fig. 3-7). This result may suggest that novelty-induced exploration activates the HPC and causes an elevation in genes known to be involved in synaptic plasticity and memory encoding. In contrast, Ncs1 and Drd2 were elevated only when novelty was coupled with a safe environment and high levels of rearing, an intriguing result that led us to wonder whether NCS-1 serves as a prerequisite for the learning bonus provided by high exploration.
Figure 3-8. Novel dim environments elevate Ncs1 and Drd2 expressions.

Comparison of various mRNA levels 30 minutes following exposure to testing environments. (A) Representative images of electrophoresed RT-PCR amplicons for indicated mRNA. (B) Ratio of Ncs1, Drd2, Fos, Creb1, and Actin mRNA expression levels normalized to Gapdh (n = 3 per group, triplicate). The expressions of those genes were measured by real-time PCR. Data are expressed as mean ± SEM. * P < 0.05, ** P < 0.01, *** P < 0.001.
3.2.3 NCS-1 is required for exploration-enhanced learning.

To test the molecular relationship between NCS-1 and exploration, I generated a mouse mutant of the protein by screening the RIKEN ENU-mutagenized genomic DNA archive. One resultant mouse, Ncs1\textsuperscript{P144S/P144S} harbored a point mutation that introduces a third degree of freedom of rotation at residue 144 and at least two additional hydrogen bonds, which could produce interference with proper protein folding and/or give rise to protein instability (Fig. 2-1 and 3-9 A). Quantitative PCR revealed no differences in whole brain Ncs1 mRNA between P144S and wild-type genotypes (Fig. 3-9 B), however, immunoblots revealed a gene dose-dependent reduction in NCS-1 protein (Fig. 3-9 C). The reduction in protein but not mRNA levels is consistent with the in silico molecular modeling predicted destabilization of protein structure (Fig. 3-9 A).

\textit{Ncs1}\textsuperscript{P144S/P144S} mice showed no abnormalities in measures of general physiology, such as walking and grooming (Fig. 3-10 A) in the open field test and motor function in the rotarod test (Fig. 3-10 B). I also examined endophenotypes of mental illness, such as anxiety (Fig. 3-10 C-E), depression and addiction (Fig. 3-10 F-G), but found no significant differences.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3-9.png}
\caption{P144S point mutation of NCS-1 reduces protein stability.}
\end{figure}

(A) Models of wild-type (left) and P144S (right) NCS-1 protein. Taken from Saab, 2009. (B) Ncs1 mRNA levels between genotypes. (C) Representative immunoblot (upper) of NCS-1 using tubulin as loading control with densitometric quantification (lower). Data are expressed as mean ± SEM. * P < 0.05, ** P < 0.01.
Figure 3-10. No changes in motor function and in performance for anxiety or depression related behavior measures of Ncs1P144S/P144S mice.

(A) In the open field, in the first 5 minutes, regardless of lighting conditions, no differences were found in walking and grooming between Ncs1P144S/P144S mutants and littermate controls. (B) The rotarod test showed no genotypic differences in motor performance or motor learning (slope) (Ncs1+/+, n=6; Ncs1P144S/P144S, n=6). (C, D) In the Light/Dark box test, no genotypic differences were found in (C) latency to enter the brightly-lit chamber and (D) number of transitions between the two sides (Ncs1+/+, n=6; Ncs1P144S/P144S, n=6). (E) In the elevated plus maze, the duration spent in the open and closed arms was similar between genotypes (Ncs1+/+, n=11; Ncs1P144S/P144S, n=8). (F) No differences between genotypes in time spent swimming or immobile in the forced swim test (Ncs1+/+, n=13; Ncs1P144S/P144S, n=12). (G) No genotypic differences were found in the amount of sucrose solution (1 %) ingested in the sucrose preference test (Ncs1+/+, n=6; Ncs1P144S/P144S, n=6). Data are expressed as mean ± SEM.
However, $Ncs{1}^{P144S/P144S}$ mice reared less frequently than their littermate controls in the open field (Fig. 3-11 A) and spent less time in the center of the novel arenas (Fig. 3-11 B) despite normal horizontal activity (Fig. 3-11 C). In the New Frontier task (Saab et al., 2009), $Ncs{1}^{P144S/P144S}$ subjects explored from their home cages but showed fewer total visits to the novel frontiers (Fig. 3-11 D). Interestingly, there was no genotypic difference in the exploratory behavior when tests were performed in a threatening, low-exploration environment (Fig. 3-11 A-D), suggesting NCS-1 is specifically required for the intrinsically rewarding exploration induced learning.

I proceeded to examine whether a link between exploratory rearing and spatial memory exists in our genetic model. The NCS-1 mutants reared less without changing overall ambulation during object recognition training and showed poorer DO memory and intact NO recognition (Fig. 3-11 E-H). A correlation analysis revealed significant association between rearing activity and spatial memory in $Ncs{1}^{+/+}$ but not $Ncs{1}^{P144S/P144S}$ mice (Fig. 3-11 I; Table 3-1), suggesting NCS-1 is critical for the learning bonus afforded by self-directed exploration. Additionally, no correlation was found between horizontal movement and spatial memory (Fig. 3-11 J; Table 3-1).
Figure 3-11. *Ncs1*\textsuperscript{P144S/P144S} mice how reduced exploration and impaired spatial memory.

(A-D) Exploratory activity for *Ncs1*\textsuperscript{P144S/P144S} mice (n = 12 per group) in the open field (A–C) and New Frontier tests (D) were conducted under dim or bright lighting and the following measures were recorded: Rearing (A), ambulation in the center of (B) or over the whole arena (C), and crossing events (D). (E–H) Object recognition testing showing rearings (E) and travelled distance (F) during training phase, and preference test for displaced (G) or novel objects (H). (I) Correlation plot for displaced object preference as a function of exploratory rearing. (J) Correlation plot between horizontal movement and displaced object preference. *Ncs1*\textsuperscript{P144S/P144S}, n = 16; *Ncs1*\textsuperscript{+/-}, n = 15; Data are expressed as mean ± SEM. Pearson correlation (r\textsuperscript{2}) and slope (m) are shown. * p < 0.05, ** p < 0.01, *** P < 0.001. DO; displaced objects, SO; stationary objects, NO; novel object, FO; familiar object. See also Table 3-1.
To determine whether NCS-1 in the DG, specifically, is required for exploration, I proceeded to acutely reduce DG NCS-1 using siRNA. I initially screened a set of siRNAs targeting \( NcsI \) in mouse neuroblastoma N2A cells and found one siRNA (Freq 3) reduced NCS-1 protein levels relative to the negative siRNA (Fig. 3-12). Infusion of Freq 3 siRNA directly into the DG of cannulated wild-type C57BL/6J mice reduced the total amount of basal NCS-1 protein in the HPC to about half (Fig. 3-13 A). Rearing was reduced in the open field without changing ambulation (Fig. 3-13 B-C), and also during the training phase of the object recognition task (Fig. 3-13 F-G). Like the low-explorers and mouse line \( NcsI^{P144S/P144S} \), specific \( NcsI \) knockdown in the HPC impaired DO memory (Fig. 3-13 H) while sparing the recognition of NOs (Fig. 3-13 I). \( NcsI \) siRNA-treated mice displayed reduced rearing and hole-board test performance (Fig. 3-13 D-E). Acute reduction of \( NcsI \) in the HPC also abolished the correlation between exploration and learning (Fig. 3-13 J; Table 3-1), and again, I found no correlation between horizontal movement and spatial memory (Fig 3-13 K; Table 3-1). These data show that the DG and NCS-1 both underlie the motivation to explore novelty, as well as the associated learning bonus.
Figure 3-12. NCS-1 levels after knockdown in N2A cells.

(A) Representative immunoblot for NCS-1 and tubulin expression 1 day after siRNA transfection into N2a cell lines. Lane sequence order is as per section (B). (B) A graph of quantified relative NCS-1 protein levels in N2A cells after transfection of three different siRNAs against *Ncs1*. Data expressed as a fraction of non-transfected cell NCS-1 levels normalized to tubulin. Compared to Negative siRNA, the Freq 3 siRNA showed the best reduction (67 %), while Freq 1 siRNA showed minimal reduction and Freq 4 siRNA showed 35 % reduction of NCS-1 protein level by immunoblot. Freq 3 siRNA was chosen for further in vivo experiments. Statistical significance was determined by t-test following densitometric analysis of n=3 blots for each group. Data are expressed as mean ± SEM. *p<0.05, *** P < 0.001
Tubulin

NCS-1

Control

Negative

siRNA

Ncs1

siRNA

0 20 40 60 80 100
Total Distance (cm)

0-5
5-10
10-15
15-20
20-25
25-30

0 500 1000 1500 2000
Time (min)

Total Distance (cm)

0.0 0.5 1.0 1.5
C

c

ontro

Nega

tive siRNA

Ncs1 siRNA

***

Rearings (events)

D

0 5 10 15 20
Rearings (events)

Displaced Objects Preference (%)

Negative siRNA: m=0.26/r^2=0.11/p=0.21
Ncs1 siRNA: m=0.19/r^2=0.11/p=0.21

Displaced Objects Preference (%)

Rearings (events)

Displaced Objects Preference (%)

Total Distance (m)

Displaced Objects Preference (%)

Total Distance (m)
Figure 3-13. Ncs1 knockdown in dentate gyrus (DG) reduces exploration.

(A) Representative immunoblot for hippocampal NCS-1 and tubulin three days after the DG was infused with negative siRNA or siRNA against Ncs1, as compared to untreated control mice on the left with densitometric analysis (normalized to tubulin) showing that Ncs1 siRNA reduced NCS-1 protein level (two representative samples per group). Mice were tested in the open field (B, C) and hole-board test (D, E) under dim lighting and measured for rearing (B, D), distance (C) and holepoke exploration (E). (F–I) Relationship between exploratory rearing and spatial memory in the object recognition test for Ncs1 siRNA- and negative siRNA-treated mice. Reduced rearing (F) and normal travel distance (G) during object habituation and subsequent testing for displaced object preference (DO) over stationary objects (SO) (H). (I) Intact novel object (NO) over former object (FO) preference in all siRNA-treated mice. (J) Plot of exploratory rearing data (F) against displaced object memory (H). (K) Correlation plot between horizontal movement and displaced object preference. Negative siRNA, n = 13; Ncs1 siRNA, n = 11. Data are expressed as mean ± SEM. Pearson correlation (r²) and slope (m) are shown. *p < 0.05, **p < 0.01. See also Table 3-1.
3.3. Discussion

This study establishes that animals, actively influencing their experience of sensory information through intrinsically motivated exploration, activate specific brain structures required for efficient learning. Furthermore, I identified a molecular basis behind the LM benefit associated with self-directed exploration, demonstrating NCS-1 and the DG are both required. The findings suggest that a fundamental need to explore, likely present in many species, including humans (Archer et al., 1983), may be tied to their LM abilities. Exploration is thus a crucial facet of behavior for reasons that go beyond direct material benefits of exploring a immediate environment.

Novel stimuli can induce different forms of exploratory behavior, chiefly depending on their perceived potential to deliver pain or reward. Fear and novelty-driven exploration are thus inversely related, producing a continuum of behaviors that may belie a common neurobiological basis. Indeed, dopaminergic projections to the ventral HPC and BLA influence motivation and aversive reaction through tonic and phasic components, the effects of which appear to be D2R-dependent (Belujon and Grace, 2015). Consistent with this, our data suggest that dimly-lit environments facilitate exploration by both reducing the “threatening” properties of NOs and promoting their “rewarding” characteristics. I confirmed that behavior in brightly-lit conditions is associated with changes in anxiety-related behaviors, including reduced rearing, increased grooming activity, and increased avoidance of the center of the arena. The competing balance of survival drives, namely safety vs. exploration and their associated neural circuitries, likely determine an animal’s willingness to engage with its environment. Indeed, these drives likely act in concert to influence LM directly, but also in synergy with the animal’s sensory experience to form the basis of contextual memory in an NCS-1 dependent manner. As expected, animals with either acute *Ncs1* knock down in the DG or, or chronic and global NCS-1 reduction, do not demonstrate a self-direct exploration learning bonus. Therefore, the novel *Ncs1*P144S/P144S mice generated and characterized here may represent a powerful animal model to further study the learning bonus.
The learning bonus from SNS operates through either exploration activity, exploration drive or in tandem. To pinpoint specific mechanism involved and examine whether impaired exploration was enough to devoid the exploration learning bonus, exploration was deliberately impaired using BoNT injection while exploration drive remained intact. Although the intention of performing BoNT experiments were to impaire exploration activity without affecting motivation, BoNT-induced paralysis in legs may cause stress, which in turn affecting the exploration drive. Therefore, further experiments are required to answer this question to measure the exploratory drive in each environments as well as BoNT groups. However, we cannot directly measure exploratory drive, but indirect measurement could be used such as cortisol level or defecation for stress measurement and also DA release or high-frequency USVs could be used.

Using IEG expression, I identified neural circuitry induced by environments that favor exploration. SNS conditions that foster exploration, enhanced Fos expression in the DG, CA3 and NAc. In human fMRI studies, the NAc, the main projection target of dopaminergic neurons of the substantia nigra/ventral tegmental area, is activated for reward anticipation (Schultz, 2004). Both novelty and reward cues (as motivational factors) co-activate the substantia nigra/ventral tegmental area and HPC (Brewer et al., 1998; Wittmann et al., 2005). Interestingly, this is coherent with the impairment observed in the object recognition task after pharmacological manipulation of the NAc and DG in mice (Dees and Kesner, 2013; Sargolini et al., 2003). Specifically, novelty facilitates the induction and persistence of long-term plasticity in the DG (Davis et al., 2004; Straube et al., 2003), processes considered as cellular mechanisms for LM. Our data suggests that NAc-hippocampal communication modulates the salience of environmental features that in turn promotes fine-tuning of exploratory behavior necessary for LM.

Self-directed exploration is also important clinically. Reduced exploration of novel stimuli is prevalent in memory deficit disorders, including Alzheimer’s disease (Daffner et al., 2001), fragile X syndrome (Mineur et al., 2002), age-related cognitive deficit (Rosenzweig and Barnes, 2003), as well as other learning disabilities (Costa and Silva, 2002) including ASD (Pierce and Courchesne, 2001). In addition, patients with a number of psychiatric disorders, such
as schizophrenia (Li et al., 2007) and depression (Harwood and Agam, 2003) exhibit impairments in exploration or LM or both. Therapeutically, finding novel molecular targets, such as NCS-1, to ameliorate cognitive deficits in these disorders may be possible by focusing on the mechanisms coupling self-directed exploration to efficient learning. Some evidence from other candidate genes, notably PDE4B, suggests that this is promising, as genetic inhibition of PDE4B lead to improvements in both exploration and LM, potentially through perception of environmental threat (McGirr et al., 2015).

Non-threatening educational practices that support information gathering and investigation may be of benefit to student academic success, development of identity, and future adaptation (Flum and Kaplan, 2006; Dohrmann et al., 2007). In particular, the efficiency of language learning exhibits a reciprocal correlation with environmental stress (MacIntyre et al., 1997), and curiosity promotes life-long learning in nurses (Kedge and Appleby, 2009). Our data provide evidence that a learning bonus is produced by exploration in non-threatening novel environments and reveal insights into the molecular and anatomical basis for these benefits. Further investigation of the link between novelty-induced exploration and memory may prove fruitful in the search for better therapeutic strategies for combating cognitive dysfunction associated with aging, neurodegenerative, and neuropsychiatric disorders.
Chapter 4

4  USVs and IEG expression patterns when mice explore

Contributions to project

H.S. Mun performed all experiments and the writing of the manuscript.

T.V. Lipna and J.C. Roder helped review and edit the manuscript.

Some parts of contents of this chapter have been published in Frontiers in behavioral neuroscience: Mun H. S. et al.


A link to the published paper can be found at

http://journal.frontiersin.org/article/10.3389/fnbeh.2015.00316/abstract
Summary

Previously, I used rearing as an index of exploration and associated it with better spatial LM in non-threatening novel environments. To better understand the relationship between exploration and hippocampal-dependent spatial LM, it would be helpful to have additional indices of novelty-induced exploratory states. Here, I studied whether USVs and brain IEG expression patterns could serve as additional readouts of intrinsically motivated exploration.

4.1 Introduction

Exploration is an essential aspect of behavior, but is also risky and thus avoidance behavior may preferentially occur in organisms encountering novel stimuli (Berlyne, 1964). Approach or avoidance is one of the most basic behavioral decisions for animals facing novel environments. Frequently, approaching new environments is thought to be driven by foraging or mating needs. However, the initial motivation to approach new environments can also be independent of foraging or reproduction, as observed in mice given a choice of novelty versus food without social cues (Chance and Mead, 1955). A critical question in animal behavior is, what motivates approach behavior and what are the behavioral and neurobiological mechanisms that support such explorative behavior? Indeed, it is currently unknown how to reliably measure motivations or affective states related to approach or avoidance behavior, which is not associated with food or reproduction. Here, I have tried, first, to establish the qualitative and reliable measurements for such phenomena by measuring USVs, and to provide underlying mechanisms by determining the activated brain regions.

Precise characterization of USVs is fundamental to ascertain level of perceived threat or reward during exploration novel environments since growing studies of vocal communication of rodents linking emission of USVs during or in anticipation of threats or rewards (Wöhr and Schwarting, 2013). Also, comparing neuronal activities, as detected by expression of a neuronal activity marker, c-fos, when mice explore novel environments will help us determine how different brain regions are recruited to benefit LM. Further application of StEM enabled to correlate and compare the derived direction of effects between activated brain regions:
Hippocampus and its associated structures.

USVs, which are above human’s hearing range, often reveal a great deal about their general state, including motivational or affective states in many species, including songbirds and whales (Au et al., 2006; Wilbrecht and Nottebohm, 2003). Rodents also emit USVs and studies are mainly focused in pups in response to maternal separation and stressor or adults with social rewards (Portfors, 2007). Furthermore, rats communicate with USVs by emitting 22 kHz calls during or in anticipation of threats and 50 kHz calls during or in anticipation of rewards (Wöhr and Schwarting, 2013). Although it is not extensively studied in mice, a few papers showed that mice may emit low frequency 30 kHz (26 to 36 kHz) calls with the existence of stressors (Ko et al., 2005) and emit high frequency – 40 kHz calls with novelty exploration (Chabout et al., 2012). A few studies in mice have found the communicative and affective function of USVs in situations with each developmental stage, such as pup isolations, adolescent play, and adults in social contexts (Wöhr et al., 2013; Scattoni et al., 2009; details discussed in Chapter 1-4).

However, an analysis that includes both USVs and a detailed behavioral repertoire for mice during exploration of novel environments is currently missing in the literature. Moreover, the functional and affective properties of their vocal expressions, in terms of behaviors, have rarely been studied, especially in a non-social context. Studies on USVs of adult mice were not comprehensively conducted in any context other than reproduction related contexts (Kerchner, 2004). Recently, female-induced vocalization has been used to phenotype cholinergic and dopaminergic KO adult mice (Wang et al., 2008).

Surprisingly, the analysis of behavior in association with USVs is rarely measured as an important factor in interpreting the functions of USVs. Some have tried to score a selection of behaviors together with USV emissions, but behavioral analyzes, which also encompass USVs emissions, are rarely performed in adult mice. Only in studies in pups and adolescent mice, some temporal analysis of behavior association with USVs found these cries from pups happen prior to or during locomotion and head raising, a feature, which appear to encourage approach behaviors (Branchi et al., 2004). HF calling in adolescent mice is positively correlated with social investigation (Panksepp et al., 2007). To remedy this shortcoming, the current study founded on the premise that consistent recording of USVs coupled with distinct behavioral expressions will
allow us to accumulate behavioral correlates of a broad range of USVs and ultimately build the library of the “mouse language.” While much of the literature has focused on USV emissions in social contexts or threat-reward related settings, the behavior of adult male mice emitting USVs during exploration of novelty has not been studied extensively. What is generally and curiously missing is a simultaneous analysis between USVs and detailed mouse’ behavioral repertoire during exploration of novelty, an account that will help “decode” mouse’ USVs and provide new insight into mouse language.

Furthermore, the specific brain structures are engaged in different types of psychological processes in the organism. Cognitive processing of novelty recruits some essential brain regions, such as HPC (Knight et al, 1996; Nyberg et al., 2005; Yamaguchi et al., 2004; Jenkins et al., 2004; Brown et al., 2001), prefrontal cortices (Daffner et al., 2000; Dias et al., 2002; Xiang et al., 2004; Matsumoto et al., 2007), and several other regions including NAc (Ranganath et al., 2003; Yamaguchi et al, 2004). I aimed to study the patterns of activated brain regions during intrinsically motivated exploration in novel arena, with a focus on the functional link between HPC and NAc. First, lesion studies in animals (monkeys and rats) repeatedly showed that the NAc is necessary for processing unknown reward (e.g. response to novel environments) (Kelly, 1976; Fink et al, 1980; Schwarting et al., 1996). The functional links between NAc and the HPC remains highly contentious (Lisman et al., 2011).

In previous IEG studies, animals were exposed to either novel or familiar stimuli (Mishkin et al, 1975; Aggleton et al., 1985; Mumby et al, 1990) with spontaneous exploration (Ennaceur et al., 1988) without consideration of levels of threat or stress that mice encounter when they are exposed to novelty. One of the shortcomings of these studies is the difficulty in determining whether an increased IEG expression was a consequence of exposure to stress, novelty, or the effect of an enhanced motor activity (Montag-Sallaz et al., 1999). Therefore, the association between IEG expression and distinct neural coding for an event has remained unclear.

The general aim of the present study is to characterize precisely the relationship between USV emissions/activated brain regions and exploratory behaviors in adult male mice in novel...
environments. Specific aims for this study are (a) to provide a USV feature analysis of mice exploring environments to ascertain whether USVs predict emotional states as shown in rats, (b) to do a simultaneous analysis of describe exploratory behavioral patterns in mice that display HF vs. LF calls, (c) to evaluate whether rearing on hind limbs is associated with certain types of USVs, (d) to discrete stress, novelty or the motor activity effects on the IEG expression, multiple conditions were tested; high exploration (novel dim), low exploration (novel bright), novelty control (familiar dim), stress control (familiar bright) and motor control (wheel running in the homecage), and (e) to analyze the connectivity among activated brain regions, StEM was applied to the results of counted c-Fos positive cell numbers in each brain area. In brief, I found that HF calls were emitted and the function link between NacC and Hip was strengthened during exploratory behavior in dim novel environments. In addition, LF calls and the connection between Amyg and HPC were associated with escape responses in brightly lit novel environments.

4.2 Results

4.2.1 Spatial novelty in dimly lit arena maximize rearings

To enhance ability to score vocalization and identify relevant brain regions, I modified the experimental set up by adding more training days. Exploratory behaviors were measured in experimental paradigm where mismatch environments were introduced in either dim or brightly lit arena after habituation (Fig. 2-4). During the habituation phase, the number of rearings and horizontal movements were reduced as mice habituate to the environments and objects (Fig. 4-1). When mice exposed to familiar spatial arrangement in either dimly- or brightly- lit arena, mice reared as much as the last day of the habituation session. However, when spatial novelty was introduced after habituation, the frequency of rearing showed group and interaction effects (Fig. 4-1 A; interaction, \( F_{(15,130)}=3.39, p<0.0001 \); Group, \( F_{(3,130)}=34.00, p<0.0001 \); Time, \( F_{(5,130)}=7.07, p<0.0001 \)), as the rearing levels were increased only in dimly-lit arena, but not in the brightly lit arena by post-hoc test (Fig. 4-1 A; \( p<0.0001 \)). The level of total distance traveled in those novel contexts remained similar to familiar contexts (Fig. 4-1 B; interaction, \( F_{(15,130)}=1.05, p=0.41 \); Group, \( F_{(3,130)}=1.70, p=0.19 \); Time, \( F_{(5,130)}=6.03, p<0.0001 \)). Interestingly, the number of rearing
was double the amount in novel spatial rearrangement in the dimly-lit arena compared to that of novel environments without habituation (Fig. 4-1 B).
Figure 4-1. Spatial novelty induces vertical exploration (rearings).

(A) Mice were tested in spatial novel dim, spatial novel bright, familiar dim and familiar bright (each group, n=8). (B) Plots show vertical rearing. *** p<0.001.
4.2.2 Mouse USVs during novel environment exploration

I quantified and analyzed the number and features of USVs emitted in the five different treatment conditions: ND, NB, FD, FB, and FW (Fig. 2-4). Two mice, each from FD and FW conditions, failed to utter any calls and therefore were excluded from further analysis. The number of USVs emitted by adult male mice differed significantly among treatments (Fig. 4-2 A; Table 4-1 A; $F_{(4,48)}=24.16$, $p<0.0001$). Post-hoc comparisons showed that mice in ND condition emitted significantly more calls than mice in all other conditions (Table 4-1). I, then, examined the number of the high and low frequency calls in the five treatments. Both high and low frequency call number varied by treatment (HF calls: Fig. 4-2 B; Table 4-1 B; $F_{(4,48)}=12.93$, $p<0.0001$; LF calls: Fig. 4-2 C; Table 4-1 C; $F_{(4,48)}=12.93$; $p<0.0001$). Mice in ND condition emitted significantly more calls at both frequencies than mice in all other conditions (Table 4-1 B-C). However, the number of calls was significantly greater for mice in the NB condition compared to the FD and FB conditions only for low frequency calls (Fig. 4-2 C; Table 4-1 C). Mice in the NB condition also had the largest percentage of low frequency calls (out of total number of all calls) at 78.93 ± 5.38%, compared to ND (53.64 ± 6.17%), FD (50 ± 13.15%), FB (52.29 ± 8.91%), and FW (57.05 ± 6.91%).

There was no main effect of experimental groups on peak frequency (Fig. 4-2 D; Table 4-1 D; $F_{(4,48)}=2.23$, $p=0.079$). There did appear to be a trend in which mice in ND, FD, and FB conditions emitted somewhat higher mean peak frequency USVs (34.93 ± 2.79, 34.91 ± 5.47, and 31.04 ± 3.16 kHz, respectively), and mice in NB and FW conditions tended to emit lower mean peak frequency USVs (26.01 ± 1.16 and 28.74 ± 2.34 kHz, respectively; Fig. 4-2 D). Mean call duration differed significantly among treatments (Fig. 4-2 E; Table 4-1 E; group effect, $F_{(4,48)}=14.737$; $p<0.0001$); call duration was significantly longer in the ND group than in all other groups (Fig 4-2E). I then plotted the distribution of individual USV call durations by peak frequency for each treatment separately (Fig. 4-2F). This illustrates well the effect of experimental conditions, where calls of high frequencies USV have a longer duration in the ND treatment relative to the others (Fig. 4-2F).
Figure 4-2. Ultrasonic vocalizations (USVs) in adult male mice.

(A) Total number of USVs, (B) number of high frequency calls (HFC; >35 kHz), and (C) number of low frequency calls (LFC; 20-35 kHz) were displayed. For the acoustic features, (D) mean peak frequency, (E) call duration, and (F) distribution of calls with frequency and call durations were shown peak in each environments, including novel dim (ND), novel bright (NB), familiar dim (FD), familiar bright (FB) and water. Data are expressed as mean ± SEM. * P < 0.05, *** P < 0.001.
Table 4-1. The summary of statistical analysis of the results in Figure 4-2.

Details of statistical analyses for group effects by one-way ANOVA and for paired comparisons of total number of calls for each call types in the five different conditions by Bonferroni’s post-hoc tests. ND: Novel Dim, NB: Novel Bright, FD: Familiar Dim, FB: Familiar Bright.

A. Total number of USV calls *(Group Effect: $F_{(4,48)}=24.16; p<0.001$)*

<table>
<thead>
<tr>
<th></th>
<th>NB (n=11)</th>
<th>FD (n=10)</th>
<th>FB (n=10)</th>
<th>Water (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND (n=11)</td>
<td>t=6.93, p&lt;0.001***</td>
<td>t=8.25, p&lt;0.001***</td>
<td>t=8.34, p&lt;0.001***</td>
<td>t=6.31, p&lt;0.001***</td>
</tr>
<tr>
<td>NB (n=11)</td>
<td>-</td>
<td>t=1.48, p=1.00</td>
<td>t=1.57, p=1.00</td>
<td>t=0.62, p=1.00</td>
</tr>
<tr>
<td>FD (n=10)</td>
<td>-</td>
<td>-</td>
<td>t=0.09, p=1.00</td>
<td>t=2.09, p=0.42</td>
</tr>
<tr>
<td>FB (n=10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t=2.18, p=0.34</td>
</tr>
</tbody>
</table>

B. High frequency calls *(Group Effect: $F_{(4,48)}=12.93; p<0.001$)*

<table>
<thead>
<tr>
<th></th>
<th>NB (n=11)</th>
<th>FD (n=10)</th>
<th>FB (n=10)</th>
<th>Water (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND (n=11)</td>
<td>t=6.11, p&lt;0.001***</td>
<td>t=5.52, p&lt;0.001***</td>
<td>t=5.85, p&lt;0.001***</td>
<td>t=4.55, p&lt;0.001***</td>
</tr>
<tr>
<td>NB (n=11)</td>
<td>-</td>
<td>t=0.44, p=1.00</td>
<td>t=0.11, p=1.00</td>
<td>t=1.56, p=1.00</td>
</tr>
<tr>
<td>FD (n=10)</td>
<td>-</td>
<td>-</td>
<td>t=0.32, p=1.00</td>
<td>t=1.08, p=1.00</td>
</tr>
<tr>
<td>FB (n=10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t=1.41, p=1.00</td>
</tr>
</tbody>
</table>

C. Low frequency calls *(Group Effect: $F_{(4,48)}=23.37; p<0.001$)*

<table>
<thead>
<tr>
<th></th>
<th>NB (n=11)</th>
<th>FD (n=10)</th>
<th>FB (n=10)</th>
<th>Water (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND (n=11)</td>
<td>t=4.73, p&lt;0.001***</td>
<td>t=8.41, p&lt;0.001***</td>
<td>t=8.10, p&lt;0.001***</td>
<td>t=5.71, p&lt;0.001***</td>
</tr>
<tr>
<td>NB (n=11)</td>
<td>-</td>
<td>t=3.80, p=1.00</td>
<td>t=3.49, p=0.016*</td>
<td>t=0.98, p=1.00</td>
</tr>
<tr>
<td>FD (n=10)</td>
<td>-</td>
<td>-</td>
<td>t=0.30, p=1.00</td>
<td>t=2.84, p=0.07</td>
</tr>
<tr>
<td>FB (n=10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t=2.53, p=0.20</td>
</tr>
</tbody>
</table>
D. Peak frequency \((Group\ Effect: F_{(4,48)} = 2.2; p=0.08)\)

<table>
<thead>
<tr>
<th></th>
<th>NB (n=11)</th>
<th>FD (n=10)</th>
<th>FB (n=10)</th>
<th>Water (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND (n=11)</td>
<td>t=2.12, p=0.33</td>
<td>t=0.01, p=1.00</td>
<td>t=0.88, p=0.93</td>
<td>t=1.47, p=1.00</td>
</tr>
<tr>
<td>NB (n=11)</td>
<td>-</td>
<td>t=2.01, p=0.22</td>
<td>t=1.13, p=1.00</td>
<td>t=0.65, p=1.00</td>
</tr>
<tr>
<td>FD (n=10)</td>
<td>-</td>
<td>-</td>
<td>t=0.83, p=0.65</td>
<td>t=1.39, p=1.00</td>
</tr>
<tr>
<td>FB (n=10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t=0.52, p=1.00</td>
</tr>
</tbody>
</table>

E. Call duration \((Group\ Effect: F_{(4,49)} = 14.74; p<0.001)\)

<table>
<thead>
<tr>
<th></th>
<th>NB (n=11)</th>
<th>FD (n=10)</th>
<th>FB (n=10)</th>
<th>Water (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND (n=11)</td>
<td>t=4.23, p=0.001***</td>
<td>t=5.97, p&lt;0.001***</td>
<td>t=5.90, p&lt;0.001***</td>
<td>t=6.06, p&lt;0.001***</td>
</tr>
<tr>
<td>NB (n=11)</td>
<td>-</td>
<td>t=1.91, p=0.53</td>
<td>t=1.84, p=1.00</td>
<td>t=1.78, p=1.00</td>
</tr>
<tr>
<td>FD (n=10)</td>
<td>-</td>
<td>-</td>
<td>t=0.07, p=1.00</td>
<td>t=0.22, p=1.00</td>
</tr>
<tr>
<td>FB (n=10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>t=0.15, p=1.00</td>
</tr>
</tbody>
</table>
4.2.3 Acoustic features in behaviors

In order to characterize the relationship between acoustic features of USVs and behaviors, I analyzed the duration and peak frequency of USV calls emitted while an animal was engaging into certain types of behaviors. I used only ND and NB conditions for this, based on the findings that mice demonstrated the most enriched USVs' repertoire under these conditions (Fig. 4-2). There was a significant interaction between treatment group and behavior on call duration (Fig. 4-3A; Table 4-2A; interaction, $F_{(3,302)}=17.03$, $p<0.0001$; Group, $F_{(1,302)}=127.2$, $p<0.0001$; Behaviors, $F_{(3,302)}=32.89$, $p<0.0001$). Post-hoc comparisons showed that while performing unsupported rearing or walking behaviors, call duration was longer in ND compared to NB (Fig. 4-3 A; Table 4-2 A). Similarly, for USV peak frequency, a significant interaction was present between treatment and behavior [Fig. 4-3 B; Table 4-2 B; interaction, $F_{(3,302)}=6.577$, $p=0.0002$; Group, $F_{(1,302)}=6.252$, $p=0.0129$; Behaviors, $F_{(3,302)}=41.15$, $p<0.0001$], with only mice displaying unsupported rearing showing an effect of treatment, where peak frequency was higher for mice in the ND treatment (Fig. 4-3 B; Table 4-2 B). Notably, mice did not emit any USVs while not moving (Fig. 4-3 A-B).

Next, I analyzed high and low frequency calls in the same ND and NB experimental groups made while performing four most preferable behaviors: walking, self-grooming, unsupported and supported rearings. A two-way ANOVA revealed an interaction effect between three behavioral parameters (the exception was supported rearing) and percent time mice spent giving either high frequency or low-frequency USVs (Table 4-3 C). For the supported rearing, only the main effect of frequency was significant: low frequency USVs were more frequently elicited than high-frequency USVs for both treatment conditions: ND and NB (Fig. 4-3 C-first from left; supported rearing). Mice of the ND group produced more high-frequency calls when they demonstrated unsupported rearings and walking, relative to the NB group (19 %; Fig. 4-3 C-second from left; unsupported rearing; $p<0.0001$; 15 %; Fig. 4-3C-right; walking; $p<0.0001$). During self-grooming behavior this pattern was reversed and mice (46%) produced more high-frequency calls under the NB (46 vs. 7%; Fig 4-3C) than under the ND condition.
Figure 4-3. Acoustic characteristics of USVs emitted in behaviors in novel dim and novel bright contexts.

(A) Calls durations and (B) peak frequency of USVs in all conditions. (C) Emission of “high-frequency” (HF) and “low-frequency” (LF) calls in mice demonstrating “supported rearings”, “unsupported rearings”, “self-grooming” and “walking”. Data are presented as means ± SEM. ***: p<0.0001 – in comparison between “Novel Dim” vs. “Novel Bright” condition (Bonferroni’ post-hoc analysis; two-way ANOVA).
Table 4-2. The summary of statistical results in Figure 4-3.

Details of statistical results for group effects by two-way ANOVA and for paired comparisons of call duration (A) and peak frequency (B) in the Novel Dim and Novel Bright groups by Bonferroni’s post-hoc tests.

**2-way ANOVA results from Figure 4.3 A and B**

<table>
<thead>
<tr>
<th></th>
<th><strong>A. Call Duration</strong></th>
<th><strong>B. Peak Frequency</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interaction</strong></td>
<td>$F_{(3,302)}=17.03$, p&lt;0.0001***</td>
<td>$F_{(3,302)}=6.577$, p=0.0002**</td>
</tr>
<tr>
<td><strong>Group</strong></td>
<td>$F_{(1,302)}=127.2$, p&lt;0.0001***</td>
<td>$F_{(1,302)}=6.252$, p=0.0129*</td>
</tr>
<tr>
<td><strong>Behavior</strong></td>
<td>$F_{(3,302)}=32.89$, p&lt;0.0001***</td>
<td>$F_{(3,302)}=41.15$, p&lt;0.0001***</td>
</tr>
</tbody>
</table>

**Bonferroni posttest (Novel Dim vs. Novel Bright)**

<table>
<thead>
<tr>
<th></th>
<th><strong>A. Call Duration</strong></th>
<th><strong>B. Peak Frequency</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supported Rearing</td>
<td>$t=4.85$, p&lt;0.001**</td>
<td>$t=0.49$, p&gt;0.05</td>
</tr>
<tr>
<td>Unsupported rearing</td>
<td>$t=10.04$, p&lt;0.001**</td>
<td>$t=4.36$, p&lt;0.001**</td>
</tr>
<tr>
<td>Grooming</td>
<td>$t=1.45$, p&gt;0.05</td>
<td>$t=0.29$, P&gt;0.05</td>
</tr>
<tr>
<td>Walking</td>
<td>$t=9.99$, p&lt;0.001**</td>
<td>$t=0.01$, p&gt;0.05</td>
</tr>
<tr>
<td>Immobile</td>
<td>$t=0.00$, p&gt;0.05</td>
<td>$t=0.00$, p&gt;0.05</td>
</tr>
</tbody>
</table>
C. 2-way ANOVA results from Figure 4.3 C

<table>
<thead>
<tr>
<th></th>
<th>Supported rearings</th>
<th>Unsupported rearings</th>
<th>Grooming</th>
<th>Walking</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Interaction</strong></td>
<td>$F_{(1,40)}=0.08$,</td>
<td>$F_{(1,40)}=89.81$,</td>
<td>$F_{(1,40)}=136.1$,</td>
<td>$F_{(1,40)}=16.9$,</td>
</tr>
<tr>
<td></td>
<td>p=0.7707</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
</tr>
<tr>
<td><strong>Group</strong></td>
<td>$F_{(1,40)}=5.51$,</td>
<td>$F_{(1,40)}=144.1$,</td>
<td>$F_{(1,40)}=81.26$,</td>
<td>$F_{(1,40)}=16.9$,</td>
</tr>
<tr>
<td></td>
<td>p=0.0239</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
</tr>
<tr>
<td><strong>Frequency</strong></td>
<td>$F_{(1,40)}=166.7$,</td>
<td>$F_{(1,40)}=144.1$,</td>
<td>$F_{(1,40)}=18.00$,</td>
<td>$F_{(1,40)}=135.7$,</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
<td>p&lt;0.0001***</td>
</tr>
</tbody>
</table>

**Bonferroni posttest (Novel Dim vs. Novel Bright)**

<table>
<thead>
<tr>
<th></th>
<th>High Frequency</th>
<th>Low Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High Frequency</strong></td>
<td>$t=1.45$, p&gt;0.05</td>
<td>$t=1.86$, p&gt;0.05</td>
</tr>
<tr>
<td><strong>Low Frequency</strong></td>
<td>$t=19.15$, p&lt;0.001**</td>
<td>$t=1.78$, p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>$t=14.63$, p&lt;0.001**</td>
<td>$t=1.88$, p&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>$t=5.81$, p&lt;0.001**</td>
<td>$t=0.00$, p&gt;0.05</td>
</tr>
</tbody>
</table>
4.2.4 Identification of activated brain regions during spatial novelty exploration

I measured c-fos expression in the striatum, hippocampus and prefrontal regions when spatial novelty was introduced (Fig. 2-4). The main finding of this study showed that different levels of exploration differentially affected c-fos expression in selected brain regions. High exploration environments induced significantly higher c-fos protein synthesis in the DG and NacC (Fig. 4-4) than in low exploration environments. Low exploration environments were associated with more c-fos levels in CgC. Both high and low exploration environments activated CA1, CA3, mPFC, and PeriC to a greater extent than familiar environments. The introduction of bright light in the familiar environments did not alter the c-fos expression level compared to familiar dim environments.
Figure 4-4. Effects of exposure to a spatial novelty in the dim vs. bright light conditions on c-fos expression.

Counts of c-fos-stained nuclei in brain regions at 90 minutes post-exposure to a novel dim, novel bright, familiar dim or familiar bright environment. Three sections per mouse, n = 3 per group. (A–D) Counts of c-fos-stained nuclei in the HPC (A), the prefrontal cortex (B), the striatum (C) and the parahippocampal cortex (D). ND = Novel Dim; NB = Novel Bright; FD = Familiar Dim; FB = Familiar Bright; WR = Wheel Running; DMS = Dorsomedial striatum; DLS = Dorsolateral striatum; NacC = Nucleus accumbens core; NacS = Nucleus accumbens shell; DG = Dentate gyrus; mPFC = Medial prefrontal cortex; CgC = Cingulate cortex; EntC = Entorhinal cortex; PeriC = Perirhinal cortex. Data are expressed as mean ± SEM. * P < 0.05, ** p<0.01.
4.2.5 Modeling brain circuits during spatial novelty exploration

Using c-fos counts for StEM offers a potentially useful way of revealing the relative activated brain regions and of testing the direction of putative effects. This allows for an evaluation of the feasibility of network (or model) dynamics (McIntosh et al., 1991; Friston et al., 1993; Jenkins et al., 2003; Poirier et al., 2008). The initial assumption was that all models should conform to well-established patterns of connectivity between the regions of interest. Models for Novel Dim and Novel Bright environments were derived from the correlations across each brain region for the mean and absolute c-fos counts. Models were rejected if they were not based on established neuronal connectivity within the hippocampal structure, or if they did not have statistically significant pathways interlinking structures across the model.

**Novel Dim** Figure 4-5 A shows the optimal model that matched my criteria. The Group Novel model was heavily dependent on the route via the perforant path and tri-synaptic circuit. Thus, the network is essentially comprised of lateral EntC→DG→CA3→CA1. The NacC plays an integrative role in this network. The EntC→DG→CA3 pathways, pathways from mPFC, HPC and EntC to nucleus accumbens, mPFC→HPC were significant and the chosen model had a good fit ($x^2 = 9.797$, d.f. = 6, $P = 0.133$; CFI = 0.96; RMSEA = 0). However, reversing the direction of the paths between structures yielded poorer results (data not shown), with the exception of the Amyg, where no particular direction was optimal.

**Novel Bright** While the initial mPFC of the optimal model for Novel Bright (mPFC→HPC and mPFC→NacC) was the same as those for Novel Bright, the model changed radically afterwards (Fig. 4-5 B). The tri-synaptic path was less engaged, as there was no longer an influence of the lateral EntC on DG, of the DG on CA3 and of CA3 on CA1. In the model, the HPC→Amyg pathway was significant where the NacC no longer played an integrative role. Instead, CA1 acted in an integrative role where it had strong connections with the EntC and mPFC. The direction of effect was now directly from the lateral to CA1; i.e. the temporo-ammonic projection was engaged instead of the perforant pathway. The resulting model for Novel Bright had a poor fit ($x^2 = 21.018$, d.f. = 6, $P = 0.033$; CFI = 0.89; RMSEA = 0.17). Most importantly, this model highlights the dominance of the temporo-ammonic pathway over the perforant pathway, and also
the HPC to Amyg pathway. The covariance of NacC and Amyg was significant, which means they mutually impact each other.

![Diagram of temporal lobe interactions derived from structural equation modeling.](image)

**Figure 4-5. Temporal lobe interactions derived from structural equation modeling.**

(A) Optimal Group Novel Dim model and (B) optimal model of Novel Bright by StEM of c-fos positive cell numbers. mPFC=Medial prefrontal cortex; DG=Dentate Gyrus; NacC=Nucleus accumbens core; Amyg=Amygdala; EntC=Entorhinal cortex
4.3 Discussion

In this chapter, the vocal behavior and relevant brain regions of self-directed exploration in adult male mice are investigated in detail, by utilizing modified experimental protocol which maximize vertical exploratory behaviors, as shown by Lever (2006). Clear differences in self-directed vertical exploration occurred in the ND group compared to the NB group, indicative of the robustness of this new experiment and its potential for examining the neural basis of exploratory behavior. As shown in Figure 4-6, mice in response to ND showed more frequent rearings and HF calls and strengthened hippocampal–NacC connectivity, while mice in exposure to NB showed less frequent rearings, but more LF USVs and enhanced HPC-Amyg connectivity. While the 50 kHz playback induced approach behavior and sparse Fos-like immunoreactivity in the NAc, thalamic parafascicular and paraventricular nuclei, 22 kHz playback did not produce behavioral responses but elicited c-fos expression in the Amyg nuclei and the periaqueductal gray (Sadananda et al., 2008). The correlation between USVs and c-fos readouts provides clues to the mechanism of the learning bonus produced in non-threatening novel stimuli.

I explored mouse USVs in non-social contexts, manipulating the level of aversive condition by adding a bright light or shallow water to the experimental chamber. We show that mouse vocalizations vary in a context and/or behavior dependent manner. While exploration of a novel environment with a potentially aversive factor, a bright light, appears to differentially influence mouse' vocal behavior compared to novel environments without aversive factors, adult male mice emitted fewer calls in familiar conditions relative to novel condition. More specifically, mice were engaged in unsupported rearing behavior with emission of high frequency calls during exploring a novel dim light environment. In contrast, animals demonstrated avoidance, such as unsupported rearing, in the more stressful situation (novel bright light), which was coupled with low frequency calls. A detailed characterization of mouse vocalization in association with particular behavioral performance offers a unique opportunity to decipher vocalizations in mice.
Figure 4-6. Summary of results.
While novel dim group showed correlation between unsupported rearing and 50 kHz USV emissions with hippocampal-nucleus accumben connectivity, novel bright grouped illustrated correlation between supported rearing and 20 kHz USVs with the hippocampal and Amygdala connection.
Notably data on whether mice can emit USVs in a non-social context is severely lacking, thereafter still debatable. One report showed that mice would not produce USVs during exposure to aversive stimulation such as physical restraint or electric shock (Portfors, 2007). However, another study recorded USVs in adult mice in non-social contexts such as exploration of a novel environment or restraint stress (Chabout et al., 2012), showing USVs in both novel and aversive environments. The current study revealed that adult male mice are able to emit calls during exploration in both familiar and novel environments, as well as brightly-lit novel environments.

Moreover, mice differentially emitted USVs depending on the environment, although the total number of calls I saw emitted during exploration was generally lower than the number observed during social interactions (Holy and Guo, 2005; Chabout et al., 2012). As expected, adult male mice exposed to a familiar environment elicited very few calls, regardless of the light treatment. This may indicate that USVs reflect physiological conditions, given that mice are typically less aroused and active after habituation (Harris, 1943). However, the habituation effect does not explain the few calls made in familiar bright environment, since I assumed that bright light could elicit aversive states, therefore, the number of low frequency call should have increased. To unveil the possibility of the bright light being not enough to elicit aversive states, I used another stronger aversive factor, water. However, I still found low number of USVs similar to that of brightly-lit familiar conditions. This finding is in line with a study that found fewer USV calls in restrained stress compared to in novel environments (Ko et al., 2005; Chabout et al., 2012). Notably, exploration of novelty under less stressful conditions (dim light) triggered the largest number of USVs in mice; this condition presumably reflects pure exploration, without aversion, since the impact of stress was minimized. The bright light given in the novel environment significantly reduced the number of USVs, which support the idea that aversive states reduce USVs. Overall, our findings indicate that USVs might serve as a robust index of an animal's response to the stress.

The detailed characterization of acoustic signals in association with exploratory behavior reveals that acoustic features of mouse USVs are distinctive depending on context. In rats, acoustic calls are divided into high frequency calls (50 kHz) emitted during anticipation of reward or approach behavior and low frequency calls (22 kHz) emitted during anticipation of punishment or avoidance behavior (Knutson et al., 2002). Recent studies characterized USVs in
mice, and focused on vocal repertoire while excluding USVs < 25 kHz (Holy and Guo, 2005; Wang et al., 2008). In this study, by recording the whole spectrum of USVs, we found that mice in the “bright light group” (higher stress) emitted USVs at a mean frequency of 26.01 ± 1.16 kHz. These results are similar to recent findings that adult male mice may emit low frequency 30 kHz (26–36 kHz) calls when stressors are present (Ko et al., 2005) and emit high frequency 40 kHz calls with social rewards (Chabout et al., 2012). The distinctive call patterns may be aligned with the 50 kHz reward calls and 22 kHz aversive calls as shown for rodents, since bright light is aversive *per se* to mice and exploration under dim light is likely self-rewarding for mice.

While other studies scored a selection of behaviors, including USV emission, in a given period and showed correlation between the two, I analyzed simultaneously the different behavioral patterns accompanying the USVs. USVs were emitted in conjunction with most behavioral categories involving movements, and were not detected in immobile states. These findings agree with other studies, where high vocalization rates correlated with high levels of locomotor activity in rats (Fu and Brudzynski, 1994) and in mouse' pups (Branchi et al., 2004). Behavioral patterns also depended on the context. Mice from the “bright light group” spent more time in the corner and were more likely to rear against the wall, reflecting escape-oriented behavior, whereas the “dim light group” showed more exploratory behavior, such as unsupported rearing and time spent in the chamber's center. Further, our analysis revealed that mice were more likely to show unsupported rearing behavior when eliciting high frequency calls and more likely to rear against a wall, when emitting low frequency calls. Given that high-frequency calls are associated with pleasure (Chabout et al., 2012), this association between the exploratory unsupported rearing behavior and high frequency calls suggests that exploration with minimal stress levels is likely self-rewarding for mice.

I propose here first insight into how emotional and motivational individual states on novelty exploration are connected with emission of USVs in adult male mice. The widespread application of USVs in characterizing neuropsychiatric mouse models has been hampered by the use of pups (immature brain) and a lack of comprehensive studies in mice (Portfors, 2007). We argue that this framework is important for exploring mouse models of neuropsychiatric disorders (e.g., depression, autism, Rett syndrome) by characteristics of USVs during exploration of novel environments because failure to be engaged in such activity may reflect consequences of the
profound defects of sensory-motor and cognitive functions, so deficits in sensory-motor or cognitive functions may contribute to the reduced exploratory behavior in children with autism spectrum disorder (Pierce and Courchesne, 2001) and in elderly people with Alzheimer's disease (Daffner et al., 2001). Conversely, the increased novelty seeking properties might contribute to such mental disorders as substance abuse (See Review, Bardo et al., 1996) or manic episodes of bipolar disorder (Regier et al., 2013). The association we found in this study, between certain types of USVs and behavioral exploratory patterns, demonstrate a novel way to study USVs in animal models of neuropsychiatric disease. This approach will help us to understand the acoustic capacities of mice and may ultimately allow us to select novel vocal phenotypes for animal models of mental disorders, to which various behavioral designs and multiple genetic mouse lines can be applied.

High levels of rearing are seen in environmental mismatch novelty (Sutherland et al., 1983), including studies in which strong CA1 place cell remapping is elicited. These findings suggest that rearing can profitably be used as a marker of environmental novelty and are consistent with an environmental information-gathering function. This has led to the assumption that environmental mismatch novelty is mainly dependent on the HPC, and can be assayed using rearing. An early study, examining the effects of different kinds of hippocampal lesions on Morris water maze learning, noted post-trial platform rearing in this task (Sutherland et al., 1983). When the location of the hidden platform was shifted from its previous consistent location, all three types of lesioned mice (DG, CA1, and CA3) failed to increase rearing behavior and had mild impaired spatial learning. The HPC and NAc were associated with exploration in NSD while the HPC and Amyg were associated with exploration in NSB. Although it is well known that the detection of spatial novelty is associated with the HPC, the function of the striatum in spatial novelty detection is rarely studied.

StEM of c-fos activation highlighted the difference in brain connectivity during processing of novel spatial stimuli in dim vs. bright conditions. In particular, the hippocampal-NAc effective connectivity was observed in NSD and hippocampal-amygdaloid effective connectivity occurred in NSB. Additionally, a switch in parahippocampal–hippocampal effective connectivity from the temporal-ammonic pathway in the NSD group to the perforant (DG) pathway in NSB group was found. This switch was also observed between novel and familiar environments (Aggleton and
The correlated activity between the HPC and NAc may offer a pathway for the enhancing effects of motivation on spatial memory formation. The results, like most imaging findings, are correlative and care must be taken to avoid misinterpreting results to draw causal conclusions. However, c-fos antisense infusions reveal that c-fos activity in the HPC is a critical requirement for efficient, stable spatial object recognition memory in rats (Aggleton and Brown, 2005). Thus, the present study used a marker that may have a causal role for spatial recognition memory.

The role of novelty in eliciting neural responses is supported by intracranial EEG recordings from the HPC and the NAc in humans (Axmacher et al., 2010). Interestingly, work in animals and humans indicate that the enhanced memory for the novel situation is mediated by the midbrain dopamine regions (Fenker et al., 2008; Li et al., 2003). Recent studies suggest that memory formation in the HPC is modulated by the motivational significance of events, which enhance long-term memory (Adcock et al., 2006; Tse et al., 2007; Wittmann et al., 2005; Wolosin et al., 2012) for review see (Shohamy and Adcock, 2010). The effects of motivation on memory are thought to depend on interactions between the memory circuit, including the HPC and its surrounding cortices, and a reward circuit in the midbrain that includes the NAc (Lisman et al., 2011; Shohamy and Adcock, 2010). Animal research has revealed significant modulatory inputs on the HPC coming from the NAc, both anatomically and pharmacologically (Greenberg et al., 2006). The NAc itself has a high number of anatomical links to the (Groenewegen et al., 1987). In addition, neuroimaging studies in humans have begun to reveal functional interactions between midbrain dopamine regions and the HPC during novelty exploration and memory formation (Adcock et al., 2006; Shohamy and Adcock, 2010; Shohamy and Wagner, 2008).

The NB condition did not lead to c-fos activation in the HPC and mPFC to the same extent as in the ND condition, supporting the idea that novelty, but not aversion, leads to c-fos activation (Yochiy et al., 2012). Direct connectivity of the ventral HPC-Amyg-mPFC was revealed by StEM in NB conditions. Anatomically, ventral HPC projects to both mPFC and Amyg (Pitkanen et al., 1997). The Amyg receives massive projections from the mPFC (Orsini et al., 2011), and projects back to the mPFC. The excitatory and inhibitory inputs from the HPC and Amydala interact in the mPFC (Ishikawa and Nakamura, 2003).
The HPC appears to be involved in both ND and NB conditions. The HPC has been suggested to be a comparator of the existing information with the incoming sensory information. When a possible threat or novelty (mismatch between observed and stored) is detected, the HPC may take over control of the behavior (Gray et al., 2003). Thus, the HPC plays a crucial role in novelty-induced exploratory behavior and familiarization (Eichenbaum et al., 2007; Manahan-Vaughan and Braunewell, 1999). The Amyg, although best known as a fear center, also plays a role in novelty-induced exploration and anxiety-like behavior with a connection to the ventral HPC, by sending information to HPC heightening arousal (McGaugh, 2005) and modulating affective memory (Roozendaal et al., 2009). Indeed, lesions of the ventral HPC and BLA reduce anxiety-like behavior across various behavioral tests (Bueno et al., 2005; Deacon et al., 2002a). Differential modulation between CA1 and DG, as detected in the present study, has been found in other studies. For example, decreased and increased firing rates were registered in the CA1 and DG interneurons of rats, respectively, under the same novel condition (Nitz, 2003). In another study, c-fos expression in the CA1 and DG areas increased and decreased, respectively, after exposure to novel environment (Albasser et al., 2010). Finally, in a study of neuroplasticity, environmental novelty was associated with an increased c-fos expression in the CA1, but not in the DG (VanElzakker et al., 2008). The present results support a relationship between an increased c-fos expression in the DG and a novel environment (Aggleton and Brown, 2005). It appears that different types of novel stimuli, stressors, and learning conditions described, may each activate specific brain mechanisms; nonetheless, other possible explanations for the present results cannot be excluded without further investigation.

A limitation of this study is that the assumption that exposure to FO, used as a control procedure, does not induce new learning is suspect. Studies into the phenomenon of reconsolidation highlight the ways in which each repeated exposure to an item may recapitulate aspects of the learning processes associated with its first exposure (Finnie and Nader, 2012; Nadel et al., 2012), and that reconsolidation may require IEG expression (Bozon et al., 2003; Lee et al., 2004).

Our results provide insight into how individual emotional and motivational states and novelty exploration are connected to the emission of USVs and activated brain regions in adult
male mice. The widespread application of USVs in characterizing neuropsychiatric mouse models has been hampered by the use of pups (immature brain) and a lack of comprehensive studies in mice (Portfors, 2007). We argue that this framework is important for exploring mouse models of neuropsychiatric disorders by characteristics of USVs during exploration of novel environments because failure to be engaged in such activity may reflect consequences of profound defects of sensory-motor and cognitive functions. Deficits in sensory-motor or cognitive functions may contribute to the reduced exploratory behavior in children with ASD (Pierce and Courchesne, 2001) and in elderly people with Alzheimer's disease (Daffner et al., 2001). Conversely, increased novelty seeking properties might contribute to such mental disorders as substance abuse (See Review, Bardo et al., 1996), and manic episodes of bipolar disorder (Regier et al., 2013). The association we found between certain types of USVs and behavioral exploratory patterns demonstrate a novel way to study USVs in animal models of neuropsychiatric disease. This approach will help us to understand the acoustic capacities of mice and may ultimately allow us to select novel vocal phenotypes for animal models of mental disorders, to which various behavioral designs and multiple genetic mouse lines can be applied.

Previously, I used rearing as an index of exploration and associated it with better spatial LM in non-threatening novel environments. To better understand the relationship between exploration and hippocampal-dependent spatial LM, it would be helpful to have additional indices of novelty-induced exploratory states. Here, I studied whether USVs and brain IEG expression patterns could serve as additional readouts of exploration.
Chapter 5

5 Discussion and Future Directions

In Chapter 1, I outlined a brief history of LM research with a focus on factors that affect learning efficiency. I also described the synaptic plasticity focusing on long-term enhancement and depression of transmission, known as LTP and LTD, respectively, followed by a brief introduction of NCS-1. Finally, I introduced USVs and putative brain regions, which might be a useful index of exploration bonus, which may benefit learning.

In Chapters 3 and 4, I present evidence that animals actively influence their sensory information experiences through intrinsically motivated exploration, which co-occurred with the activation of activating specific brain pathways that are required for learning and/or retaining spatial information. Furthermore, this study identified the molecular basis of the LM benefit associated with self-directed exploration. In addition to demonstrating that exploration requires NCS-1 and the DG, I conclude that exploration is a crucial facet of behavior that can be quantified accurately and meaningfully. During exploration in a safe novel environment, HF USVs, and hippocampal-NAc connectivity was found to be associated with information gathering exploratory behavior. Conversely, during exploration in the fearful novel environment, LF calls, and hippocampal-amygdala connectivity were associated.

In this chapter, I place the findings of this research within the context of the current body of literature, commenting on the significance of the results obtained in this thesis.

5.1 Open Field Test

The main finding of this study is that exploration behavior in a safe novel environment is associated with enhanced learning ability. Further, the number of rearings in a safe novel environment could be used as an index of hippocampal-dependent learning. I measured this phenomenon using DO recognition tests in an open field test.
The open field test is one of the most widely used behavioral tests in rodent research and is also used to interpret the various domains of behavior, including motor function, novelty exploration, and anxiety which result in its benefits and disadvantages at the same time. The open field test serves as a starting point for assessing novel environment exploration and general locomotor activity as well as initial screening for anxiety-related behaviors in rodents (Prut and Belzung, 2003). In addition, repeated exposure or extended session length allows refinement to assess habituation to the increasingly familiar chamber environment. Although the primary information obtained in an open field test is beneficial for planning subsequent testing, it has been criticized for confounding factors such as locomotor, anxiety and novelty-induced exploration and a lack of standardization, including lighting levels.

In the analysis of primary open field data, it can be difficult to distinguish a high anxiety-like phenotype from impairment in novelty-induced exploration as both can manifest as reduced locomotion (Blanchard et al., 1990; File et al., 2001; McNaughton et al., 2000; Crawley, 2007). For example, the researchers that first described DRD4 KO mice (Dulawa et al., 1999) described the phenotype as reduced exploration of novelty, including less center exploration in a novel arena, reduced NO exploration, and greater preference for home base in the emergence test. However, Holmes et al. (2001) characterized the same DRD4 KO mice as exhibiting increased anxiety-related response. Results from my studies suggest that lighting level in the open field might be a key to differentiating reduced novelty exploration from anxiety-related behavior; using the proposed new method described in Figure 5-1 to distinguish between these two interpretations will allow more precise definitions for phenotypes of genetically modified mouse lines.

Although the stress created by the brightly lit novel test environment is known to influence anxiety-like behaviors (Prut et al., 2003), the level of lighting is often unreported in open field tests. Experimental conditions have used dim light (-40 lux), medium light (100-200 lux), or bright light (300-600 lux) (Table 5-1). Table 5-1 summarizes current findings on gene used to research pharmacological treatments for anxiety disorders, with a focus on studies using the open field test. In the majority of cases, lighting levels were not provided. The center time is often the behavioral response reported that is used to indicate anxiety-related behaviors. The number of rearings is rarely reported. In addition to the open field test, one or two other measures
were used to determine the anxiety-related phenotypes in the mutant mice.

I suggest that the number of rearings in dimly lit environments is an indicator of novelty exploration, which facilitates learning processes, as discussed further in section 5.2. As presented in Table 5-1, an increased number of rearing movements were observed in DRD3 KO mice and 5-HT5A KO mice. Aged DRD3 KO mice outperformed age-matched WT mice on the measures of exploration and spatial memory. This is accompanied by a higher degree of hippocampal CREB phosphorylation, which may have neuroprotective effects on memory consolidation (Hardingham et al., 2002). Researches, utilizing 5-HT5A receptor antagonist SB-6995516 and serotonin precursor l-tryptophan, suggests that the blockage of 5-HT5A receptors appears to impair exploration, STM, and LTM in an autoshaping task, while stimulation of the same receptors might facilitate exploration, STM, and LTM (Gonzalez et al., 2013). While further investigation is required, studies from DRD3 and 5-HT5A genes suggest that the number of rearings might predict learning success.

High light levels typically suppress locomotor activity in rodents because rodents exposed to bright light have increased stress levels. In mice, light levels above 200 lux significantly reduce locomotor activity in the open field compared to activity at light levels of 2–10 lux (Blizard, 1971; Krsiam and Janku, 1971). Consistent with this, a dimly lit novel environment facilitated exploration and enhanced contextual memory on the DO memory test in this study. Additionally, high light levels may provoke latent anxiety-related behaviors (Gould et al., 2009). Therefore, behaviors in dimly lit novel environments may be more reflective of novelty exploration while in a brightly lit environment, behaviors may be reflective of an anxiety-related response.

This conclusion is supported by neural activity scored according to IEG expression. Using c-fos expression, I identified neural circuitry activated by environments that favor exploration. In particular, dimly lit novel conditions enhanced c-fos expression in the DG, CA3, and NAc regions of the brain. In human fMRI studies, the NAc, which is the primary projection target of dopaminergic neurons of the substantia nigra/ventral tegmental area, is activated in anticipation of a reward (Schultz, 2004). Both novelty and reward cues (as motivational factors) co-activate the substantia nigra/ventral tegmental area and HPC in humans (Brewer et al., 1998; Wittmann et
al., 2005). Interestingly, this is consistent with the impairment observed in an object recognition task after pharmacological manipulation of the NAc and DG in mice (Dees and Kesner, 2013; Sargolini et al., 2003). Specifically, novelty facilitates the induction and persistence of long-term plasticity in the DG (Straube et al., 2003; Davis et al., 2004), that is the processes considered cellular mechanisms for LM. StEM analysis revealed that the NAc-hippocampal connection is strengthened in the dimly lit environment, and that this enhanced communication may modulate the salience of environmental features that, in turn, promote the fine-tuning of exploratory behavior necessary for LM. Brightly lit novel conditions, which induce anxiety-related behavior, had enhanced c-fos expression in the BLA. The amygdala is related to fear and also plays a role in both novelty-induced exploration and anxiety-like behavior. The amygdala most likely conveys important processed threat stimulus information to the ventral HPC, heightening arousal (McGaugh, 2005) and modulating emotional memory (Roozendaal et al., 2009). Indeed, lesions of the ventral HPC and BLA reduce anxiety-like behavior across various behavioral tests (Bueno et al., 2005; Deacon et al., 2002b). In line with these reports, StEM analysis of NB groups found strengthened connectivity between HPC and BLA.
Table 5-1. Representative behavior testing results for genetically engineered mice.

<table>
<thead>
<tr>
<th>Genetic Mutant</th>
<th>Anxiety-related phenotype</th>
<th>OF: Lighting</th>
<th>OF: Locomotion</th>
<th>OF: Center Time</th>
<th>OF: Rearings</th>
<th>Other Behaviors</th>
<th>Ref(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD 65 KO</td>
<td>↑</td>
<td>Bright (w/o level)</td>
<td>—</td>
<td>↓</td>
<td>EZM (↓)</td>
<td>(Kash et al., 1999)</td>
<td></td>
</tr>
<tr>
<td>MAOA KO</td>
<td>↓</td>
<td>↑</td>
<td>Agression (↑)</td>
<td>(Cases et al., 1995)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAOB KO</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>EPM (-) FST (mobility ↑)</td>
<td>(Grimsby et al., 1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRD4 KO</td>
<td>↑ (?), —</td>
<td>↓</td>
<td>Emergence (↓), Novel Object Exploration (↓)</td>
<td>(Dulawa et al., 1999)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRD3 KO</td>
<td>↓</td>
<td>↑ ↑</td>
<td>EPM (↑)</td>
<td>(Steiner et al., 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A OE</td>
<td>—</td>
<td>↑ ↑</td>
<td>—</td>
<td>EPM (↑)</td>
<td>(Kusserow et al., 2004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A KO</td>
<td>↑ (?), —</td>
<td>↓</td>
<td>EPM (↓), Novel Object Exploration (↓)</td>
<td>(Heisler et al., 1998)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A KO</td>
<td>↑</td>
<td>↓ ↓ ↓</td>
<td>EPM (↓)</td>
<td>(Ramboz et al., 1998)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A KO</td>
<td>↑</td>
<td>↓</td>
<td>FST (↓)</td>
<td>(Parks et al., 1998)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT1A Autoreceptor KO</td>
<td>↑</td>
<td>↓ ↓</td>
<td>Novel Object Exploration (↑), EPM(-)</td>
<td>(Richardson et al., 2011)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT5A KO</td>
<td>—</td>
<td>↑ ↑</td>
<td>↑</td>
<td>Novel Object Exploration (↑), EPM(-)</td>
<td>(Graihle et al., 1999)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH OE</td>
<td>↑</td>
<td>400-600 lux</td>
<td>↓ ↓</td>
<td>No habituation</td>
<td>(Kasahara et al., 2007)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH OE</td>
<td>—</td>
<td>500 lux</td>
<td>↑ ↑</td>
<td>D/L (↓), Vogel conflict (-), Rotarod (↓)</td>
<td>(van Gaalen et al., 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRHR2 KO</td>
<td>↑</td>
<td>120 lux</td>
<td>—</td>
<td>EPM (↓), D/L (-)</td>
<td>(Bale et al., 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRHR2 KO</td>
<td>—</td>
<td>175 lux</td>
<td>—</td>
<td>EPM (-)</td>
<td>(Coste et al., 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRHR2 KO</td>
<td>↑</td>
<td>650 lux</td>
<td>—</td>
<td>EPM (↓)</td>
<td>(Kishimoto et al., 2000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH-BP OE</td>
<td>—</td>
<td>↑ ↑</td>
<td>↑ ↑</td>
<td>EPM (-)</td>
<td>(Burrows et al., 1998)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH-BP KO</td>
<td>↑</td>
<td>—</td>
<td>↓ ↑</td>
<td>EPM (↓)</td>
<td>(Karolyi et al., 1999)</td>
<td></td>
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</tr>
</tbody>
</table>
5.2 Ultrasonic Vocalizations (USVs)

Procedures developed to date to measure anxiety-like responses have focused on exploratory movements in an open field test (Boissier et al., 1968; Christmas and Maxwell, 1970), into open arms of a plus maze (Lister, 1987), and into the light portion of a two-chambered apparatus (Crawley, 1981), along with behaviors related to defense or flight behaviors away from a predator in the mouse defense test battery (Griebel, 1995). These procedures rely on locomotor behaviors as the unique index of anxiety-like responses. In section 5.1, I suggested that varying lighting levels in the open field test might differentiate exploration from anxiety-like behavior since the two types of behavior respond differently to varying light levels and produce distinct patterns in activated brain regions. Nonetheless, normal locomotor function is still required to assess either behavior. The ability to examine anxiety-like states in tests that are independent of locomotor function is important given that assuming normal locomotor function in KO mice is not always reasonable. To address this, I used USVs to assess processing of primary sensory information.

Previously, USVs have been analyzed to screen anxiolytic drugs, mostly in rats and mouse pups and to examine emotional states during maternal separation distress in mutant mice (Scattoni et al., 2009). For example, classic anxiolytic drugs with potent effects in humans, such as benzodiazepines, decrease USV in the newborn rodents (Carden and Hofer, 1990). Several studies have focused on the impact of serotonergic drugs on USV in rat (Winslow and Insel, 1990a; 1990b; 1991; Mos and Olivier, 1989) or mouse pups (Benton and Nastiti, 1988; Nastiti et al., 1991). Brunner et al. (1999) utilized maternal separation-induced USVs as part of a battery test to examine the development of anxiety-like responses in 5-HT1B KO mice. Wild-type mice show more USVs within the 20–40 kHz range than prenatally stressed animals of both 5-HTT+/+ and 5- HTT+/− genotypes as well as non-stressed 5-HTT++ animals (Jones et al., 2010). However, results appear to vary since Felix-Ortiz and Febo (2012) observed an increase in three distinct forms of USVs among PND5 of, HTT agonist, VPA-treated mice.

Measuring USV emission rate with maternal separation limits widespread use of this measure for assessing anxiety-related phenotype since it requires conducting experiments in mice pups whose brains are immature. Further, a comprehensive understanding of mouse vocalization
in response to stressors is currently lacking. Until recently, it was not clear whether adult mice emit USVs in a non-social context. One report found that mice would not produce USVs in response to aversive situations, such as physical restraints or electric shock (Portfors et al., 2007). Another study recorded USVs generated in adult mice in non-social contexts, such as exploration of a novel environment or during restraint stress (Chabout et al., 2012). I investigated USV emissions during exploration in a non-social context in brightly lit and dimly lit open field tests as a measure of anxiety-related behavior, given the fact that the brightly lit open field leads mice to behave in a stressed manner and activates the fear center, the amygdala. The study revealed that adult male mice emit calls during exploration in both familiar and novel environments and they emit these calls differentially depending on the level of light. However, the total number of USVs uttered during exploration was lower than that of emitted during social investigation in other studies (Chabout et al., 2012). In this study, similar to studies done using rats, I found distinct differences among treatment groups in frequencies and duration of USVs produced by mice. The “novel dim group” (high exploration) emitted 34.93 ± 2.79 kHz calls for longer duration (37.36 ± 4.13 ms), while the “novel bright group” (low exploration) emitted 26.01 ± 1.16 kHz calls for shorter duration (22.98 ± 2.53 ms). The distinctive call patterns may be aligned with the 50 kHz reward calls and 22 kHz aversive calls observed in previous studies since bright light is aversive to mice and exploration under dim light is likely to be rewarding for mice.

As outlined briefly in Chapter 1, anxiety procedures based on USV may offer some advantages over other methods. The brief isolation of pups that elicits USV is a typical component of any rodent’s history and, therefore, seems to be an appropriate, nonintrusive measure of emotionality. The present data support previous findings obtained using various experimental procedures and strengthen the suggestion that stress-induced USV may be a suitable model for the assessment of anxiolytic drugs (Cuomo et al., 1988; Gardner et al., 1985). In conclusion, the presently described USV test appears to be a simple, rapid, and reliable anxiety test that partially overcomes the shortcomings of the existing models and is suitable for screening purposes although further validation remains to be performed by examining the effects of known anxiolytic drug.

The most interesting finding in the USV analysis during exploration in the open field
under either bright or dim lighting is the association of specific behaviors with distinct patterns of USV frequency. Specifically, analysis revealed that mice are more likely to rear against a wall when emitting low frequency calls in the brightly lit open field and are more prone to show unsupported rearing behavior when eliciting high frequency calls in the dimly lit open field. Although further investigation is necessary to assert the distinction in function of supported and unsupported rearing movements, the possibility of the difference, as well as the future experiment to resolve the question, is discussed in the following section.

5.3 Rearing

The object recognition test, which consists of three sessions, is used widely to assess LM in genetically modified mice. For the research presented in this thesis, the first (training) session included two light levels, and followed by two subsequent memory tasks, namely, an object memory and spatial memory task. I found that the number of rearings during the training session was correlated with performance in the subsequent spatial memory task.

In addition, safe vs. fearful novel environments during the training session led to similar efficiency of spatial learning, as assessed by the DO memory task. Although it seems to imply that anxiety is expressed only in the open-field, the plus-maze, and the light–dark box because these are tests of anxiety, and not in a working memory task or an object recognition task because these are LM tasks, there is no reason animals would not express fear and anxiety when introduced to a spatial navigation or an object recognition task, especially when the object recognition task involves an open field. Therefore, it is worthwhile to record and analyze training session data. It is also more efficient and useful if subjects from both treatment groups, i.e., dim and bright light groups, are introduced to the acquisition of a memory task without habituation, to understand novelty exploration and anxiety states. More importantly, rearing behavior should be recorded and analyzed as an indicator of information gathering for spatial memory during the training session. Rearing behavior has been associated with motivational state and general arousal level in different learning situations (Lever and Burton, 2006). USV recordings can provide an index of novelty exploration and/or anxiety states as discussed in section 5.2. I posit, therefore, that there are four ways of measuring exploration that is distinct from locomotion and anxiety: (a) object contact; (b) rearings (especially unsupported rearings); (c) number of center
entries; and (d) high frequency USV calls, measured in novel dim environments. In all cases, interpretation of any change in the measure of exploratory behavior must be made while considering any changes in locomotor activity except for USV calls. Recording both the number of rearings and USV calls during the training session will provide more precise information about exploratory responses to novel environments and anxiety states, which can be confounding variables in LM tests related to locomotor and exploratory activity of animals. Therefore, I propose using the manipulated object recognition test or open field test equipped with USV detectors to measure exploration or anxiety-related phenotypes and to identify cognitive enhancers. I discuss how to interpret the data acquired in those tests in section 5.4.

Here, I illustrate how modification of open field testing can generate more conclusive and precise results. Testing in both brightly-lit and dimly-lit (20-40 lux) or even a completely dark (-2 lux) open field test equipped with USV detector provides an accurate way to ascertain whether the phenotype is based on anxiety, exploration or motor function (Fig. 5-1).
Figure 5-1. A possible interpretation of the behavior output data taken from the novel dim and novel bright groups.

Motor function, novelty exploration, or anxiety related behaviors could be assessed through this single experimental paradigm. Depending on the results, further experiments could be planned. Arrows denote variation from the behavior observed in wild-type littermates. Dashes signify that the mouse behavior is indistinguishable from that of wild-type littermates.
Rearing consists of animals standing on both hind paws in an upright posture. Unlike locomotion, this behavioral response reflects not only exploratory activity but also emotionality (Gironi Carnevale et al., 1990). It is regarded as an exploratory behavior (Carli et al., 1989; Fernández Espejo, 1997) because it allows the animal to sample or scan the environment (McGregor et al., 2002; van Abeelen, 1970), may be a marker of environmental novelty (Ho et al., 2002; Lever and Burton, 2006). Moreover, rearing is used as an orienting behavior; orienting or non-selective attention is associated with the duration of a rat’s rearing episode, with longer rearing indicating more orienting behavior (Colorado et al., 2006). Rearing is also regarded as an indicator of anxiety, in not only the open field (Carli et al., 1989; Lamprea et al., 2008), but also other exploratory based anxiety tests, such as in the EPM (Escorihuela et al., 1999; Lepicard et al., 2000; Rodgers et al., 1997) or the light-dark box (Costall et al., 1989; Crawley et al., 1984; Lepicard et al., 2000). Reductions in the number of rearings have been interpreted as heightened anxiety responses (Belzung and Griebel, 2001; Rodgers et al., 1997), and like grooming, they have been reported to occur mostly in the enclosed areas of the EPM (Adamec et al., 2004; Carola et al., 2002; Escorihuela et al., 1999; Rodgers et al., 1997) or next to the walls and in the corners of the OF (Carli et al., 1989; Carola et al., 2002; Choleris et al., 2001; Lamprea et al., 2008). My results are in keeping with these observations from other researchers, as this study found that rearings in enclosed areas (supported rearings) were correlated with low frequency USVs, suggesting that both might be outputs of anxious states.

One possible reason for this is that rearing itself may be a means to express various states. In other words, rearings could be occurring for different purposes in different contexts and as shown in this study, rearing in the dimly lit environment was associated with increased learning, while rearing in a brightly-lit environment was not. Rearing in brightly-lit environments might instead be an expression of anxiety states.

In a number of studies, rearing is considered a general locomotor or exploratory activity and a correlation between the rearing and locomotory activity is observed in the EPM (File, 1982), the light-dark box (Crawley, 1981) and the open field test (Kondratova et al., 2010; Thiel et al., 1999). However, this correlation was not tested in most studies and in others, it proved neither consistent nor reliable (Belzung and Griebel, 2001; Kim et al., 2002; Milner and Crabbe,
In this study, I also found no correlation between rearing and preferential exploratory response to DO in the brightly lit environment in which most supported rearings occurred. However, I found a correlation between rearing and exploratory behavior in the novel dim conditions, in which mainly unsupported rearing occurs. Since high frequency USVs are related to unsupported rearing in novel dim environments, anxiety tests may not be able to detect a correlation between rearing and exploratory activities. However, in exploratory-based LM tests, such as the object recognition task, the unsupported rearing in dimly lit novel environments is correlated with exploratory activity.

In Chapter 3, I focused mainly on the total number of rearings to determine their effects on LM. However, from the analysis of USVs and their relationship to behavior, I hypothesize that unsupported rearing and rearing to the walls might have different functions. Therefore, I reanalyzed Figures 3-2 and 3-5 with a focus on unsupported rearing versus supported rearing. In Figure 5-2 A, during the habituation/training phase, I measured rearings divided into rearings to the wall, rearings to the objects and unsupported rearings. Mice in the novel dim conditions showed significantly more frequent number of rearing to the objects and unsupported rearings, which I defined as investigatory rearings (Fig. 5-2 B). Furthermore, mice trained in a novel dim environment showed better spatial memory than mice trained in bright light (Fig. 5-2 C) but the lighting did not affect NO memory (Fig. 5-2 D). Finally, I plotted the investigatory rearings to DO preference. The regression line between investigatory rearings and DO preference showed similar correlation value ($r^2 = 0.462$) to that of total rearings ($r^2 = 0.44$; Fig. 5-2 E). No correlation was found between rearing to the walls and preference for the DO (Fig. 5-2 F).
Figure 5-2. Exploratory rearing promotes spatial learning.

(A) Diagram showing object recognition procedure. (B-F) Relationship between exploratory rearing and spatial memory in the object recognition test. Mice were trained in an arena with four objects and under dim (n=14) or bright (n=15) lighting while number and types of rearing (B) were recorded. (C) Later, preference towards two displaced objects (DO) over stationary objects (SO) was evaluated. (D) Ability to recognize a novel object (NO) over a familiar object (FO) was evaluated. (E-F) Relationship between exploratory rearing and spatial memory in the object recognition test for mice in Novel Dim and Novel Bright groups. The solid line represents Novel Dim group, while the dash line represents Novel Bright group. (E) Plot of Investigatory rearings data (which combined number of unsupported rearings and rearings to objects) against DO preference (C). (F) Plot of number of rearings to the walls (B) against DO memory (C) as a spatial memory index. Data are expressed as mean ± SEM. Pearson correlation ($r^2$) was used and slope ($m$) is shown. * P < 0.05, ** P < 0.01, *** P < 0.001.
My results suggest that future analyses of rearing (and USVs) may provide insight into the neurochemical mechanism underlying behavioral response to novelty. Locomotor responses to novel stimuli have previously been linked to mechanisms responsible for addiction and stress (Gray and McNaughton, 2003): Animals with high responses to novelty were found to show higher locomotor responses to the psychostimulant amphetamine (Hooks et al., 1991), a greater predisposition to drug self-administration (Pierre and Vezina, 1997), and higher sensitivity for natural reinforcers and stressors (Dellu et al., 1996; Rouge-Pont et al., 1993).

Hippocampal effects were stronger in animals that showed a higher rearing response in the novel open field test and thus higher rearing was linked with higher cholinergic reactivity in the forebrain of these rats (Thiel et al., 1999). Hippocampal acetylcholine (ACh) is thought to play a critical role in mechanisms that are particularly relevant for information processing, including novelty processing, attention, learning, and memory (Blokland, 1995; Fibiger, 1991). My data indicate that hippocampal activation of ACh does not reflect the detection of novelty since the magnitude of the cholinergic response did not differ between the novel and the familiar situation. Results here agree more with a presumptive function of ACh in attention. Such attentional mechanisms are necessary not only when the animal is exposed to a novel environment, but also when it is re-exposed to it. Handling may also involve attention, possibly to a lesser degree, compared to handling followed by open field exposure. Furthermore, hippocampal ACh might be able to affect the detection of novelty and/or the behavioral responsiveness to it, which might explain the individual relationships between cholinergic and behavioral responses to open field exposure and to being handled. Several neurochemical studies have shown cholinergic activity changes in LM paradigms.

The neurochemical analysis reveals higher dopamine levels in the ventral striatum of frequently rearing rats together with lower serotonin levels in the medial frontal cortex. These behavioral differences were found to be related to systematic differences at the level of the brain, especially on the transmitter dopamine. NCS-1 may play a role in deregulating the dopaminergic neurotransmission, leading to altered behavioral outputs (Kabbani et al., 2002). NCS-1 interacts with D2R (Kabbani et al., 2002; Lian et al., 2011) and attenuates D2R internalization in a Ca$^{2+}$-dependent manner by interacting with GRK2 (Kabbani et al., 2002). I will further discuss the
role of NCS-1 in novelty exploration as well as anxiety states in the following section.

5.4 Neuronal Calcium Sensor-1 (NCS-1)

The necessity of NCS-1 in the contexts of learning benefits during exploration in dimly lit environments was determined by multiple independent means that provided convergent results. Whole brain NCS-1 reductions since birth (\textit{Ncs1}^{P144S/P144S} line) and acute disruption of NCS-1 in adulthood by DG-specific \textit{Ncs1} knockdown abolished the memory enhancement resulting from high exploration. Previously reported \textit{Ncs1} KO mice displayed impairments in exploratory behavior, memory, and in mood-related phenotypes (de Rezende et al., 2014), although the \textit{Ncs1}^{P144S/P144S} line did not show any anxiety or depressive phenotypes. Taken together, these data provide evidence that NCS-1 is a molecule that links exploratory drive and memory.

It may also be argued that physical activity, rather than exploration itself, might be a relevant function of NCS-1. Chronic exercise or the 8-week swimming treatment, but not acute exercise or a single swimming treatment, enhanced object location memory tasks without affecting memory performance during NO tests. Chronic exercise promoted a significant increase in hippocampal NCS-1 levels (Drumond et al., 2012). Conflicting results have been observed for forced treadmill running with some studies showing improved object recognition memory (Griffin et al., 2009; Hopkins and Bucci, 2010), while others show impairment (Molteni et al., 2002). However, the exercise effects were seen in Morris water maze memory evaluation (Molteni et al., 2002; O'Callaghan et al., 2007). While the locomotor activity of \textit{Ncs1}^{P144S/P144S} mice appeared normal in the Rotarod test, they exhibited lower physical activity in single acquisition stage and affected spatial learning in DO recognition tasks. I have not yet examined whether chronic exercise might compensate (partially or completely) for NCS-1 reduction or loss, thereby boosting LM.

Another confounding factor might be anxiety feature since \textit{Ncs1}^{P144S/P144S} mice show reduced center traveling time in the open field, which indicative of anxiety. However, \textit{Ncs1}^{P144S/P144S} mice did not show changes in EPM and light dark box. Additionally, no changes in low frequency USV calls were observed.
When subjecting the Ncs$_{1}^{P144S/P144S}$ mouse line to the system introduced in Figure 5-2, mice had reduced exploratory locomotion and high frequency USVs only in dimly lit novel environments and not in brightly lit environments. Therefore, I conclude that this mouse line is impaired in novelty exploration (Fig. 5-3). Further, hippocampal-dependent LM performances were also reduced in the Ncs$_{1}^{P144S/P144S}$ mouse line.

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**Figure 5-3. Application of a method described in Figure 5-1 in NCS-1 (P144S) mouse line.** Ncs$_{1}^{P144S/P144S}$ mice showed reduced numbers of rearings and high-frequency USVs. According to Figure 5-1, learning & memory might be affected. Indeed, Ncs$_{1}^{P144S/P144S}$ mouse line is impaired in spatial learning and memory tests but normal in both motor function and anxiety related behavior tests.

5.5 Limitations

Most findings in the thesis are based on correlation analysis. Thus, while it is clear that, for
example, exploratory rearings are associated with enhanced spatial learning, it remains uncertain whether one variable causes the other, or whether both are connected to a third, unmeasured variable. However, I tried to overcome the shortcoming of these correlative assays by using a different approach, which might explain the causal relationship although further analysis or experiments are required to determine directionality.

First, self-directed exploration is correlated with learning. I tried to decipher the effects of self-directed exploration on learning by comparing learning efficacy in a low exploration environment (dim lighting) and a high exploration environment (bright light) in the object recognition test. Here, two factors exist: exploration level and lighting level. To look at whether exploration itself depends on lighting and then causes learning efficiency, I applied BoNT to block hind leg muscle movement, thereby reducing exploration level; if this led to decreased learning efficiency, I could conclude that exploration itself might have influenced learning. However, blocking hind leg movements might also create another stress factor, similar to bright light; hence, this experiment may not be able to answer the question fully, although the test sessions were done three days after the BoNT injection when the stress level might be reduced. To answer this question fully, measurements of stress level, such as the glucocorticoid or its metabolites, should be done in all treatment groups to provide information on stress levels. Additionally, since I have USV analysis done in ND, NB, FD, and FB to understand the emotional states of mice being exposed in those environments, measuring USVs in BoNT-treated group would be helpful to gauge a stress level of mice in this group.

Second, I observed a correlation between the vocalization features and self-directed exploration. I also analyzed the vocalization effects simultaneously with the behavior to look for further supporting correlative evidence. Whether mice vocalize specific types of USVs due to the particular movement remains unknown. Further analysis with playback experiments might be able to answer the question.

Finally, neural network association based on the c-fos expression in self-directed exploration was first determined in a separate correlative assay. Later, I tried StEM analysis, which has advantages over correlation coefficients when analyzing the relationship in neural networks. With this analysis, I found stronger connectivity in HPC-NacC during self-directed
exploration. To further confirm these results, optogenetic approaches can be used to determine the causal relationships.

Currently, methods to measure the strength of neural connectivity are not available. However, brain activity can be measured by fMRI, and further connectivity can be inferred from statistical analysis of fMRI results. Similarly, in this study, I used IEG, c-fos, as a neuronal marker to map activated brain regions, and later, the connectivity of the neural network was analyzed statistically using StEM. However, several potential limitations exist in c-fos based functional mapping. First, it is not clear whether the capacity of c-fos expression is universal among central neurons and whether the activated neurons express the c-fos gene with comparable sensitivities. Second, the temporal dynamics of gene regulation limit the detection of fluctuations in activity levels associated with specific external events.

The environment can also play a role in finding differences between controls and mutants in any one of these tests. For example, Crabbe et al. (1999) found that different laboratory settings can affect behavioral differences between control and mutant mice. The exact reason for these lab-to-lab discrepancies is not known, but it is most likely due to subtle differences in environmental or test conditions. Although every effort was made to control for these factors (for example, all tests were performed using similar apparatuses and identical instructions), the potential for such discrepancies still exists. Thus, some of the differences between laboratories in finding a mutant as behaviorally deficient may be due to environmental effects. However, this does not negate the findings within an individual laboratory. When proper controls are performed, simultaneously or in parallel with the mutants, conclusions can be made appropriately.

5.6 Future Directions and Applications

The assay outlined in Figure 5-1 has proven useful in identifying enhanced LM, but has not yet been tested on other strains with learning impairments. In at least some cases, novelty exploration and curiosity may be the same as in wild-type littermates while the ability to retain information may be compromised. The LM process consists of three stages: encoding (gathering sensory information), consolidation, and retrieval. A learning impairment can be caused during
any one or more of those stages. Engineered mice with cognitive enhancements are generated by manipulating genes affecting either the acquisition or consolidation of memory. For example, NR2B, calcineurin and H-ras are thought to be involved in the acquisition of memory, whereas ORL1 (Opioid receptor-like 1), CaMKIV, eIF2α (Eukaryotic translation initiation factor 2A), and cbl-b seem to modulate memory consolidation (Silva, 2003). NR2B-deficient mice lack a habituation response (Badanich et al., 2011). Calcineurin mutants showed high locomotion in the novel open field test. It would be interesting to apply the assay used in this thesis to engineered mice with memory enhancement due to each step of memory formation. While such a study may not have the ability to distinguish between mice with deficits in consolidation and retrieval stages, it would provide a useful tool to identify cognitive enhancers that promote curiosity and novelty exploration.

I have not used this assay on anxious mice. In many cases of engineered mice models designed to target anxiety-related genes, the mice do not show behavioral differences in the EPM or the light and dark box. The assay used here may be more sensitive and applicable to anxiety-related behaviors and thus able to distinguish behavioral differences. Further, I suspect that anxious animals may exhibit deficits/variations in dim condition behaviors and that this might serve as a measure of the degree of anxiety.

In conclusion, my work provides important extensions and refinements to behavioral analyses that can inform the design of testing environments. It takes a further step towards elucidating animal communication. The inability to communicate effectively can accompany human disorders, and most notably ASD. Hence refinements, such as the USVs offered here, may be particularly useful for the ASD model. Aside from its disease oriented applications, this work and addition of further technological innovation to the methods proposed can begin to give us insight into the depth of complexity of animal language - a fascinating result in itself.
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