CHARACTERIZATION AND INTERVENTIONAL STRATEGIES IN NOVEL TRANSGENIC RAT MODELS OF AUTISM SPECTRUM DISORDERS

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BY

VIJAYAKUMAR K

UNDER THE GUIDANCE OF

PROF. SUMANTRA CHATTARJI

NCBS, GKVK POST BELLARY ROAD BANGALORE

NATIONAL CENTER FOR BIOLOGICAL SCIENCES

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Private University Established in Karnataka by ACT 35 of 2013

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DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**CHARACTERIZATION AND INTERVENTIONAL STRATEGIES IN NOVEL TRANSGENIC RAT MODELS OF AUTISM SPECTRUM DISORDER**" submitted for the award of Doctor of Philosophy to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of my guide SENIOR PROF.SUMANTRA CHATTARJI. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged.

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

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Date: 25-1-2023

Name of candidate: VIJAYAKUMAR K

Reg. No.: 21117020491

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BENGALURU - 560064

CERTIFICATE

This is to certify that the work incorporated in this thesis "**CHARACTERIZATION AND INTERVENTIONAL STRATEGIES IN NOVEL TRANSGENIC RAT MODELS OF AUTISM SPECTRUM DISORDER**" submitted by VIJAYAKUMAR K was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Research Supervisor:

Date: 25-1-2023

Senior Prof. Sumantra Chattarji

National centre for Biological Sciences,

GKVK post, Bangalore

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List of Figures and tables

Abbreviations

AFC: Auditory fear conditioning

Akt: AK strain transforming

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Amph-1: Amphiphysin-1

ANOVA: Analysis of variance

APA: Active place avoidance task

ASD: Autism spectrum disorder

BDNF: Brain-derived neurotrophic factor

BLA: Basolateral amygdala

BRRS: Bannayan–Riley–Ruvalcaba syndrome

CA2: Cornu ammonis 2

CACNAC: Calcium Voltage-Gated Channel Subunit Alpha1 C

CAMKII: Ca^{2+} /calmodulin-dependent protein kinase II

Cas9: CRISPR associated protein 9

CDC: center for disease control

CDD: CDKL5 deficiency disorder

CDKL5: Cyclin dependent kinase like 5

C-elegans: Caenorhabditis elegans

CFC: Contextual fear conditioning

CNTNAP2: Contactin-associated protein-like 2

CNV: Copy number variation

CPCSEA: Committee for the purpose of control and supervision of experiments on animals

CRISPR: clustered regularly interspaced short palindromic repeats

CS: Conditional stimulus

CS: Cowden syndrome

Cyfip-1: Cytoplasmic FMR1-interacting protein 1

DNA: Deoxy ribonucleic acid

DNMT-1: DNA-methyltransferase 1

dPAG: Dorsal periaqueductal grey

DSM-V: Diagnostic and statistics manual – V

ERK: Extracellular-signal-regulated kinase

fMRI: Functional magnetic resonance imaging

FMRP: Fragile X mental retardation protein

FXS: Fragile X syndrome

GAP: G-protein activating protein

GF: Growth factor

GPE: Glycine-proline-glutamate

GSK-3: Glycogen synthase kinase 3

GTPase: Guanosine triphosphatase

HDAC4: Histone deacetylase-4

Het: Heterozygous

ID: Intellectual disability

IGF: Insulin like growth factor

iPSC: Induced pleuripotent stem cells

IQ: Intelligence quotient

ITI: Inter trial interval

KO: Knockout

LE: Long Evans

LTD: Long term depression

LTP: Long term potentiation

L-VSCC: L-type voltage-sensitive calcium channel

MAGI: Membrane-associated guanylate kinase

MAPK: Microtubule associated protein kinase

MDS: MECP2 domain syndrome

MECP2: Methyl CpG binding protein 2

MGluR5: Metabotropic glutamate receptor 5

MoSeq: Motion sequencing

mTOR: Mammalian target of rapamycin

NDD: Neurodevelopmental disorder

Ngl-1: Neuregulin-1

NLGN3: Neuroligin 3

NMDA: N-methyl-D-aspartic acid or N-methyl-D-aspartate

OC: Object context

OLR-LTM: Object location recognition- Long term memory

OP: Object place

OPC: Object place context

OR: Object recognition

PET: Positron emission tomography

PI3K: Phophoditidyl inositol 3 kinase

PIP2: Phosphotidyl inositol bisphosphate

PIP3: Phosphotidyl inositol phosphate 3

PND: Postnatal day

PSD 95: Post synaptic density protein 95

PTEN: Phosphatase and tensin homolog

Rac1: Ras-related C3 botulinum toxin substrate 1

RPM: Rotations per minute

SCN1A: Sodium channel protein type 1 subunit alpha

SD: Sprague Dawley

SEM: Standard error mean

SHANK: SH3 and multiple ankyrin repeat domain

SNP: Single nucleotide polymorphism

SNV: Single nucleotide variation

SYNGAP: Synaptic Ras GTPase activating protein

TS1: Training session 1

TS2: Training session 2

US: Unconditional stimulus

VTA: Ventral tegmental area

WT: Wildtype

List of publications

1. Natasha J. Anstey*, **Vijayakumar Kapgal***, Shashank Tiwari, Thomas C. Watson, Anna K. H. Toft,Owen R. Dando, Felicity H. Inkpen, Paul S. Baxter, Zrinko Kozić, Adam D. Jackson, Xin He,Mohammad Sarfaraz Nawaz, Aiman Kayenaat, Aditi Bhattacharya, David J. A. Wyllie, Sumantra Chattarji, Emma R. Wood, Oliver Hardt and Peter C. Kind. Imbalance of flight–freeze responses and their cellular correlates in the *Nlgn3*$^{-/y}$ rat model of autism. *Molecular Autism*, 2022;13:34. https://pubmed.ncbi.nlm.nih.gov/35850732/

2. D. Katsanevaki, SM. Till, I. Buller-Peralta, TC. Watson, MS. Nawaz, D. Arkell. Tiwari, V. Kapgal, S. Biswal, JAB. Smith, NJ. Anstey, L. Mizen, N. Perentos, MW. Jones, MA. Cousin, S. Chattarji, A. Gonzalez-Sulser, O. Hardt, ER. Wood, PC. Kind. Heterozygous deletion of SYNGAP enzymatic domains in rats causes selective learning, social and seizure phenotypes. bioRxiv preprint doi: https://doi.org/10.1101/2020.10.14.339192

Chapter 1.Introduction

Autism was first described as “infantile autism” by Leo Kanner (Kanner, 1943) because of the distinct social isolation profile in a group of children. The word autism *per se* was used first by Austrian scientist Eugene Bleuler for lack of social interests in patients with schizophrenia (Bleuler, 1911). Further, German scientist Hans Asperger had described similar symptoms in patients as “autistic psychopathy” (Asperger, 1944). Autism spectrum disorders (ASD) are group of neurodevelopmental disorders which gets detected as early as 1-3 years during childhood. Children with ASD present with distinct set of problems pertaining to social interactions, verbal/ non-verbal language and repetitive or restrictive type of behaviors (DSM-V). Additionally, they display wide variety of associated symptoms like anxiety, sensory-motor deficits, hyperactivity and cognitive deficits. Every 1:100 children have ASD across globe (CDC). Global disease burden for ASD is on rise and poses a challenge for both developed and developing countries. Pathobiology of ASD is still not clear and hence there is only symptomatic treatment available to manage the behavioral problems faced by these children.

Genetic basis of Autism was first substantiated by Susan Folstein and Michael Rutter’s study which found that there is a high chance of occurrence of autism in monozygotic twins but not dizygotic twins (Folstein and Rutter, 1977). Subsequent studies led to listing of autism in Diagnostic and Statistical Manual of Mental Disorders for the first time in 1980. At this point of time, autism was categorized as subtypes which included infantile autism, residual autism, childhood-onset pervasive developmental disorder and atypical autism. Later in 1994 the autism was brought under broad umbrella term as autism spectrum disorder which presents with triad of core features i.e. social interaction deficits, communication deficits and restricted, repetitive, and stereotyped behavior, interests, and activities. Latest revision of DSM-V (2013) included dyad of core features like difficulties in social communication and social interaction and restricted and repetitive behavior, interests, or activities.

1.1. Genetic etiology of Autism spectrum disorders

Early studies on karyotyping resulted in identifying regions with chromosomal abnormalities in the genome implicated in Autism (Gillberg and Wahlström, 2008). Further genetic loci screens for susceptible chromosomes were found in multiple chromosomes (Buxbaum *et al.*, 2001; Liu *et al.*, 2001; Risch *et al.*, 2014). Candidate gene approach led to linking of various genes like *NLGN 3*, *MeCP2*, *TSC*, *UBE3A* to ASD etiology (Carney *et al.*, 2003; Jamain *et al.*, 2003; Serajee, 2003; Jiang *et al.*, 2004).The advent of genome sequencing further revolutionized ASD research which helped in realizing that etiology of ASD is highly heterogeneous and multigenic in nature. Among them, very few cases like Fragile X, Rett syndrome and tuberous sclerosis emerged as monogenic causes of syndromic Autism (Artuso *et al.*, 2011;Woodbury-Smith and Scherer, 2018). Till date many large scale genome wide association studies on patients and their relatives found hundreds of risk genes associated with ASD. Studies which used whole exome sequencing in large cohorts of affected individuals implicated three broad categories of proteins involved in synaptic function, transcriptional regulation and chromatin remodeling (O'Roak *et al.*, 2011; De Rubeis *et al.*, 2014). Furthermore, copy number variations (CNV) and single nucleotide polymorphisms (SNP) in chromosomes would occur because of duplications, deletions, translocations, and inversions of base pairs in genes coding synaptic proteins, transcriptional regulators, cell cycle and chromatin regulators. Thus, CNV's and SNP's are factors responsible for the heterogeneity of the observed phenotypes in ASD (Marshall and Scherer, 2012; Pizzo *et al.*, 2019).

1.2. Epigenetic and environmental factors contributing to ASD etiology

Despite ASD's being highly heritable there are phenotypic variances among monozygotic twins (Ronald and Hoekstra, 2011) which points towards the epigenetic involvement in Autism. Evidence from clinical studies indicate role of methylation and histone modification leading to ASD phenotypes in patients with Fragile X syndrome, Rett syndrome, CDKL5 disorder and PTEN hamartoma syndrome (Ladd-Acosta *et al.*, 2014;

Wong *et al.*, 2014; Trazzi *et al.*, 2016; Ho *et al.*, 2020). Another study on post mortem brain tissue uncovered a distinct epigenetic signature like histone acetylome abberations which links to the ASD phenotypes (Sun *et al.*, 2016). The fact that the environmental factors were indeed responsible for Autism came into existence from controversial studies involving thimerosol based vaccines resulting in autism in children (Wakefiled *et al*., 1998- Retracted citation). However, this theory has been debunked by other studies over many years (Parker *et al.*, 2004). Further evidences from various other studies elucidated role of environmental insults as a vulnerability factor of ASD. Factors such as increased parental age, sodium valproate intake for epilepsy treatment or toxic chemical exposure during pregnancy, maternal diabetes, enhanced steroidogenic activity, viral infections during pregnancy, antidepressant drug usage etc., would contribute to ASD etiology (Modabbernia, Velthorst and Reichenberg, 2017; Bölte, Girdler and Marschik, 2019). Hence, epigenetic and environmental factors add complexity to already complex genetic etiology of ASD's. No wonder the phenotypic heterogeneity observed in patients is resultant of its complex etiology makes it more difficult in understanding pathophysiology and developing treatment strategies to combat the disease.

1.3. Syndromic and non-syndromic autism

Clinically, ASD's are generally classified as syndromic or non-syndromic. When ASD coexists with other phenotypic and dysmorphic characteristics, this condition is described as syndromic. Most often, these conditions have a known etiology which can include chromosomal abnormalities, copy number variations, and are generally monogenic in nature. For example the fragile X syndrome (FXS), MECP2 duplication syndrome (MDS) and PTEN macrocephaly syndrome (Amir *et al.*, 1999; Butler, 2005; Van Esch *et al.*, 2005; Verkerk and Sutcliffe,1991) etc. In contrast to non-syndromic ASD, syndromic types of ASD are distinct clinical entities with distinct developmental trajectories. The non-syndromic or idiopathic autism the etiology is unknown. The patients may or may not express all the phenotypes like in syndromic autism but might

display wide range of heterogeneity among them. De novo / germ line mutations in various genes specifically encoding synaptic proteins are now very much known to be responsible for non-syndromic autism (Caglayan, 2010). The whole exome sequencing studies over last few decades have detected the genetic link in these cases and many of them now are understood to be monogenic in nature (Yu *et al.*, 2013; C Yuen *et al.*, 2017; Satterstrom *et al.*, 2020).

1.4. Male bias in Autism

According to recent data from Center for disease control (CDC), ASD is 4.2 times more prevalent in males than in females (Maenner *et al.*, 2021). It is interesting to note that male bias exists in most of the neurodevelopmental disorders (NDD) which includes intellectual disability, attention deficit hyperactivity disorder (ADHD) etc., (Werling and Geschwind, 2013). The sex-specific single nucleotide polymorphisms (SNPs), single nucleotide variants (SNVs), microdeletions, copy number variants (CNVs), and proteins have all been implicated in the increased male prevalence with ASD (Mitra *et al.*, 2016; Werling, Parikshak and Geschwind, 2016). A recent review on the male bias in Autism discusses multiple factors like sex chromosomes, fetal testosterone, female hormones, neuro peptides like oxytocin and corticotrophin releasing factors, serotonin and BDNF levels etc. (Ferri, Abel and Brodkin, 2018). A similar review summarized sex specific behavioral characteristics in male and female rodents of genetic, epigenetic and environmental models of ASD (Jeon *et al.*, 2018). Both the studies mentioned above highlights multiple factors which places males at an increased risk quotient than females. However, more research is required to ascertain if the females are underdiagnosed or late diagnosis results in this skewed prevalence in ASD.

1.5. Synaptopathies in autism

The term synaptopathy refers to the neurological, neuropsychiatric or developmental disorders which occur due to loss or deficiency of a synaptic protein (Grant, 2002). The

human synapse proteome comprises of rich collection of proteins with myriad functions which are disrupted by several gene mutations causing multiple brain disorders. A typical synapse consists of proteins like cell adhesion molecules, scaffolding proteins, translational regulators, receptors, ion channels, kinases etc (Figure 1). Almost all of the proteins mentioned above have been implicated in autism. Whole genome sequencing had led to finding new risk genes associated with ASD (Satterstrom *et al.*, 2020). Mutations in many such genes are highly penetrant and would result in monogenic form of ASD. The developmental dysregulation of synaptic function and homeostasis indeed appears to be causative factor in ASD along with the epigenetic and environmental influence. Following clinical studies are few examples of synaptopathies resulting in ASD. Stephane Jamain's group first identified a point mutation in gene coding for cell adhesion molecule neuroligin 3 (NLGN3) where arginine was replaced with cysteine on 451 base pair in two patients diagnosed with ASD (Jamain *et al.*, 2003). Further studies attributed neuroligin 3 roles in synaptogenesis, synapse maintenance and function. Another case report revealed mutations in gene coding for cyclin dependent kinase like 5 (*Cdkl5*) formerly called as serine threonine kinase 9 resulted in severe infantile spasms and mental retardation (Kalscheuer *et al.*, 2003). CDKL5 has role in dendritic growth, synaptic transmission, gene expression etc., CDKL5 is an upstream molecule for MeCp2 which is implicated in Rett syndrome. Further, the CDKL5 deficiency is identified as independent clinical entity resulting in early-onset encephalopathy (Fehr *et al.*, 2013). Another study identified de novo truncating mutations in *SynGAP1* gene with non syndromic mental retardation (Hamdan *et al.*, 2009).SYNGAP1, encodes a ras GTPase-activating protein which is crucial for cognition and synapse function. Furthermore, a clinical case report with patients suffering from Cowden syndrome showed germline mutations in phosphatase and tensin homolog (*Pten*) displayed autistic features (Zori *et al.*, 1998; Goffin *et al.*, 2001). PTEN has role in cell cycle progression, cell death, cell migration, transcription, translation etc. Apart from these many more synaptic proteins like neurexins, SHANK, mGlur5, CATNAP2, CACNAC, etc., have been implicated in autism (Chen *et al.*, 2014a; Howell and Smith, 2019; Bonsi *et al.*, 2022; Jiang *et al.*, 2022). These

human studies resulted in further modeling in mice to ascertain cellular, molecular, electrophysiological and behavioral manifestations of the synaptopathies (Murdoch and State, 2013; Bey and Jiang, 2014; Howell and Smith, 2019).

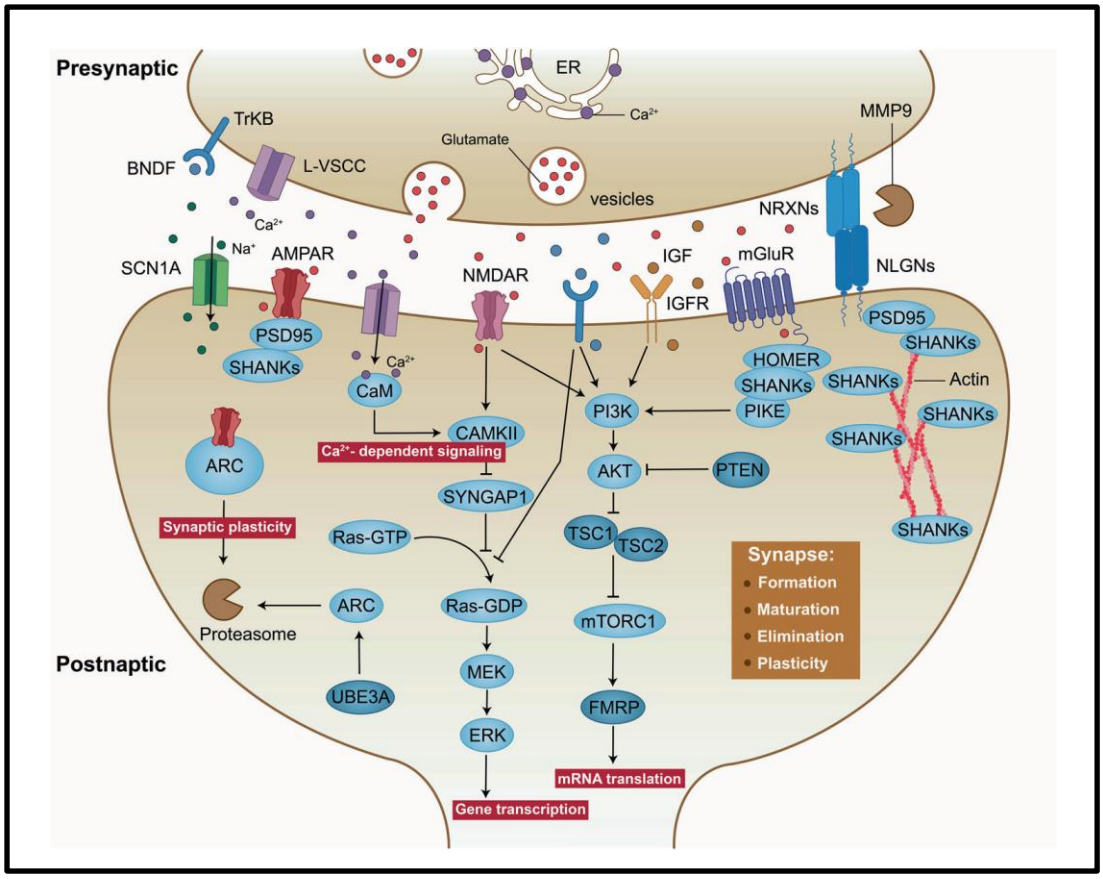


Figure.1.5.Molecular pathways implicated in synaptic function for ASD. Adapted from Jiang et al., 2022. At a typical excitatory synapse, proteins encoded by autism risk genes involve synaptic scaffold proteins (for example, SynGAP and SHANKs), neurotransmitter receptors (for example, NMDARs, AMPARs and mGluRs) and cell adhesion molecules (NRXNs and NLGNs). Further, activation of cell surface receptors leads to activation of the Ras/ERK and PI3K/AKT/mTOR pathways which are further regulated by kinases like CDKL5 and PTEN. In addition, mutations in ion channels, such as L-VSCCs and sodium channel protein type 1 subunit-α (SCN1A), both of which have been implicated in synaptic dysfunction and autism-like behavior

1.5.1. Neuroligin 3 mutation in ASD

Neuroligins are post synaptic cell adhesion molecule which binds to presynaptic neurexins. There are 5 isoforms of neuroligins in humans, i.e. NLGN1, NLGN2, NLGN3, NLGN4X, NLGN4Y. In vitro studies have emphasized role of neuroligins in synapse formation and necessary for synapse maturation and maintainance (Scheiffele *et al.*, 2000; Südhof, 2008; Singh and Eroglu, 2013). Among them NLGN3 protein is present both in excitatory neurons and inhibitory neurons (Budreck and Scheiffele, 2007). Stephane Jamain's group first reported the single nucleotide polymorphism (SNP) in exon 6 which resulted in replacement of arginine with cysteine on 451 position leading to 90% reduction in NLGN3 protein (Jamain *et al.*, 2003). Another study reported truncation in exon 4 in 9 individuals with ASD which resulted in premature stop codon and loss of exon 7 and 8 (Talebizadeh, 2005). Further reports in ASD patients found SNP in 3'UTR which resulted in very mild 9% reduction NLGN3 protein (Steinberg *et al.*, 2012). Studies on different cohorts of ASD patients revealed SNP in exon 6 and 7 which either led to loss of function mutation or complete reduction of NLGN3 protein (Volaki *et al.*, 2013; Redin *et al.*, 2014; C Yuen *et al.*, 2017; Quartier *et al.*, 2019). *Nlgn 3* mutations have been modeled in many organisms like *Drosophila*, *C. elegans*, mice and rats. Vast majority of work has been done mice with complete deletion or R451C knock in as described in the first study linking *Nlgn3* to autism (Jamain *et al.*, 2003). Alternatively, *Nlgn3* R704C knock in mouse is also used which was adapted from the clinical study implicating *Nlgn 4X* to autism (Yan *et al.*, 2005). Another study showed hyperactivity, reduced ultrasound vocalization, lacked social novelty preference, reduced fear memory and normal spatial learning and memory in *Nlgn3* KO mice (Radyushkin *et al.*, 2009). Other studies have also reported reduced sociability during the three chamber task, increased locomotor activity in an open field, and enhanced spatial navigation learning in the Morris water maze in comparison to WT littermates (Tabuchi *et al.*, 2007; Etherton *et al.*, 2011; Jaramillo *et al.*, 2018). Furthermore, studies from same group have shown that the phenotypes observed in these NLGN3 deficient mice were indeed influenced by genetic background on which they are bred. Moreover,

another study described the effect of housing environment on the phenotypes observed in WT and *Nlgn3* KO mice (Kalbassi *et al.*, 2017). Recent studies in Nlgn3 KO rats have showed some similar and contrasting behavioral phenotypes when compared to mice studies (Hamilton *et al.*, 2014; Thomas *et al.*, 2017). This alludes to the influence of environment, age, genetic background etc., on the behavioral phenotypes. Hence, results obtained should be carefully interpreted providing the detailed information about the timeline, order and protocols used for behavioral phenotyping of these animal models.

1.5.2. Cdkl5 mutation in ASD

Cyclin dependent kinase like 5 (CDKL5) previously known as serine threonine kinase 9 was first isolated and sequenced during transcriptional mapping effort in the Xp22 chromosomal region (Montini *et al.*, 1998). First case study in two females reported early infantile spasms, severe developmental delay with mutations in gene coding for CDKL5 (Kalscheuer *et al.*, 2003). Another clinical study identified the CDKL5 deficiency in siblings with infantile spasms and autistic traits (Weaving *et al.*, 2004). Further studies reported boys with mutations in *Cdkl5* presented with more severe form of disease than girls (Elia *et al.*, 2008; Siri *et al.*, 2021). Subsequent studies in large cohorts of patients with Cdkl5 mutation displayed autistic features along with epilepsy and intellectual disability (Bahi-Buisson *et al.*, 2008, 2012; Szafranski *et al.*, 2015). CDKL5 gene transcript contains 23 exons, coding exons start from exon 2 (Bahi-Buisson and Bienvenu, 2011; Williamson *et al.*, 2012). Cdkl5 is an upstream molecule to MeCP2 and the phenotypes in patients with Cdkl5 mutations were initially described atypical Rett like features (Mari *et al.*, 2005). However, later CDKL5 deficiency disorder was considered as a separate entity with distinct clinical profile compared to Rett syndrome (Fehr *et al.*, 2013). Further molecular characterizations of CDKL5 transcript isoforms from human, mouse and rat have revealed more information on new exonic regions and a range of novel splice and UTR isoform (Hector *et al.*, 2016, 2017). Over years, multiple hemizygous, heterozygous mice and conditional knock out models of CDKL5 deficiency have been

generated by targeting exon 6, exon 4 and exon 2 respectively (Wang *et al.*, 2012; Amendola *et al.*, 2014a; Fuchs *et al.*, 2014; Jhang *et al.*, 2017; Tang *et al.*, 2017; Okuda *et al.*, 2018). These mice models of CDKL5 deficiency claim phenotypic similarity with humans in regard to cognitive deficits, social deficits, motor deficits and stereotypic repetitive behaviors. However, none of these mice show seizure phenotype which is the most debilitating symptom observed in humans. High phenotypic variability among these mice lines poses a problem with respect to reproducibility of the data obtained. Hence, it is necessary to test effects of genetic perturbations observed in ASD in new models to develop therapeutic strategies.

1.5.3. SynGAP mutations in ASD

Synaptic ras GTPase activating protein (SynGAP) is post synaptic scaffolding protein which is associated with the postsynaptic density protein (PSD 95) and is required for excitatory neuron plasticity (Chen *et al.*, 1998). Further to elucidate the physiological role of *SynGAP* knock-out mice model was generated (Kim *et al.*, 2003). These mice die within week after birth which indicates the essential role of SYNGAP for postnatal development. Additionally, the neuronal cultures obtained from these mice showed increased clusters of AMPA receptors which indicates at involvement of SYNGAP in receptor trafficking. Since then, many clinical studies have implicated *SynGAP* mutations resulting in severe mental retardation and ASD. Heterozygous mutations in *SynGAP* were first identified in patients with severe intellectual disability, ASD and epilepsy (Hamdan *et al.*, 2009). Subsequent human study genotyped large cohort of ASD patients using SNP-microarray technique and found *SynGAP* mutations among different de novo mutations (Pinto *et al.*, 2010). Further case reports with with large patient cohorts have revealed truncating and missense mutations, chromosomal aberrations and microdeletions (Krepischi *et al.*, 2010; Rauch *et al.*, 2012; Writzl and Knegt, 2013; Parker *et al.*, 2015; Vlaskamp *et al.*, 2019; Verma *et al.*, 2020). Alongside human case reports there were multiple behavioral characterization studies in mice with heterozygous *SynGAP* mutation. *SynGAP* heterozygous mice display severe impairments in cognitive,

social and emotional domains (Guo *et al.*, 2009; Muhia *et al.*, 2010; Clement *et al.*, 2012; Ozkan *et al.*, 2014; Berryer *et al.*, 2016). Other study in heterozygous SynGAP mice demonstrated reduced activity in sensory cortical neurons responsible for touch processing which may contribute towards above mentioned phenotypes (Michaelson *et al.*, 2018). Furthermore, *SynGAP* heterozygous mice show reduced seizure threshold which is an established phenotype in human (Sullivan *et al.*, 2020). Overall, mice models of *SynGAP* heterozygosity have reproduced cellular, molecular, physiological and behavioral phenotypes which are observed in humans. But there is variability with respect to site of mutation (conditional knockout vs global heterozygous mutation) and the behavioral protocols used. In this thesis I have employed comprehensive behavioral assays to assess behavioral phenotypes in novel *SynGAP* heterozygous rat model of synaptopathy. It would be interesting to evaluate if there occurs any mechanistic validations between two species which would further help to develop interventional strategies in treating the synaptopathy in humans.

1.5.4. Pten *mutations in ASD*

Phosphatase and tensin homolog (*Pten*) initially was identified as tumor suppressor gene which appears to be mutated at considerable frequency in human cancers (Li *et al.*, 1997). PTEN has many biologic roles in cellular function. In the cytoplasm PTEN dephosphorylates PIP3 to PIP2, acting as a negative regulator of PI3K and thus affecting all downstream functions controlled by the AKT/mTOR axis. Thus, controls cell cycle progression, induction of cell death, transcription, translation, stimulation of angiogenesis, and stem cell self-renewal. Nuclear functions of PTEN include maintaining genomic integrity, DNA repair, and degradation of onco proteins and chromatin remodeling (Song, Salmena and Pandolfi, 2012; Milella *et al.*, 2015; Enriquez-Barreto and Morales, 2016). *Pten* mutations were first observed in cancer patients showing autistic traits with macrocephaly (Zori *et al.*, 1998; Goffin *et al.*, 2001; Butler, 2005). Multiple case reports then described *Pten* germline mutations in Cowden syndrome, Proteus syndrome or Bannayan-Riley-Ruvalcaba Syndrome collective called as PTEN

hamartoma syndrome show autistic features (Busch *et al.*, 2013). Recent high throughput genomic studies in large cohorts of patients with germline *Pten* mutations have been identified with autistic traits (Frazier *et al.*, 2015; Hansen-Kiss *et al.*, 2017; Busch *et al.*, 2019; Cummings *et al.*, 2022). Several mice models have been generated over years delineating PTEN functions. Loss of PTEN function leads to hyperactivation of PI3K/AKT/mTOR pathway in forebrain specific conditional knockout *Pten* mice (Lugo *et al.*, 2014). *Pten* heterozygous mice display reduced social behaviors, increased repetitive behaviors (Lugo *et al.*, 2014; Clipperton-Allen and Page, 2015; Smith, White and Lugo, 2016; Chai *et al.*, 2021). The behavioral characteristics of heterozygous *Pten* mice and conditional knockout mice varied significantly in the majority of mouse studies. In this thesis I have carried out behavioral assessment of novel transgenic rat with heterozygous mutations in *Pten*.

1.6. Models of Autism

Translation relevant animal models are required to identify the underlying pathology and to develop the therapeutic strategies to bridge the gap between bench and bedside research. An ideal model animal would facilitate researcher to integrate behavior, in vivo neuroimaging, and pathology which is very rarely possible with human subjects. This would result in “forward translation” where basic science findings are incorporated into novel clinical therapies and in “reverse translation” where one looks for mechanistic explanation of the clinical findings in the model organism. Technological advances in genetic manipulation have led to the generation and characterization of multiple models of ASD. ASD being a human specific heterogenic neurodevelopmental disorder with wide range of behavioral symptoms is difficult to model in any organisms. However, the genetic orthologs are similar for most of the genes implicated in autism and hence a mechanistic understanding of loss of function of any gene related to ASD in any model would be beneficial in understanding the neurobiology or pathophysiology of the disease. Choosing a specific model depends on the specific question the researcher wants to address. Multiple model systems like *in vitro* human stem cells, non-human

primates, *drosophila*, *C-elegans*, aplysia, mice and rats. Each model system has its own advantages and disadvantages.

1.6.1. Human stem cells as model of ASD

Human *in vitro* models offer advantage in overcoming species barrier, gain mechanistic insights of disease mechanism, provides high – throughput experimental platform to test novel candidate drugs and can be used in isogenic cell based therapies. Shinya Yamanaka and Kazutoshi Takahashi in 2006 invented the technology to develop induced pluoriptent stem cells (iPSC). Mouse iPSC lines were generated by reprogramming a mouse fibroblast by retroviral delivery of Yamanaka factors (Takahashi and Yamanaka, 2006). Further in 2007, Yamanaka and colleagues applied the same technology to generate human induced pluripotent stem cells (Omole and Fakoya, 2018). These cells are self-replicating in cultures and can give rise to cells from the three germ layers. Use of iPSCs do not warrant rigorous ethical considerations, hence use of iPSCs in ASD modelling has been very encouraging. Many studies since then have used hiPSC to model ASD (Das Sharma *et al.*, 2020). However, disadvantages of *in vitro* models include immaturity of neurons after prolonged time in cultures and might be difficult to study developmental time points. Cellular niche for complex cultures with astrocytes and other supportive cells are lacking and difficult to maintain. *In vivo* synaptic connectivity is absent in *in vitro* system and cannot account for specific circuit dysfunctions observed in rodent models (de la Torre-Ubieta *et al.*, 2016).

1.6.2. Non-human primate models of ASD

Non-human primates offer advantage to ASD research as they are nearest in phylogenetic lineage compared to rodents. Rodents are separated by 70 million years and non-human primates like macaques or marmosets by 25 million years (Kumar and Hedges, 1998) and hence exhibit greater similarity in genetics, neurobiology and behavior. Recent research in non-human primates such as monkeys with MeCP2 over

expression show reduced social interaction and increased repetitive behaviors (Liu *et al.*, 2016). Another study used non genetic approach in marmosets using valproic acid revealed altered synaptogenesis and vocalizations (Watanabe *et al.*, 2021). Further, a recent study used CRISPR/Cas9 to induce heterozygous reduction in SHANK3 which resulted in decreased spine density and matured neurons in prefrontal cortex. Also, they observed reduction in levels of postsynaptic PSD95 protein and a subset of glutamate (Zhao, Jiang and Zhang, 2018). Though these studies are encouraging but have huge input cost and difficult ethical requirements. Hence, cannot be used to avail high throughput benefit as in rodent studies.

1.6.3. Fly model of ASD

Drosophila melanogaster is a well-known model organism for genetic research. *Drosophila melanogaster* is being used in order to identify novel genes associated with ASDs to understand their molecular functions in synaptogenesis, synaptic function, synaptic plasticity, and the formation and consolidation of brain circuits. *Drosophila* genome is very simple compared to any mammalian organism genome and availability of genetic toolkit to alter the multiple gene results in wide variety of mutants with low input cost, more off-springs in less time and lesser ethical requirements. There is no dearth to the genetic analysis studies in *Drosophila* models of ASD. Interestingly, the behavioral assays like social space assay, activity assay, odor-taste learning assays have been used in ASD research (Tian, Zhang and Han, 2017; Ueoka *et al.*, 2019). However, research in *Drosophila* could cater to our understanding of genetics by revealing distinct molecular etiologies underlying ASDs but not towards complex behavioral profiles shown by humans.

1.6.4. Invertebrate models of ASD

Invertebrates like *Aplysia californica* and *C-elegans* are also being used as model systems for ASD research. *Aplysia californica* is a gastropod also called sea slug. *Aplysia*

contains a small group of neurons related to specific behaviors related to learning and memory and social behaviors. A previous study tested the effect of depletion of neurexin and neuroligin at sensory-to-motor neuron synapses of the gill-withdrawal reflex in *Aplysia* (Choi *et al.*, 2011). *Caenorhabditis elegans (C-elegans)* is a nematode consisting of simple nervous system with 302 neurons and well characterized synaptic functions (Wang, Sliwoski and Buttner, 2011). *C-elegans with Nrx-1* mutation show defective exploratory capacity, sinusoidal postural movements and gentle touch response (Calahorro and Ruiz-Rubio, 2013). However, these invertebrates are hermaphrodites and are not social animals and thus cannot match with the complex social behavioral profiles in humans.

1.6.5. Mouse models of ASD

Mus musculus is the model of choice for ASD researchers over last few decades which is reflected when one reviews vast literature on rodent models of Autism (Zoghbi and Bear, 2012; Pasciuto *et al.*, 2015; Ueoka *et al.*, 2019). The genetic tool box available to precisely generate specific gene mutation in mice made them valued preclinical models of ASD. They provide platform to study cellular, molecular, systems and behavioral manifestations of specific mutations responsible for mutations and rescue of phenotypes observed by therapeutically using novel candidate drugs. Many established assays like juvenile social interaction, three chambered test to assess deficits in sociability and social novelty recognition are presumed to be correlates of human social deficits observed in ASD population. Altered ultrasonic vocalizations in mutant mice are being considered as human correlates of speech delay and deficits. Repetitive behaviors such as grooming, jumping, marble burying etc., are considered correlates of repetitive or restrictive behaviors observed in human patients with ASD. Cognitive deficits in mice models are mirrored using Morris water maze, radial arm maze and T- maze. However, there are limitations with respect to anthropomorphizing the symptoms observed in humans to the behavioral deficits observed in mice. Firstly, the interpretation could be confounded based on the observed behavioral deficits. For example, social and

cognitive deficits observed could be due to impairment in sensory processing, altered fear and anxiety, hyperactivity, motor deficits etc. One needs to be cautious while interpreting the behavioral outcomes in these models because of cross contamination of the modalities could affect the measured parameters. Secondly, the behavioral capacities of mouse and humans are very different for example the ability of verbal recognition, recognition of facial emotions, sustained and divided attention etc., is very difficult to assess in mouse. Thirdly, the anatomy of mouse is simple compared to highly developed prefrontal cortex, fusiform gyrus etc., in humans. Lastly, the behavioral symptoms in humans is acquired by patient reports depicting the internal states but measured as an outward behavior in mice. Finally, the variability in the behavioral readouts of mice with specific mutations causing ASD between different research groups could be attributed to genetic background of mice, inbreeding, age of the mice and experimental methods and behavioral scoring methodology. Translating the findings in mice to humans is very difficult as various clinical trials have failed over the last decade. A recent example to this is failed clinical trial of mavoglurant in humans failed to be efficacious in human patients with Fragile X syndrome (Berry-Kravis *et al.*, 2016). This clinical trial was based on the mGluR theory in Fragile X mice which proposed that the absence of FMRP can lead to over activation of mGluR signaling, leading to enhanced hippocampal LTD which contributes to the features of the FXS phenotype (Bear, Huber and Warren, 2004) and subsequent studies used novel drug mavogurant which is a non-competitive inhibitor of mGluR5 rescues molecular, neuronal spine, and behavioral phenotypes in the mouse model of Fragile X syndrome (Levenga *et al.*, 2011; Gantois *et al.*, 2013; Pop *et al.*, 2014). Overall, mouse models have added a lot to our understanding of molecular and cellular basis of ASD but most studies have anthropomorphized the behavioral phenotypes as correlates for human phenotypes. The behavioral phenotypes observed in mice must pass the test of mechanistic translatability across the vertebrate-mammalian models like rats and non-human primates which are more superior.

1.6.6. Advantages of Rat models of ASD

Rat models of ASD have been generated recently which have gained traction in the field due to the novel gene editing technological advances like CRISPR/Cas 9 and zinc finger nucleases. Rats and mice are separated evolutionarily by 12 million years (Gibbs *et al.*, 2004). It is vital to establish the conserved patho-physiologies between both the mammalian species for the sake of translational applicability in humans. Though both rats and mouse are collectively called rodents, there are many ethological and evolutionary differences between the two species. Rats are less prone to experimenter-induced anxiety which facilitate in depth examination of multiple complex behaviors (Meijer *et al.*, 2007; Ellenbroek and Youn, 2016). Larger brain size of rats is advantageous for accurate surgical manipulations like microinjection, cannulation, and implantation of probes to facilitate recordings in rat pups as early as 14^{th} day of postnatal life for studying developmental changes in neural activity (Lipska *et al.*, 2002; Langston *et al.*, 2010; Zeeb and Winstanley, 2013). Further rats are approximately 10 times larger than mice and possess 25 ml of circulating blood volume compared to 1.5-2.5 ml of circulating blood volume in mice. More blood volume allows re-sampling of blood for biomarker or metabolite estimations and hence is advantageous for diagnostic preclinical tests (Parasuraman et al, 2010). Rats' larger brains have a 10 fold higher spatial resolution in PET imaging than mice, which is advantageous. (Zheng *et al.*, 2016). Additionally, compared to mice, evidence from advanced imaging techniques like resting state fMRI is less varied in rats. (Jonckers *et al.*, 2011). Moreover, the rat's metabolic physiology and biochemistry is closer to that of humans compared to mice (Goutianos *et al.*, 2015) which makes them a better model for studying drug pharmacodynamics while developing putative treatment strategies. Besides, drug efficacy studies had reported high variability in mice (Fattore, 2002; Paterson *et al.*, 2003). Moreover, neurotransmission and neuromodulation between rats and mouse appears to be different (Blanchard *et al.*, 1997; Konstandi *et al.*, 2000). Indeed, rats are better performers in behavioral tasks which makes the data to be reliable and robust than that of mice (Whishaw and Tomie, 1996; Colacicco *et al.*, 2002; Kummer *et al.*, 2014;

Ellenbroek and Youn, 2016). Most of the behavioral tests were mainly designed considering ethology of rats which were then modified for mice (Jaramillo and Zador, 2014). Temperament of mice is territorial and aggressive whereas rats are highly social with floating hierarchy and show rare aggression on conspecifics (Wöhr and Scattoni, 2013). The biological individual variance in mice is more compared to rats as they are socially rigid and more aggressive which results in great individual variation in behavior, physiology and immune function (Hendrie, Weiss and Eilam, 1996; Phifer-Rixey and Nachman, 2015). Rats show rich and complex social repertoire compared to mice, which is very evident in the form of transmission of social food preference, emotions and pain sensitivity (Kim *et al.*, 2010; Knapska *et al.*, 2010; Fanselow, 1985; Strupp and Levitsky, 1984). Whereas, mice exhibit less contact social play (Siviy and Panksepp, 2011). Further, rats show pro-social behaviors like empathy (Atsak *et al.*, 2011; Bartal, Decety and Mason, 2011; Sato *et al.*, 2015) and cognitively more flexible compared to mice (Cressant *et al.*, 2007). Hence, with all the evidences listed above rats would be a better option to model ASD than mice.

In this thesis I have evaluated the behavioral characteristics of four novel transgenic rat models of synaptopathies i.e *Nlgn3*$^{-/y}$,*Cdkl5*$^{-/y}$, *SynGAP*$^{+/-}$,*Pten*$^{+/-}$, which are known to cause ASD. I have used modified well established rigorous behavioral assays for rats from extensive behavioral neuroscience literature. I observe some convergent and some divergent behavioral profiles between the rat lines as well in comparison to their respective mice studies. Identifying stable behavioral phenotypes across the mammalian models adds value to the translational modeling of ASD. Additionally, I have also used a novel therapeutic approach to alleviate behavioral deficits in *Nlgn3*$^{-/y}$ rats. The results obtained in this study will lead to identification of mechanistic insights behind the observed behavioral deficits. Circuit specific dysfunction, molecular and cellular alterations behind the observed behavioral deficits would further help in ascertaining the pathobiology behind the specific mutations leading to ASD. Furthermore, this study provides platform to test various novel drugs to alleviate the behavioral deficits which would be a step towards bench to bedside application.

Chapter.2. Materials and Methods

2.1 Genotyping

Ear tissue was collected on postnatal day 21 from all the transgenic rats. For convenience sake the samples were sent to *Transnetyx,* Cordova, USA for genomic DNA extraction and genotype detection. See appendix for the western blots confirming the protein levels in each rat model.

2.2 Experimental animals

Rats aged postnatal day (PND) 60-90 was used for all behavior experiments. Breeder pairs for each transgenic line were procured from *Horizon Discovery*, US (Now *Inotiv.Inc*, Indiana, US). Transgenic rats were bred onto the *Sprague-Dawley* (SD) / *Long Evans* (LE) background. *Sprague-Dawley Nlgn3*$^{-/y}$ (58 base pair deletion in Exon 5) / *Pten*$^{+/-}$ (7 BP deletion in exon 7) and their respective wild type male rats were obtained from heterozygous crossings (Percie du Sert *et al.*, 2020). *Long Evans Cdkl5*$^{-/y}$ (10 base pair deletion in Exon 8)/ *SynGAP*$^{+/-}$ (1 base pair deletion in Exon 8) and their respective wild type male rats were obtained from heterozygous crossings. All transgenic rats except *Cdkl5*$^{-/y}$ were housed heterogeneously at the age of PND 30 post weaning in a combination of 2 WT + 2 KO/Het per cage. *Cdkl5*$^{-/y}$ were rats were housed homogenously i.e. 4 rats of same genotype per cage. This was done do avoid frequent fight resulting in injuries when they were housed in heterogeneous housing. Animals were kept in a 14h light/10h dark cycle and had ad-libitum access to standard diet and water. All animals were handled by the experimenter over 3 days before starting the behavioral experiments. Their body weight was monitored throughout the experimental days. All procedures involving animals were conducted in accordance with the guidelines of the CPCSEA, Government of India and approved by the Institutional Animal Ethics Committee of National Centre for Biological Sciences, Bangalore, India.

In this thesis I have investigated if the genetic perturbation in rats with *Nlgn3, Cdkl5, SynGAP and Pten* resulted in altered behavior. The term face validity is used in the field to see if these mutations results in corresponding human condition. However, Autism being a human disorder with altered behavior reflecting their internal states and measuring outward behavior in rodents cannot be justified as human correlate. Since, these synaptopathies would have developmental impact on different brain regions and their connectivity. Thus, current approach towards this should be, do the mutations in these transgenic rats affect natural spontaneous behaviors of rats which are dependent on multiple brain regions. I address this by using series of behavioral experiments which are divided over two behavioral test batteries. Behavioral test battery 1 included experiments like marble interaction test to assess repetitive behavior, object location recognition to assess long term location memory, auditory fear conditioning task to assess the emotional learning and memory, rotarod test to assess the motor co-ordination and leaning and tail flick test to assess the pain sensitivity. Behavioral test battery 2 included another series of behavioral assays like object location recognition to assess short term location memory, spontaneous alternation tasks like object recognition, object place, object context and object-place-context which are complex short term memory tests, three chambered social interaction task to assess the social behavior and active place avoidance task using rotational platform/carousel maze to assess avoidance learning and memory. These experiments were conducted in defined sequence accounting for amount of stress induced by the protocol i.e. least stressful to most stressful (McIlwain *et al.*, 2001). These experimental cohorts broadly cover tests to identify behavioral deficits in multiple domains of social, cognitive, sensory motor and repetitive behaviors which share similar neural circuitry in mammalian organisms. Thus, in some way provide mechanistic insights into behavioral deficits observed in autism/neuro-developmental disorder.

2.3. Methods

Some of the methods except object location recognition, rotarod, social interaction & object place context tasks were adapted from the manuscript titled:

> Natasha J. Anstey*, **Vijayakumar Kapgal***, Shashank Tiwari, Thomas C. Watson, Anna K. H. Toft,Owen R. Dando, Felicity H. Inkpen, Paul S. Baxter, Zrinko Kozić, Adam D. Jackson, Xin He,Mohammad Sarfaraz Nawaz, Aiman Kayenaat, Aditi Bhattacharya, David J. A. Wyllie, Sumantra Chattarji, Emma R. Wood, Oliver Hardt and Peter C. Kind. Imbalance of flight–freeze responses and their cellular correlates in the *Nlgn3*$^{-/y}$ rat model of autism. *Molecular Autism*, 2022;13:34. https://pubmed.ncbi.nlm.nih.gov/35850732/

2.3.1. Marble interaction task

Rats were habituated to open field (45 × 60 cm) arena with fresh bedding (2 inch) for 20 min on two consecutive days (**Schematic-2.3.1**). On day 3, the rats were allowed to explore the same arena with 20 equidistantly placed opaque glass marbles (6 cm) arranged in 4 rows and 5 columns, respectively. The procedure was recorded with the overhead camera and the analysis was done using Boris v 2.98 behavior analysis software (Friard and Gamba, 2016). The light intensity throughout was uniformly maintained at 20 lx. Parameters like distance travelled, digging (Duration, Latency & frequency), marble interaction (Duration, Latency & frequency) and number of marbles buried (Criteria: 75% of the surface of marble is covered by bedding) were measured.

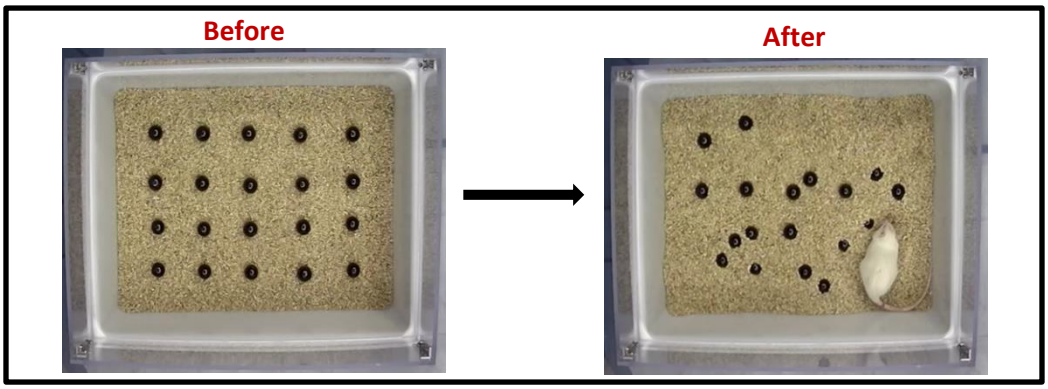


Figure.2.3.1. Schematic of marble interaction task

2.3.2. Object location recognition task - Long term memory

Rats were habituated to open field (60*60) arena with fresh corncob (2 inch) bedding on the floor and white walls. The light intensity was uniformly maintained at ~ 20 lx. Rats were allowed to explore for 10 min before returning to their home cage. This was repeated for a total of 4 days. On day 5-7, the rats were allowed to sample two identical but diagonally placed ceramic objects for accumulated exploration of both the objects for 90 seconds. This was to facilitate equal sampling of the object location in both the genotypes. Post sampling, rats were left undisturbed in their home cage for over next three days i.e. day 8-10. Further, on the day 11 the rats were allowed to probe the same arena with one of the object displaced to a novel location for 3 minutes (**Schematic-2.3.2**). Preference index was calculated using the formula (time exploring object at novel location - time exploring object at old location/ total exploration time). Both after sampling and probe trials the animals were placed in a separate cage. Rats were re-introduced into their home cage an hour after sampling or probe trials. The procedure was recorded with the overhead camera and the analysis was done in Z score behavior analysis software (Courtesy: Prof. Oliver Hardt, University of McGill, Canada).

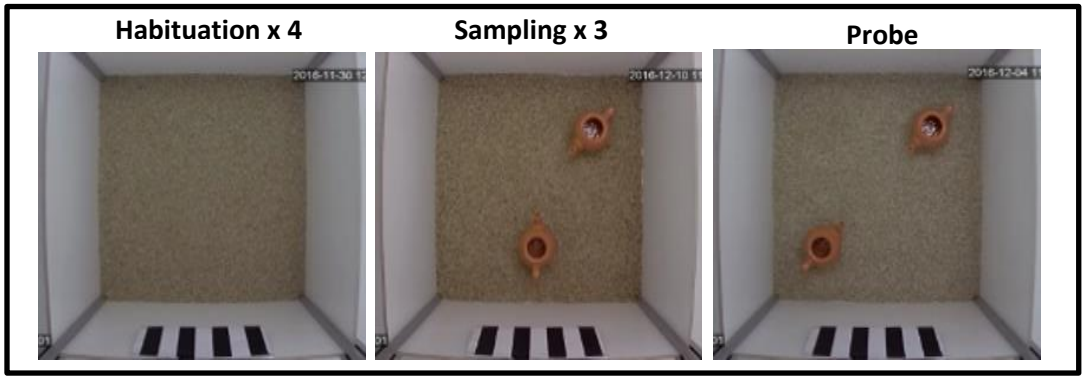


Figure.2.3.2. Schematic of the object location recognition task

2.3.3. Fear conditioning

2.3.3.1. Auditory fear conditioning, recall- extinction and extinction recall

Fear conditioning (context A, aluminum fear conditioning chamber with grid flooring, black/white horizontal-striped cue, and ~ 5 lx blue light) and recall (context B, 35 cm wide, 20 cm deep, 40 cm high arena with fresh bedding, mint odor, ~ 20 lx yellow light, and a transparent Perspex lid) took place in sound isolation cubicles (Coulbourn Instruments, Whitehall, Pennsylvania, USA).The behavior of the animals was recorded using a video camera and a frame grabber (30 Hz sampling). The apparatus was cleaned with 70% ethanol before and after experiments. Context habituation involved exploration of context B for 20 min on 2 consecutive days (**Schematic 2.3.3.1A**). On day 3, the rats were subjected to auditory fear conditioning in context A. After a baseline exploration time of 2 min, rats were presented with 3 pairings of conditioned stimulus (CS) (continuous tone, 30 s, 5 kHz, 75 dB) co-terminating with a scrambled foot-shock (unconditioned stimulus, US, 0.9 mA for 1 s, Habitest system, Coulbourn Instruments, Whitehall, Pennsylvania, USA). Each CS–US pairing was separated by inter-tone interval (ITI) of 1 min (modified from [69]. On days 4 and 5, to determine fear memory recall and extinction, rats were given 2 min to explore context B, then presented with 13 CS, with a 30 s ITI. Fear behavior was evaluated during pre-tone, tone, and ITI (**Schematic-2.3.3.1B**). Experimental videos were scored for freezing behavior using the BORIS software v2.98 (Friard and Gamba, 2016).

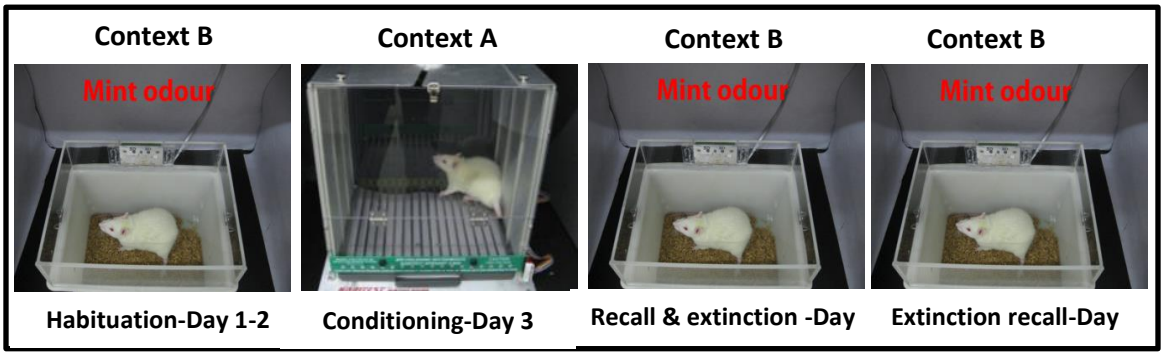


Figure.2.3.3.1A. Schematic of auditory fear conditioning apparatus

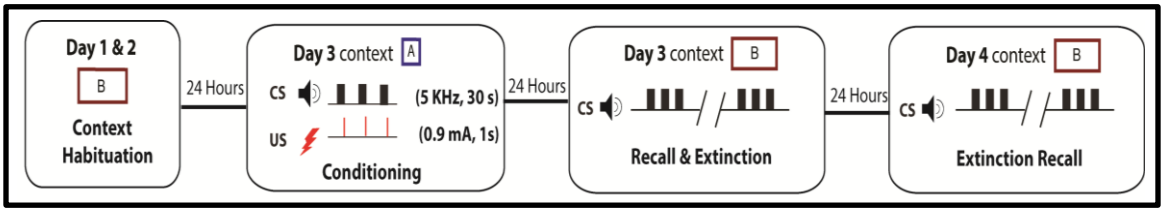


Figure.2.3.3.1B. Schematic of auditory fear conditioning protocol

2.3.3.2. Contextual fear conditioning

Rats were introduced to context A and given 2 min to explore. They were then presented with 3 unconditioned stimuli (US) of 0.9 mA scrambled foot-shock for 1 s, with a 90 s ITI. The following day, rats were reintroduced to context 'A' and were allowed explore for the durations 10 minutes (**Schematic 2.3.3.2A & 2.3.3.2B**). Experimental videos were scored for freezing behavior using the BORIS software v2.98 (Friard and Gamba, 2016).

Note: Contextual fear conditioning was only assessed in *Nlgn3*$^{-/y}$ rats.

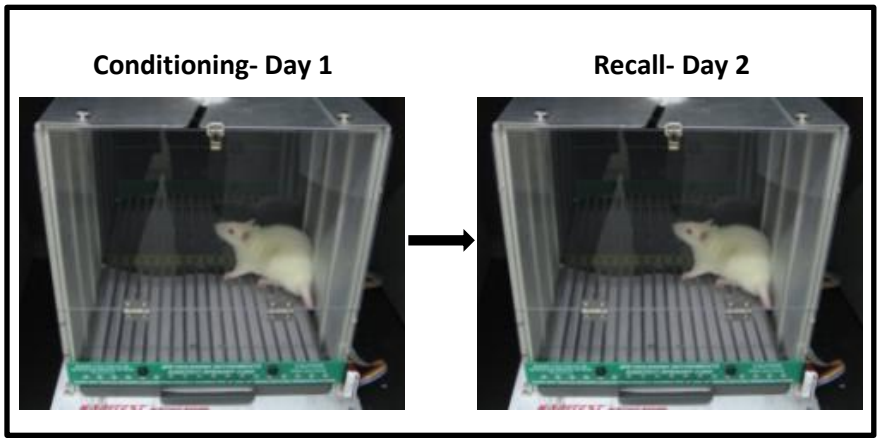


Figure.2.3.3.2A. Schematic of contextual fear conditioning apparatus

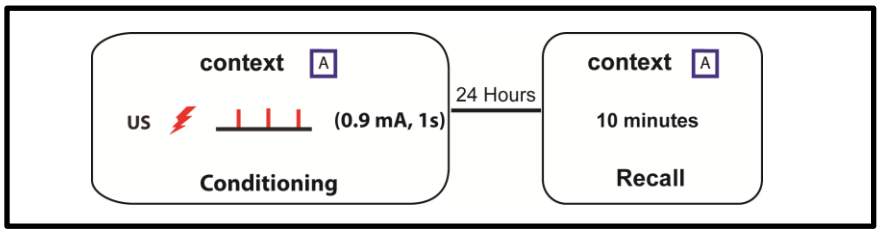


Figure.2.3.3.2B. Schematic of contextual fear conditioning protocol

2.3.3.3. Shock-ramp test

Rats were placed within context A from the fear conditioning task. The rats were given 2.5 min to explore their environment, and then were presented with 3 scrambled foot-

shocks (0.06 mA, 1 s, 1.5-min intervals). After a further 1.5-min interval, a further 3 scrambled foot-shocks were given with the intensity increased to 0.1 mA (1 s, 1.5-min intervals). This was repeated with the foot-shock intensity increasing in increments (0.2, 0.3, 0.5, 0.7, 1 mA). Following this, after another 1.5-min interval the foot-shock amplitude was then dropped back to 0.1 mA and again 3 scrambled foot-shocks were given (1 s, 1.5-min intervals) (**Schematic 2.3.3.3**). Experimental videos were scored for parameters like paw withdrawal, backpedalling, forward or backward running, and jumping behaviors.

Note: Shock ramp test was only assessed in *Nlgn3*$^{-/y}$ rats.

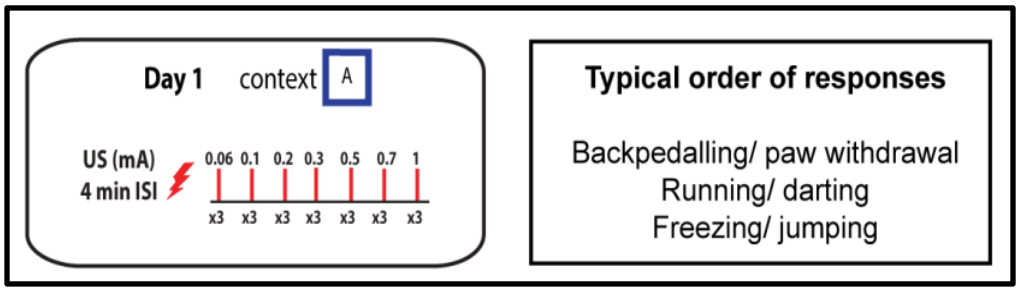


Figure.2.3.3.3.Schematic for shock ramp test

2.3.3.4. Short term fear recall and rescue of freezing deficits by IGF in Nlgn3$^{-/y}$ *rats*

Rats were placed in Context A for 20 min during habituation session. The next day (day2), post baseline of 3 minutes in the chamber, rats were subjected to tone habituation where rats were exposed to 3 CS (75 dB, 20 s) presentations before they were conditioned with 6 presentations of same CS (75 dB, 20 s, pretone-20 s) co-terminating with a foot shock (US, 0.6mAmp, 500 ms; pseudo-randomised inter-trial interval (ITI) of 70 sec). On day 3, the rats were placed in a different context (B) for ~ 9 min for recall protocol (3 min baseline, 20 sec pre-tone, 3 x CS of 20 sec each; pseudo-randomised ITI of 90-110 sec) (**Schematic-2.3.3.4**). For separate set of rats short term recall of fear memory was tested on day 2 after 6 hours after conditioning. Experimental videos were scored for freezing behavior using the BORIS software v2.98 (Friard and Gamba, 2016).

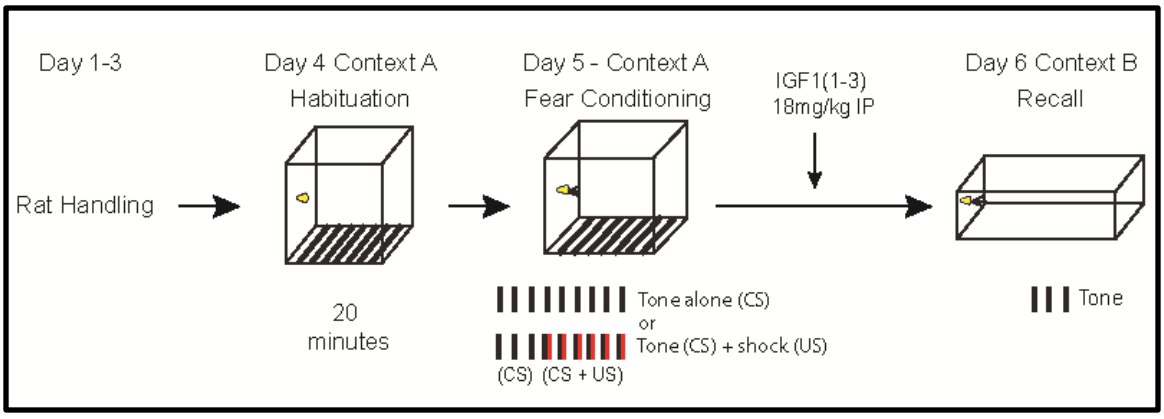


Figure.2.3.3.4 Schematic of auditory fear conditioning protocol

2.3.3.5. IGF1 (1-3) intra-peritonial injections

Injection dosage and strategies were adapted from a study from Baker and colleagues (Baker *et al.*, 2005). Commercially available IGF1 (1-3) (Bachem, cat # 4026104) was dissolved at a concentration of 18mg/ml in freshly prepared ice cold 0.1% BSA in sterile saline. A dose of 30mg/kg body weight was administered intraperitoneally at 45 mins before the recall experiment. All subsequent experimental conditions and protocols remained the same.

2.3.4. Motor coordination in Rotarod

Motor coordination was assessed in the accelerating Rotarod task using Rotamex-5^{TM} (Columbus Instruments, USA). Rotarod consists of rotating spindle (diameter- 7 cm) with 4 lanes separated by circular plastic walls. The floor was 48.3 cm below the spindle. Fall of the subject was detected by infrared beam sensors located on either side of the spindle. The rotational speed of rotarod spindle is controlled by system software. The latency to fall in both tests was measured to assess motor coordination and motor skill learning.

Rats were tested on Rotarod for 5 consecutive days. Rotarod training consists of two phase's i.e base line (constant rotation of 4 RPM) and accelerating (4-40 RPM). Rats

were rested for 30 minutes between these two phases. Baseline phase included two trials (90 s/trial, ITI 45 s). Rats were placed on constantly rotating spindle and the latency to fall from the spindle was recorded. Accelerating phase included 4 trials (90 s/trial, ITI 45 s). Each trial begins with placing the rats on the spindle. The spindle rotation speed was accelerated in a step of 4 RPM/8 s until it reached the maximum of 40 RPM (**Schematic-2.3.4**). Latency to fall and maximum RPM reached were recorded.

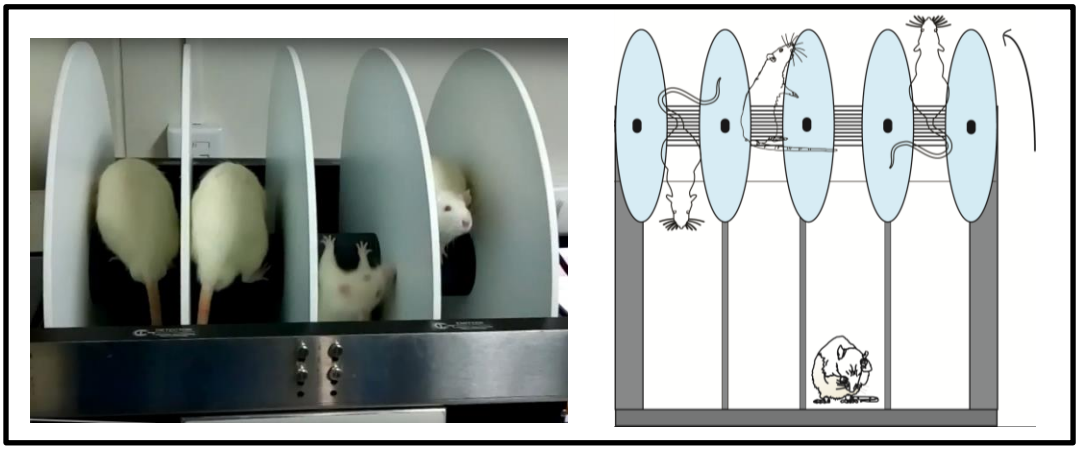


Figure.2.3.4. Schematic of Rotarod apparatus

2.3.5. Acute pain sensitivity in tail-flick analgesiometer

Thermal pain sensitivity was assessed using tail-flick analgesia meter (Columbus Instruments, USA) (**Schematic-2.3.5**). The apparatus has flat platform with a slot for the tail beneath which there is a shutter-controlled lamp as a heat source. The foot switch allows the lamp to be continuously illuminated so that the heat source provides a constant temperature to the animal's tail. The right intensity of light beam was determined to avoid tissue damage at high intensity or indefinite time to flick the tail at low intensity. Right intensity was determined which caused discomfort resulting in tail flick. Rats were first habituated in a rat strainer for three consecutive days (10 mins each) and tested with Tail flick analgesia-meter on fourth day (5 trials, 60 sec ITI). The animal's tail was kept gently over a tail slot the latency to flick the tail was measured.

Care was taken to avoid exposure of same area of tail again as it would to lead to sensitization of the thermo receptors in that area.

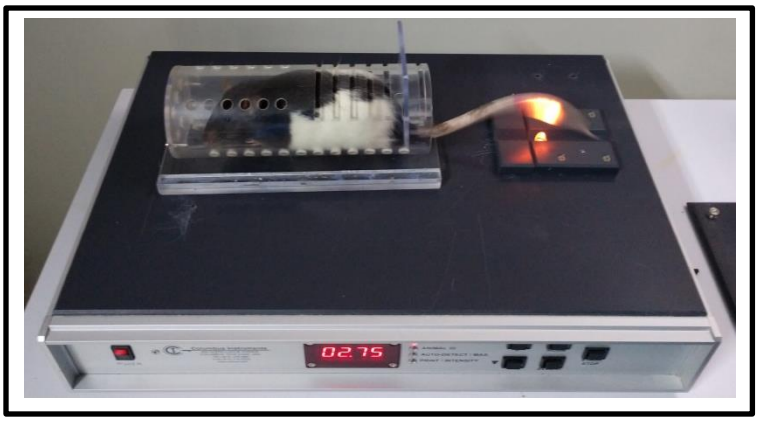


Figure.2.3.5. Tail flick analgesiometer apparatus

2.3.6. Object location recognition task – Short term memory

Rats were habituated to open field (60*60) arena with fresh corncob bedding (2 inch) on the floor and white walls. The light intensity was uniformly maintained at ~ 20 lx. Animals were allowed to explore for 10 min before returning to their home cage. This was repeated for a total of 4 days. On day 5, the rats were allowed to sample two identical but diagonally placed ceramic objects for 5 minutes. Post sampling, the rats were separately kept in a new cage for an hour. Further, the rats were allowed to probe the same arena with one of the object displaced to a novel location for 3 minutes. In between trials the objects and the arena were cleaned with 70% ethanol, fecal pellets were removed and the bedding was reshuffled to minimize the olfactory cues. The procedure was recorded with the overhead camera and the analysis was done in Z score behavior analysis software (Courtesy: Prof. Oliver Hardt, University of McGill, Canada). Refer OLR-LTM for apparatus.

2.3.7. Three chambered social interaction task

Sociability and social novelty was assessed in three chambered social interaction arena. The apparatus is a rectangular three-chambered box with transparent polycarbonate walls. Each chamber is 19 x 45 cm with an open middle section, which allows free access to each chamber. Two identical, wired cage like containers which are large enough to hold a single animal (naive/unfamiliar), one on each side were used. Test rats were habituated (2 minutes for center chamber +10 minutes for all chambers) to the arena without wired cage on first day and with wired cages in the right and left boxes for next 3 days. Stranger rats which were non littermates and at least 1 month younger compared to test rats were used for the task. These rats were also habituated to the wired cages only on day 1 and over 3 days with cage mates and dummy rats (age same as test rats) respectively. Testing was done on 5th day which included 2 phases. Phase 1 was to measure sociability (preference towards stranger animal vs novel object) and phase 2 was to measure social novelty (preference towards novel vs familiar rat). Each phase lasted for 12 minutes and inter phase interval was 5 minutes (**Schematic-2.3.7**). The apparatus was thoroughly cleaned in between phases and trials to eliminate the odor. The procedure was recorded with the overhead camera and the analysis was done in Z score behavior analysis software (Courtesy: Prof. Oliver Hardt, University of McGill, Canada).

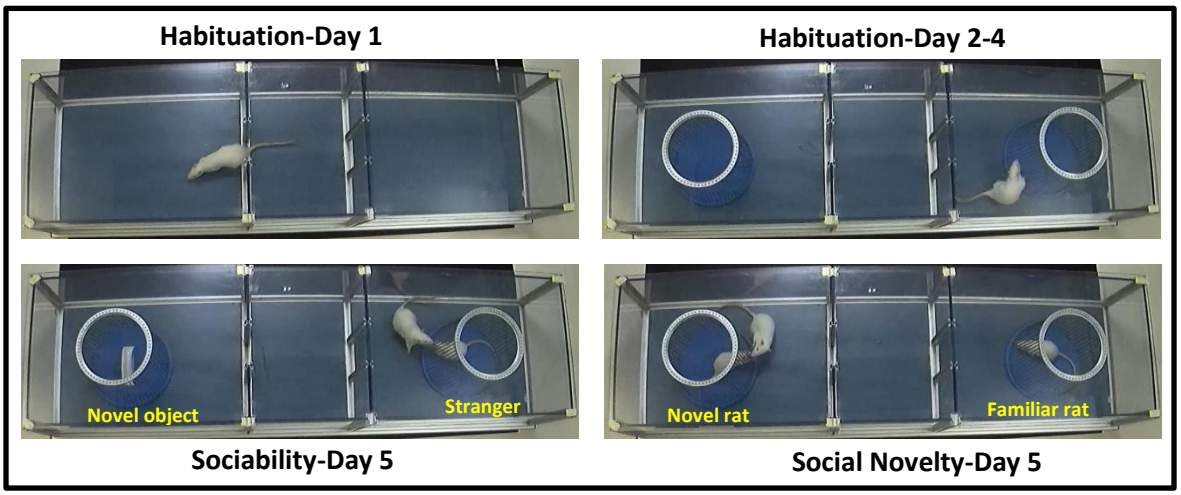


Figure.2.3.7.Schematic of three chambered social interaction task

2.3.8 Object-Place-Context Task

Spontaneous exploration tasks included group of behaviors which needs intact working memory for identification of novel object, place, context and object-place-context. All these tasks are object based novelty driven tasks. Objects were made of ceramic, metal, glass and plastic. Objects were distinct with respect to their shape, size and texture. None of the objects were repeated for a rat over different iterations. The rats were tested in square arena (60*60) made of black wooden floor & wall inserts or textured floor or wall inserts (**Schematic-2.3.8**). These tasks were adapted from (Langston and Wood, 2009; Till *et al.*, 2015; Asiminas *et al.*, 2019). Apparatus and objects were cleaned with 70 % ethanol after sampling and testing phase of the task. Behavior was recorded using overhead camera. Offline analysis was done using Zscore behavioral analysis software (Courtesy: Prof. Oliver Hardt, University of McGill, Canada) by an experimenter who was blind to genotype of rats and novel object identity.

2.3.8.1 Habituation

Rats were brought to the experimental room 30 minutes before the commencement of experiment. Rats were habituated to the two different contexts (black & textured floor/wall inserts) for 10 minutes over 4 days.

2.3.8.2 Object recognition (OR)

Rats were allowed to explore identical pair of objects for 5 minutes. Post sampling rat was placed in an opaque bucket with bedding material for the duration of 5 minutes. During the testing phase, one of the objects was replaced with a novel object and the exploration was measured over 3 minutes.

2.3.8.3 Object place (OP)

Rats were allowed to explore different objects for 5 minutes. Post sampling rat was placed in a opaque bucket with bedding material for the duration of 5 minutes. During

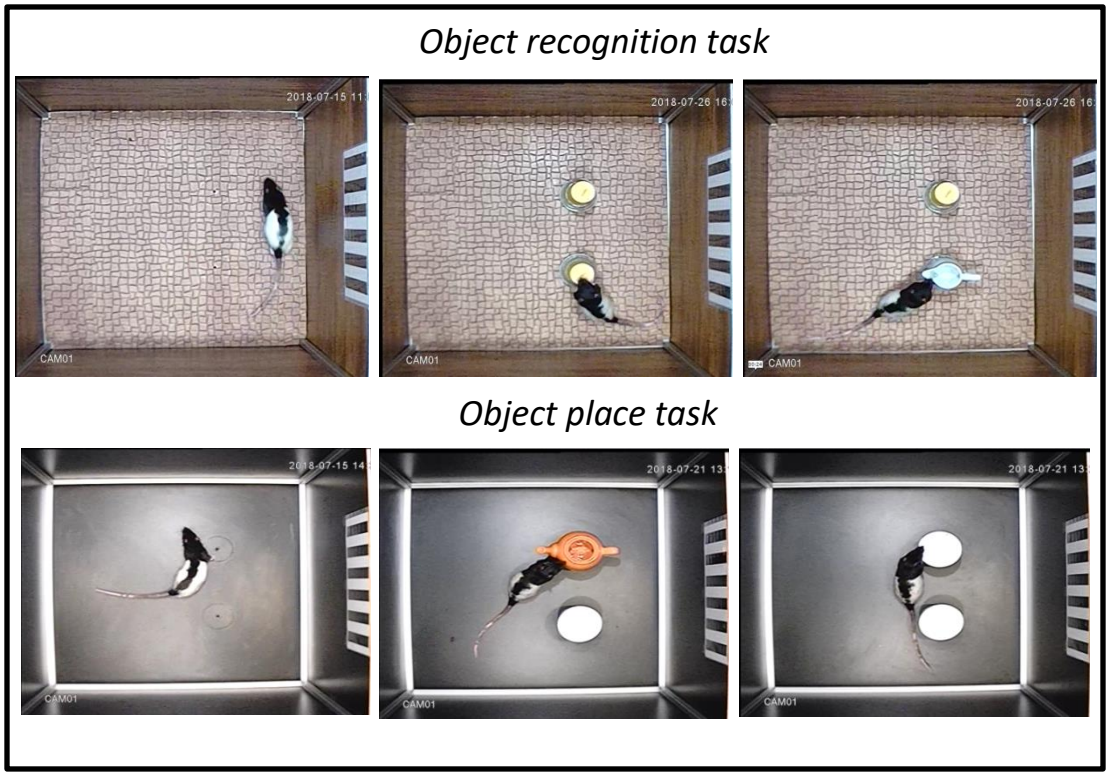
testing one of the objects was replaced with identical copy of the remaining object and exploration of rat was measured over 3 minutes.

2.3.8.4 Object context (OC)

Rats were allowed to explore identical sets of objects in arenas with two different contexts (black/textured). Post sampling rat was placed in an opaque bucket with bedding material for the duration of 5 minutes. During testing rats were allowed to explore the context where one of the identical objects was replaced with the object from the other context.

2.3.8.4 Object-place-context (OPC)

Rats were allowed to explore pair of non-identical objects in two different contexts. Care was taken to flip the position of the objects in both the contexts. Post sampling rat was placed in an opaque bucket with bedding material for the duration of 5 minutes. During testing rats were allowed to explore the context where the rat has not seen object in that particular place and context.



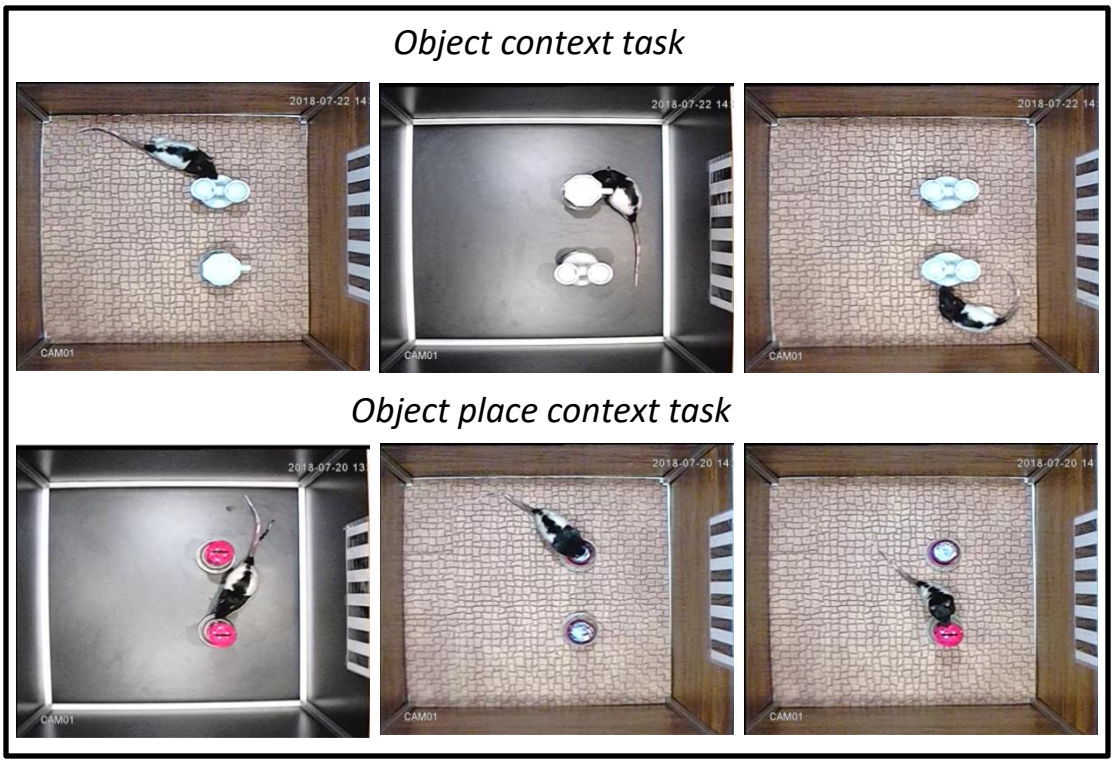


Figure.2.3.8. Schematic of spontaneous alternation tasks apparatus

2.3.9 Active place avoidance task

The rotating platform (Bio-signal group, Brooklyn, USA) (**Schematic-2.3.9.A**) has a rectangular grid floor 100 × 100 cm) connected to a constant DC current source box for shock delivery. This was on a circular aluminum base (90 cm above ground) and run by an arena motor. A circular fence made of transparent Perspex surrounded the platform (diameter: 77 cm, height: 32 cm). A transparent lid was placed on top of the circular fence. The delivery of foot-shocks (0.2 mA, 500 ms, and 1500 ms inter shock interval) was tracking based (Carousel Maze Manager). The 60° shock-zone was located on either North or South region and counterbalanced between rats. External to the arena, 3-dimensional cues were located at different distances from the apparatus. Rats were held in a cabinet for 30 min before experimentation. The protocol was adapted from (O'Reilly *et al.*, 2014). On day 1, rats were habituated to the rotating arena (1.5 RPM, 2 trials, 10-min interval in opaque bucket). The following day, rats were given two training sessions over two consecutive days (8 trials per session, 10-min intervals) in which the

shock-zone was active. On day 4 a single probe trial was given to animals without shock zone to assess their avoidance memory. Post probe trial, the rats were subjected to reversal learning in a conflict session (8 trials, 10 min ITI) where the location was switched to opposite side of the previous location (**Schematic-2.3.9.B**). An overhead ceiling camera (Firewire) connected to a frame grabber (DT3155) recorded and digitized analogue video, feeding it to the tracker software (Bio-signal group, Brooklyn, USA). Post-acquisition, files were analyzed in Track Explorer software package (Bio-signal group, Brooklyn, USA).

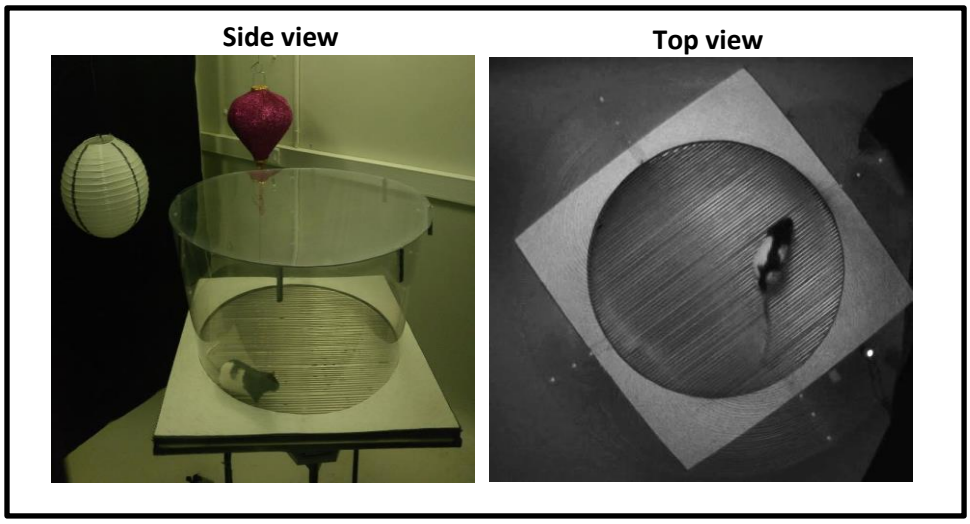


Figure.2.3.9.A. Schematic of rotational platform apparatus

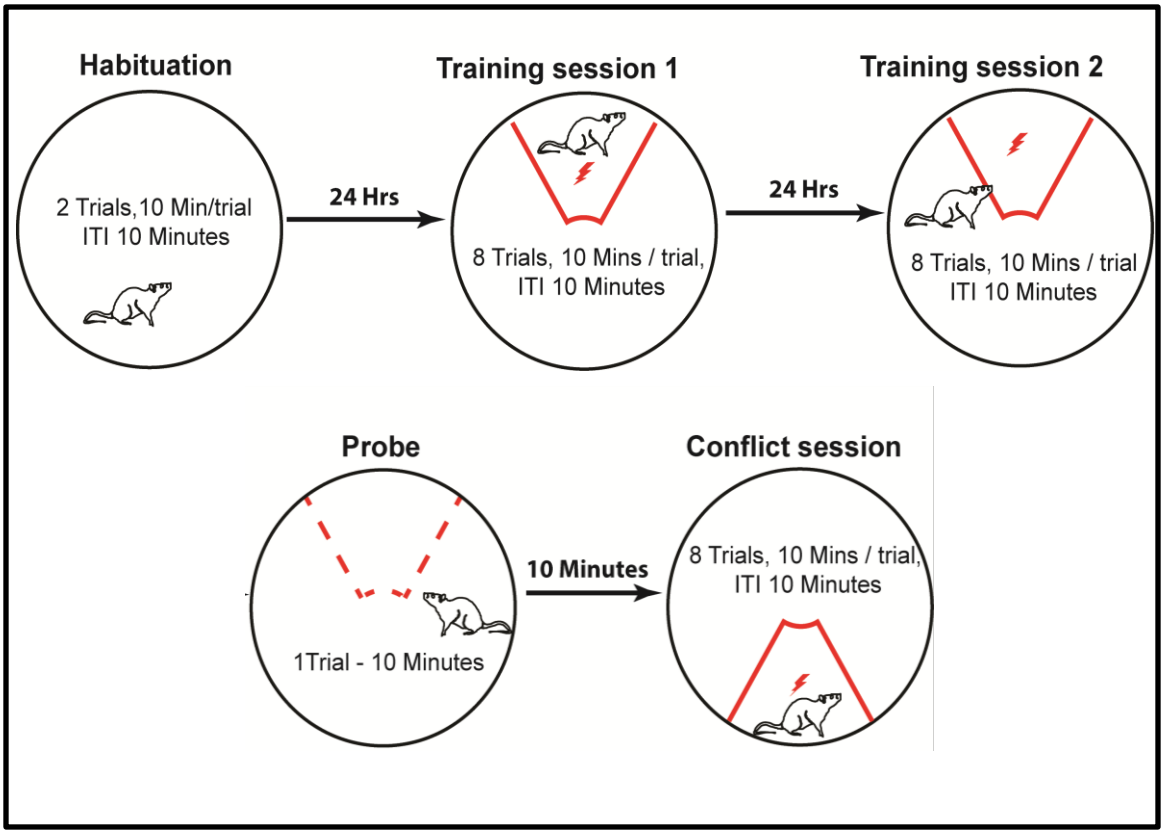


Figure.2.3.9.B. Schematic of active place avoidance task protocol.

Chapter.3. *Nlgn3*$^{-/y}$ rats show imbalance in freeze-flight responses, enhanced motor co-ordination and intervention with IGF1 rescues the fear response

Mutations in genes coding for cell adhesion molecule neuroligin-3 was first detected in twins with ASD by Jamain and colleagues (Jamain *et al.*, 2003). Point mutations in NLGN3 have since been shown to be associated with ASD/ID in several studies. The majority of *Nlgn3* mutations identified in humans resulted in complete or near-complete loss of the NLGN3 protein. NLGN3 is a scaffolding protein expressed at both excitatory and inhibitory synapses where it plays a key role in synaptic development, function, and maintenance. Mouse models of both null and point mutations in *Nlgn3* lead to behavioral phenotypes as well as alteration in synaptic function and plasticity, although the precise nature of these phenotypes differs in a mutation-specific manner. Loss of *Nlgn3* in mice led to circuit specific synaptic impairment in nucleus accumbens resulting in better acquisition of repetitive motor patterns in rotarod (Rothwell *et al.*, 2014). More recently, an *in vivo* study in Nlgn3 KO mice demonstrated an increase in excitability of neurons in CA2 linked to social cognition deficits, raising the intriguing possibility that mutations in Nlgn3 could alter the intrinsic physiology of neurons (Modi *et al.*, 2019). Phenotypic variance observed in *Nlgn3* mouse models has been attributed to the background of the mouse in which it was bred (Jaramillo *et al.*, 2018). Other models available to study autism include drosophila, zebra fish, prairie voles, rats and non-human primates. Each have their own advantages and disadvantages but are extremely important for the translatability and understanding the neurobiological mechanism leading to the observed phenotypes. In our study we have used rats as the model system because of their social complexity, cognitive superiority, offer more resolution for imaging because of the bigger brain tissue, metabolic similarity to humans and being preferred model for pharmacological testing (Knapska *et al.*, 2010; Bartal, Decety and Mason, 2011; Ellenbroek and Youn, 2016). In this thesis I have investigated if the genetic perturbation in rat *Nlgn3* resulted in behavioral alterations in domains such

as social, cognitive, motor and repetitive behaviors. The term face validity is used in the field to see if these mutations results in corresponding human condition. However, current approach towards this should be, do the mutations in *Nlgn3* affect natural spontaneous behaviors of rats which are dependent on multiple brain regions? This is because; human internal states like emotions are verbal notifications from parents and teachers in case of ASD patients. Behavioral traits like verbal and non-verbal communication, eye contact, various types of executive functions etc., cannot be quantified in rodents and it is difficult to replicate every aspect of the human disease (de la Torre-Ubieta *et al.*, 2016). So, instead of anthropomorphizing it is wise to look for species specific behavior which involves similar mammalian neural circuitry. I address this by using series of behavioral experiments which are divided over two cohorts. Behavioral test battery 1 included experiments like marble interaction test to assess repetitive behavior, object location recognition to assess long term location memory, auditory fear conditioning task to assess the emotional learning and memory, rotarod test to assess the motor co-ordination and learning and tail flick test to assess the pain sensitivity. Behavioral test battery 2 included another series of behavioral assays like object location recognition to assess short term location memory, spontaneous alternation tasks like object recognition, object place, object context and object-place-context which are complex short term memory tests, three chambered social interaction task to assess the social behavior and active place avoidance task using rotational platform/carousel maze to assess avoidance learning and memory. These experimental cohorts broadly cover most behavior tests to screen for autism-like behavioral traits in rats.

3.1. Nlgn3$^{-/y}$ rats do not show anxiety-like & repetitive/restrictive behaviors in marble interaction task

I first wanted to assess if *Nlgn3*$^{-/y}$ rats show any baseline anxiety like behavior or do they display repetitive behavior in the marble interaction/ burying assay. These rats were habituated to the marble interaction arena for over two days. On test day both WT and

Nlgn3$^{-/y}$ rats were released in same arena but with marbles arranged in 4 x 5 grid. This investigation revealed no significant difference in total number of marbles buried (**Fig. 3.1.A.** WT: 2.08±0.45 KO: 1.41 ± 0.51), marble interaction time (**Fig.3.1.B**; WT: 41.6±10.32s, *Nlgn3*$^{-/y}$: 96.01± 27.78 s), marble interaction frequency (**Fig.3.1.C.** WT: 15±1.96, *Nlgn3*$^{-/y}$: 24.33±4.34), latency to dig (Fig.1D WT: 224.6±73.23s, KO: 235.4±40.14s) and time spent in digging (**Fig.3.1.E.** WT: 31.82±6.53s, *Nlgn3*$^{-/y}$: 35.49±11.83). With all the parameters observed the *Nlgn3*$^{-/y}$ rats did not show repetitive interaction with marbles / compulsive burying compared to control rats.

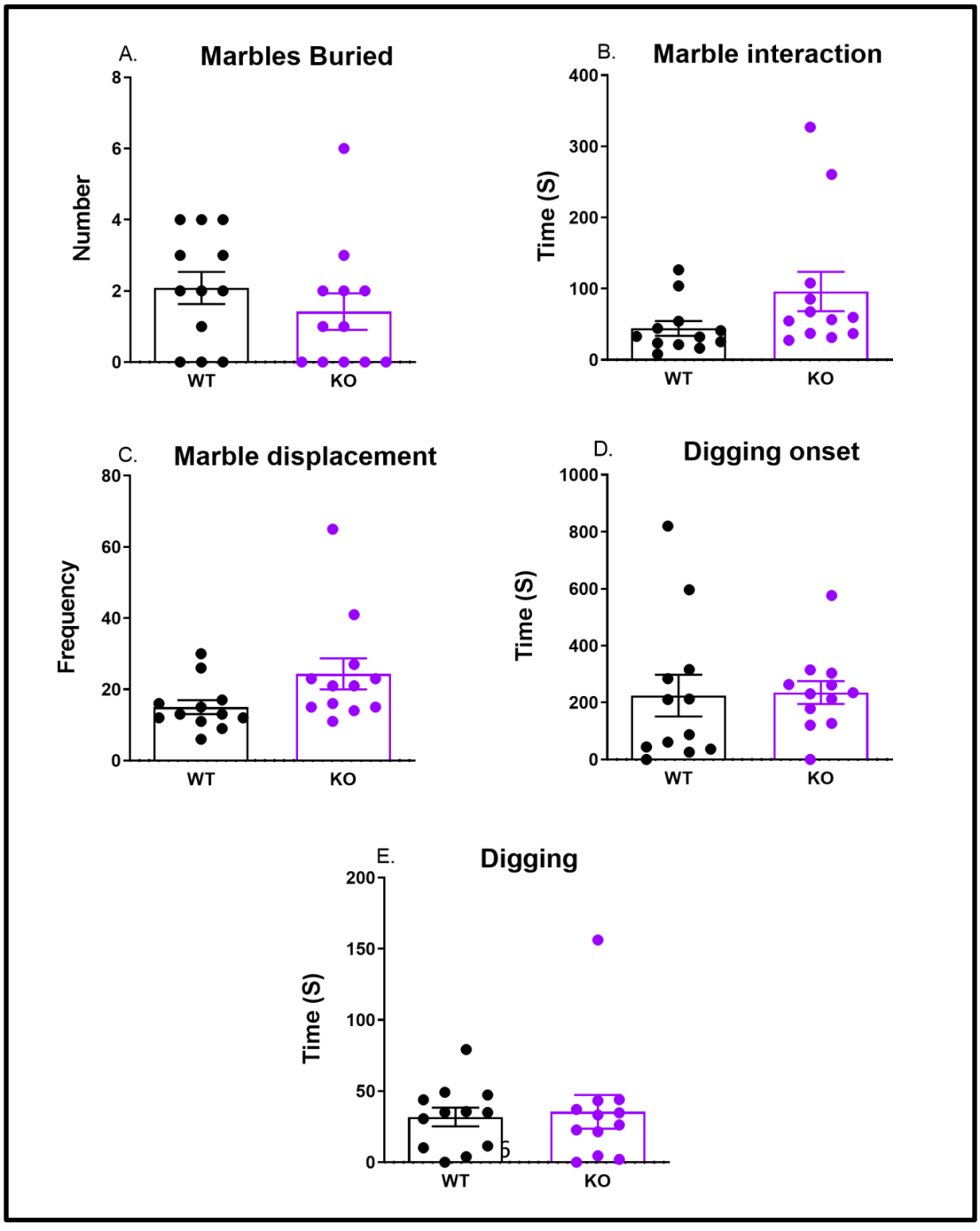
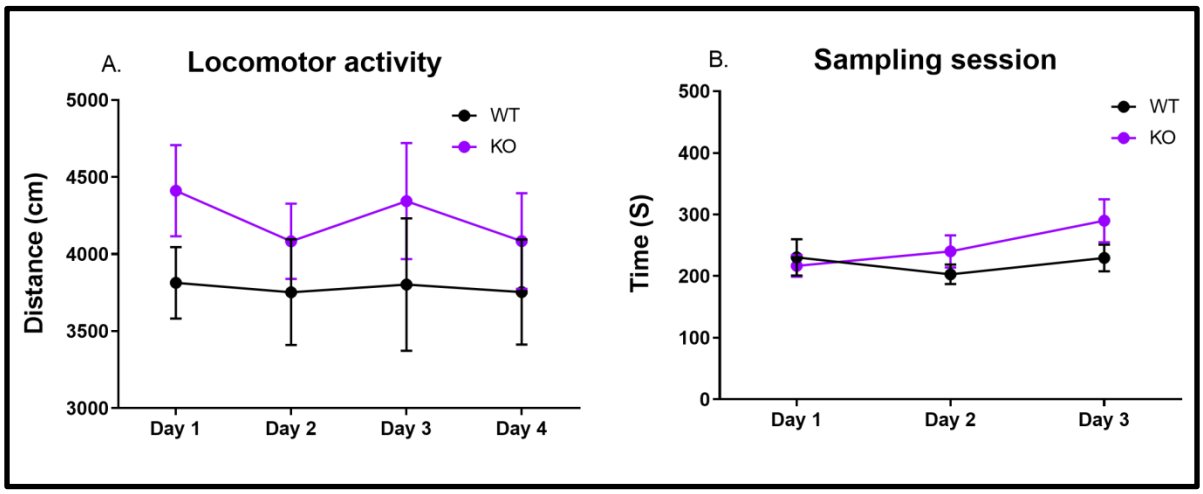


Figure.3.1. Anxiety-like behavior/ repetitive behaviors were comparable between WT and *Nlgn3*$^{-/y}$ rats. There was no significant difference in A. Marbles buried (Mann Whitney test, p=0.22 (Normality test: D'Agostino & Pearson test, alpha≠0.05 for *Nlgn3*$^{-/y}$ rats). B. Marble interaction time (Unpaired t test, p=0.26). C. Marble displacement frequency (Unpaired t test, p=0.06). D. Digging Onset (Unpaired t test, p=0.89) and E. Time spent in digging (Unpaired t test, p=0.78) between *Nlgn3*$^{-/y}$ and WT rats. WT, N=12 rats, *Nlgn3*$^{-/y}$ rats, N=12 rats.

3.2. Nlgn3$^{-/y}$ *rats show intact spatial learning and memory in object location recognition task*

Next I wanted to assess spatial learning and memory using object location recognition task. Rodents have a spontaneous explorative tendency to spend higher time with familiar but displaced object from its previous location. The behavioral analyses revealed comparable exploration in both WT and *Nlgn3*$^{-/y}$ rats during sampling session of objection location recognition task (**Fig.3.2.A**). Further, also show similar preference to the object at novel location (Fig.3.2.B. WT: 0.11±0.05 and *Nlgn3*$^{-/y}$:-0.01±0.04). Therefore, *Nlgn3*$^{-/y}$ rats show normal learning and memory in long-term object location recognition task.



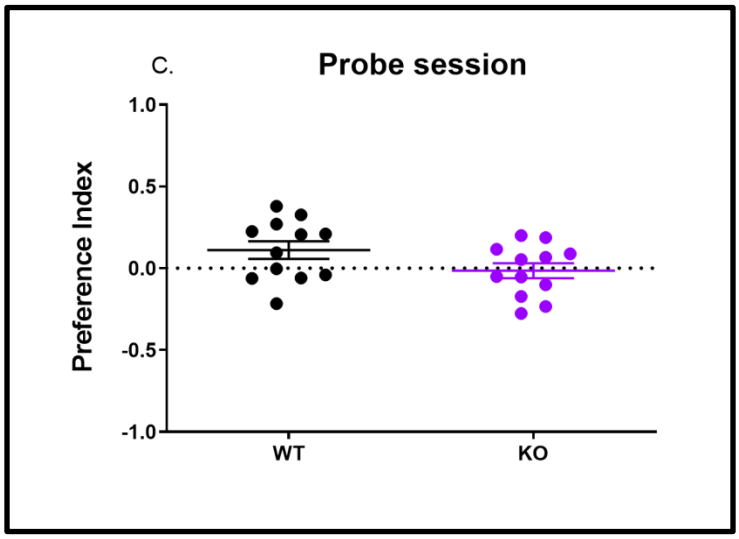
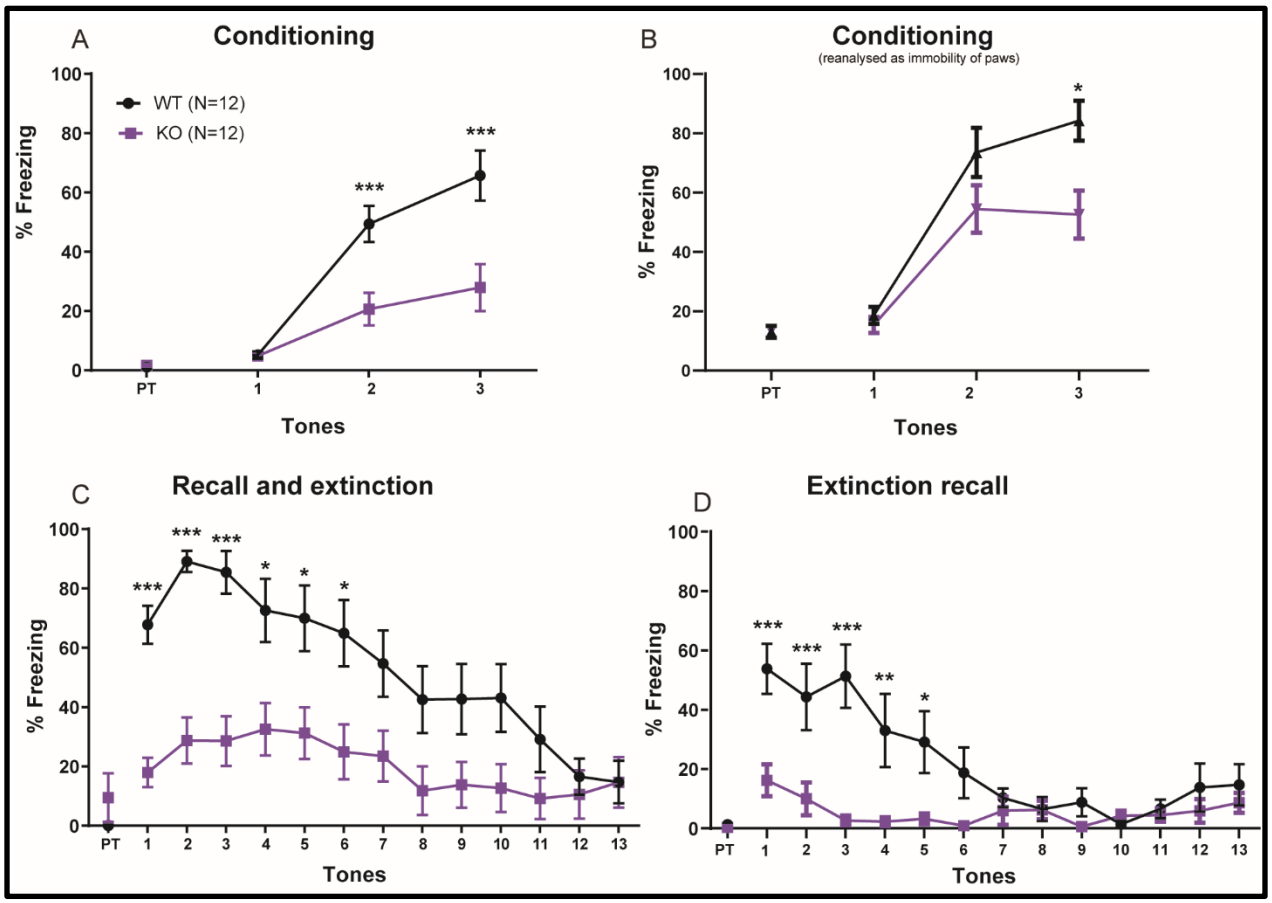


Figure.3.2. Spatial learning and memory is similar in both WT and Nlgn3$^{-/y}$ rats. A. WT and *Nlgn3*$^{-/y}$ rats showed similar habituation profile (Repeated measures two way ANOVA, Genotype: $F_{(1,22)}$=1.84, p=0.28; Interaction: $F_{(3,66)}$=0.33. B. WT and *Nlgn3*$^{-/y}$ rats explored the objects equally during sampling session (Repeated measures two way ANOVA followed by Sidak's multiple comparison test. Days: $F_{(2,44)}$=2.01 Genotype: $F_{(1,22)}$=1.19, p=0.28; Interaction: $F_{(2,44)}$=1.56. C. Preference for object placed at novel location was similar between WT and *Nlgn3*$^{-/y}$ rats. Unpaired t-test, p=0.08. WT, N=12 and *Nlgn3*$^{-/y}$, N=12

3.3-3.4. Nlgn3$^{-/y}$ rats exhibit reduced classic freezing behavior during auditory and contextual fear conditioning tasks

Further I evaluated the effect of genetic manipulation on emotional learning and memory in *Nlgn3*$^{-/y}$ rats. To study emotional learning and memory we employed Pavlovian fear conditioning tests which included auditory and contextual fear conditioning tests. Interestingly, *Nlgn3*$^{-/y}$ rats showed reduced freezing response to auditory cue during all the phases of the auditory fear conditioning task (**Fig.3.3.A, C&E**).

For statistical details refer to Table. 1. Furthermore, similar reduction in freezing response was seen during contextual fear conditioning task (**Fig.3.4.A&B**). Whilst *Nlgn3*$^{-/y}$ rats did not exhibit high levels of freezing during all the phases of fear conditioning, defined as no movement except for breathing, they did appear to respond to the tone by decreasing their overall movement. Therefore, we redefined fear response as immobility of the paws and torso but allowing for head movements. This reanalysis revealed that *Nlgn3*$^{-/y}$ rats show a more similar fear learning and extinction profile to WTs, although still significantly reduced (**Fig. 3.3.B & E**). Examination of freezing and paw immobility during the first 5 CS presentations shows significantly reduced recall in *Nlgn3*$^{-/y}$ rats relative to WTs (**Fig.3.3F**). However, *Nlgn3*$^{-/y}$ rats display a significantly higher response to the CS when considering immobility of paws only in comparison with classic freezing (**Fig.3.3F**). This effect was not seen in WT animals (**Fig.3.3.F**). Similarly, the freezing response of *Nlgn3*$^{-/y}$ rats during contextual fear recall was increased when we reanalyzed the freezing including head movements although significantly reduced (**Fig.3.4.C**). These findings indicate that *Nlgn3*$^{-/y}$ rats, despite showing reduced freezing behavior, still form the association between tone and shock but may be expressing their fear in a different manner.



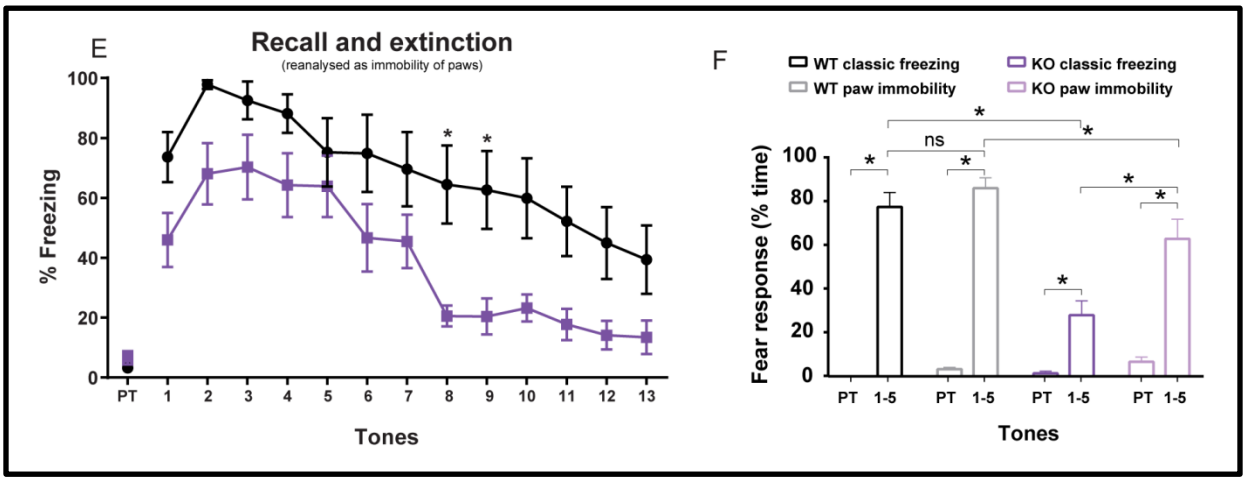


Figure.3.3. *Nlgn3*$^{-/y}$ rats display reduced classic freezing behavior in an auditory fear conditioning paradigm. A. *Nlgn3*$^{-/y}$ rats show less classic freezing behaviors during the conditioning phase ($p < 0.0001$, $F_{(1, 22)} = 6.61$, repeated measures two-way ANOVA). PT: Pre-tone. B. *Nlgn3*$^{-/y}$ rats display less paw immobility response compared to WT rats during conditioning phase of auditory fear conditioning task ($p = 0.008$, $F_{(1, 22)} = 8.333$, repeated measures two-way ANOVA. C *Nlgn3*$^{-/y}$ rats show less classic freezing behaviors during the recall and extinction phase ($p = 0.001$, $F_{(1, 22)} = 13.36$, post hoc two-way ANOVA, WT n = 12, KO n = 12). D. *Nlgn3*$^{-/y}$ y rats show reduced classic freezing behaviors during the second extinction phase ($p = 0.0009$, $F_{(1, 22)} = 14.61$, repeated measures two-way ANOVA). E. When analyzed as "immobility response" (all four paws unmoving but allowing for movement of head and neck) *Nlgn3*$^{-/y}$ rats show significantly increased response to CS in comparison with classic freezing scoring ($p = 0.004$, $F_{(1, 22)} = 13.31$, post hoc two-way ANOVA). WT rats also show significantly increased paw immobility response in comparison with classic freezing behavior ($p = 0.019$, $F_{(1, 22)} = 7.58$, post hoc two-way ANOVA). Expression of paw immobility response behavior is significantly lower in *Nlgn3*$^{-/y}$ rats in comparison with WT ($p < 0.0001$, $F_{(1, 22)} = 3.26$, post hoc two-way ANOVA). F. Percentage time exhibiting a fear response (defined as either classic freezing (black, purple) or immobility of paws (grey, pink) for pre-tone and average of tones 1–5 of recall shows a significant interaction between genotype, method of scoring, and presence of CS ($p = 0.012$, $F_{(1, 22)} = 7.52$, three-way ANOVA). Both WT and *Nlgn3*$^{-/y}$ rats display significant response to the CS (WT classic freezing: $p <$

0.0001, WT paw immobility: $p < 0.0001$, KO classic freezing: $p = 0.008$, KO paw immobility: $p < 0.0001$, post hoc Bonferroni-corrected paired t-tests). Scoring method does not affect fear response behavior during recall for WT rats ($p = 0.24$, post hoc Bonferroni-corrected paired t-test) however a significantly higher paw immobility response is expressed by *Nlgn3*$^{-/y}$ rats in comparison with classic freezing behavior ($p < 0.0001$, post hoc Bonferroni-corrected paired t-test). WT, N=12; *Nlgn3*$^{-/y}$, N=12. Data represented as mean ± SEM.

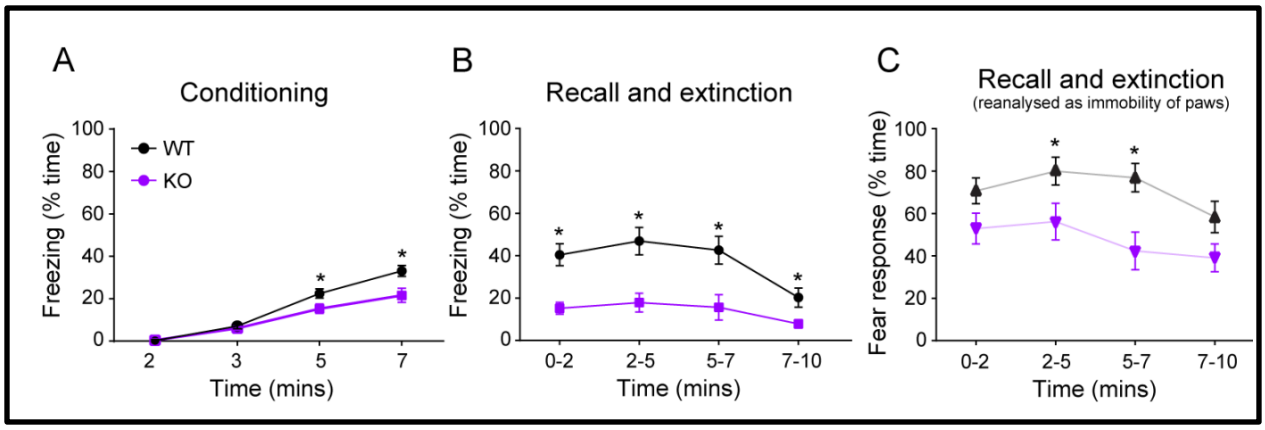


Figure.3.4. *Nlgn3*$^{-/y}$ rats display reduced classic freezing behavior in a contextual fear conditioning paradigm. (A) Classic freezing behavior is reduced in *Nlgn3*$^{-/y}$ rats in comparison to WTs during the conditioning phase of contextual fear conditioning (Genotype: $F_{(1, 25)} = 5.67$, $p = 0.025$, repeated measures two-way ANOVA). (C) Classic freezing behavior is reduced in *Nlgn3*$^{-/y}$ rats in comparison to WTs during the recall phase of contextual fear conditioning (Genotype: $F_{(1, 25)} = 26.61$, $p < 0.0001$, repeated measures two-way ANOVA). (D) When analysed as "immobility response" (i.e all four paws unmoving but allowing for movement of head and neck, shown in light purple/grey) *Nlgn3*$^{-/y}$ rats show a response to the CS significantly different to classic freezing (main effects of scoring method: $p < 0.0001$, $F_{(1, 25)} = 200.82$, and genotype: $p < 0.0001$, $F_{(1, 25)} = 20.65$, three-way ANOVA. WT, N = 13; *Nlgn3*$^{-/y}$, N = 14. Data represented as mean ± SEM.

Table 1. Statistics of auditory fear conditioning

Figure	Task/Measure	Population size	Statistical test	Results/Comparison
2B	Auditory fear conditioning (Conditioning)	WT=12 KO=12	Two-way repeated measure ANOVA	p<0.0001, $F_{(1, 22)}$=6.61
2C	Auditory fear conditioning (Recall and extinction 1)			p=0.001, $F_{(1, 22)}$=13.36
2D	Auditory fear conditioning (Recall and extinction 2)			p=0.0009, $F_{(1, 22)}$=14.61
2E	Auditory fear conditioning (Recall and extinction 1; reanalysed as immobility of paws)		Three-way ANOVA	Scoring $F_{(1, 22)}$ = 20.32, p < 0.0001; tone $F_{(12, 264)}$ = 18.75, p <0.0001; genotype $F_{(1, 22)}$ = 15.85, p = 0.001; scoring x genotype $F_{(1, 22)}$ = 0.61, p = 0.012; tone x genotype $F_{(12, 264)}$ = 1.19, p < 0.0001; scoring x tone $F_{(12, 264)}$ = 0.49, p = 0.92, scoring x tone x genotype $F_{(12, 264)}$ = 3.23, p < 0.0001 **Post hoc two-way ANOVAs:** $WT_{(classic)}$ vs $KO_{(classic)}$: Tone $F_{(12,264)}$ =12.52, p<0.0001; tone x genotype $F_{(12,264)}$ =3.871, p<0.0001; genotype $F_{(1, 22)}$ =13.36, p<0.001 $WT_{(Paw\ immobility)}$ vs $KO_{(Paw\ immobility)}$: Tone $F_{(12,264)}$ =12.33, p<0.0001; tone x genotype $F_{(12,264)}$ =0.61, p=0.83; genotype $F_{(1,22)}$=12.1,

				p<0.002 WT $_{(classic)}$ vs WT $_{(Paw\ immobility)}$ Scoring $F_{(1, 11)}$ =7.58, p<0.019; tone $F_{(12,132)}$ =12.16, p<0.0001; scoring x tone $F_{(12,132)}$=0.56, p=0.7 KO $_{(classic)}$ vs KO $_{(Paw\ immobility)}$ Scoring $F_{(1, 11)}$ =13.30, p<0.004; tone $F_{(12,132)}$ =7.43, p<0.0001; scoring x tone $F_{(12,132)}$ =5.69, p<0.0001
2F	Classic freezing Vs immobility of paws during recall		Three-way ANOVA	Scoring $F_{(1,22)}$=29.89 , p<0.0001; time $F_{(1,22)}$=191.25; genotype $F_{(1,22)}$=15.21, p=0.001; scoring x genotype $F_{(1,22)}$=8.49, p=0.007; time x genotype $F_{(1,22)}$=19.33, p<0.0001; scoring x tone $F_{(1,22)}$=15.59 p=0.001, scoring x tone x genotype $F_{(1,22)}$=7.5, p=0.012 **Post hoc test: Bonferroni-corrected paired t-tests:** WT pretone$_{(classic)}$ vs WT CS-response$_{(classic)}$ p<0.0001 WT pretone $_{(paw\ immobility)}$ vs WT CS-response$_{(paw\ immobility)}$ p<0.0001 KO pretone$_{(classic)}$ vs KO CS-

				response$_{(classic)}$ p=0.008 KO pretone$_{(paw\ immobility)}$ vs KO CS-response $_{(paw\ immobility)}$ p<0.0001 WT CS-response$_{(classic)}$ vs WT CS-response$_{(paw\ immobility)}$ p=0.24 KO CS-response$_{(classic)}$ vs KO CS-response$_{(paw\ immobility)}$ p<0.0001

3.5. Nlgn3$^{-/y}$ rats show enhanced motor learning in rotarod task

To test motor coordination and learning impairments due to underlying genetic mutation we performed a rotarod assay in *Nlgn3$^{-/y}$* rats and compared their performance to WT controls. Initially, to assess their baseline motor coordination rats were placed on the spindle rotating on fixed lower speed of 4 RPM (See methods). *Nlgn3$^{-/y}$* rats show comparable motor coordination to their controls across 5 days during the baseline session of the rotarod task (**Fig.3.5.A.** Average fall latency - WT: 74.91±8.96, *Nlgn3$^{-/y}$*: 73.65±10.55). Further, during accelerating phase of rotarod, the rats have to learn to maintain themselves on the fast moving spindle. Interestingly, *Nlgn3$^{-/y}$* rats outperformed their control littermates by maintaining themselves for longer duration at higher speeds compared to their control rats (**Fig.3.5.B.** Average fall latency - WT: 40.85±.38s; *Nlgn3$^{-/y}$*: 61.39±5.74s). This is in line with the previous reports from different research groups which have consistently replicated this phenotype in *Nlgn3$^{-/y}$* mice (Chadman *et al.*, 2008; Radyushkin *et al.*, 2009; Rothwell *et al.*, 2014). Circuit specific dysfunction in nucleus accumbens leads to increase in acquired repetitive motor routines in *Nlgn3$^{-/y}$* mice (Rothwell *et al.*, 2014). However, similar circuit specific dysfunction in this rat model needs to be validated.

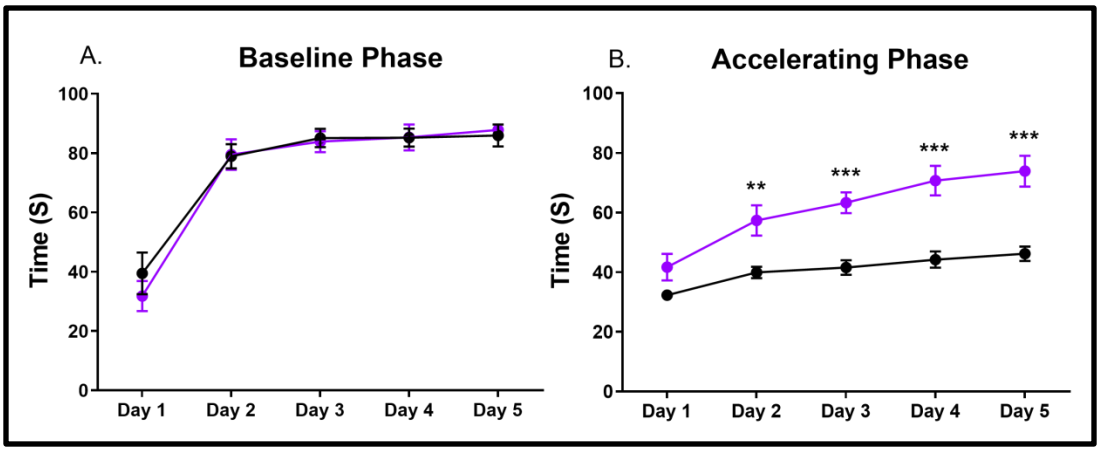


Figure.3.5.*Nlgn3*$^{-/y}$ rats show enhanced motor learning. A. Similar motor coordination during baseline phase of rotarod task (Repeated measure two way ANOVA followed by Sidak's multiple comparison test. Genotype: $F_{(1,22)}$=0.11,p=0.73, Interaction: $F_{(4,88)}$=0.47, P=0.75. B. Enhanced motor learning during accelerating phase of rotarod task (Repeated measures two way ANOVA followed by Sidak's multiple comparison test. Genotype: $F_{(1, 22)}$ = 24.66, p<0.0001***; Interaction: $F_{(4, 88)}$ = 4.663, p<0.001**. WT, N= 12 and *Nlgn3*$^{-/y}$, N=12.

3.6. Nlgn3$^{-/y}$ *display reduced acute pain sensitivity to thermal nociceptive stimulus compared to their WT control rats in tail flick test*

In order to assess the acute sensitivity to thermal pain stimulus I employed a tail flick pain sensitivity assay. Restrained rats tail was placed over the heat slot and the reflex latency to withdraw tail upon exposure to thermal heat stimuli was measured. *Nlgn3*$^{-/y}$ rats showed reduced tail flick latency compared to the WT rats (**Fig.3.6.** WT: 3.25±0.09s; *Nlgn3*$^{-/y}$: 3.68±0.16s).

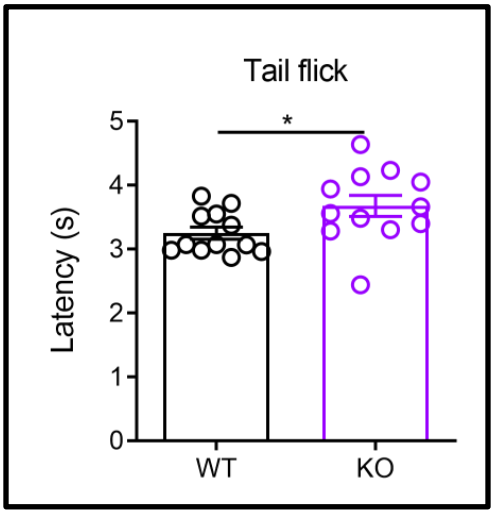


Figure.3.6. *Nlgn3*$^{-/y}$ rats show reduced pain sensitivity in tail flick test. Unpaired t-test, p=0.04*.

3.7. Nlgn3$^{-/y}$ *rats show comparable short term memory in object location recognition task*

The setup for this task was similar to the long term version which we have discussed earlier (**Fig.3.2**). Post 4 days of habituation to the open field arena the rats were given a single 5 minute sampling session. Sampling trial included exposure of rats to two similar tangentially placed objects. After 30 minutes of sampling session the rats were subjected to 3 minute probe trial where one of the objects was moved to a novel location. The preference towards the object at novel location was calculated. Preference towards the object at novel location by *Nlgn3*$^{-/y}$ rats was comparable to their WT rats (**Fig.3.7.** WT: 0.15±0.07; *Nlgn3*$^{-/y}$: 0.02±0.06).

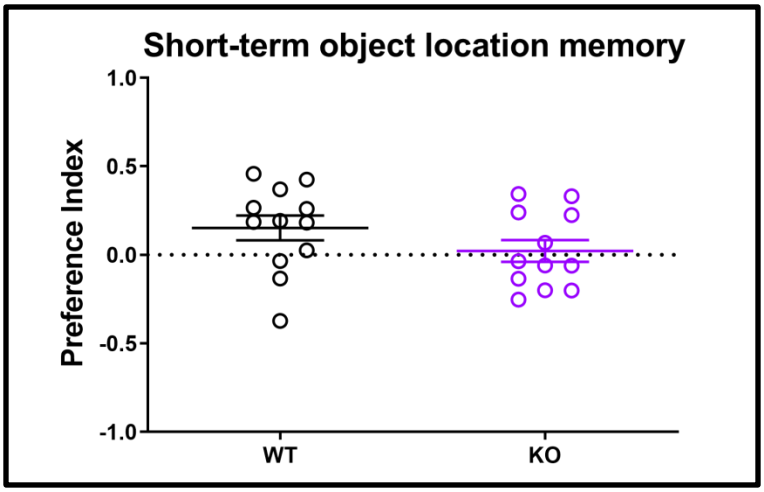
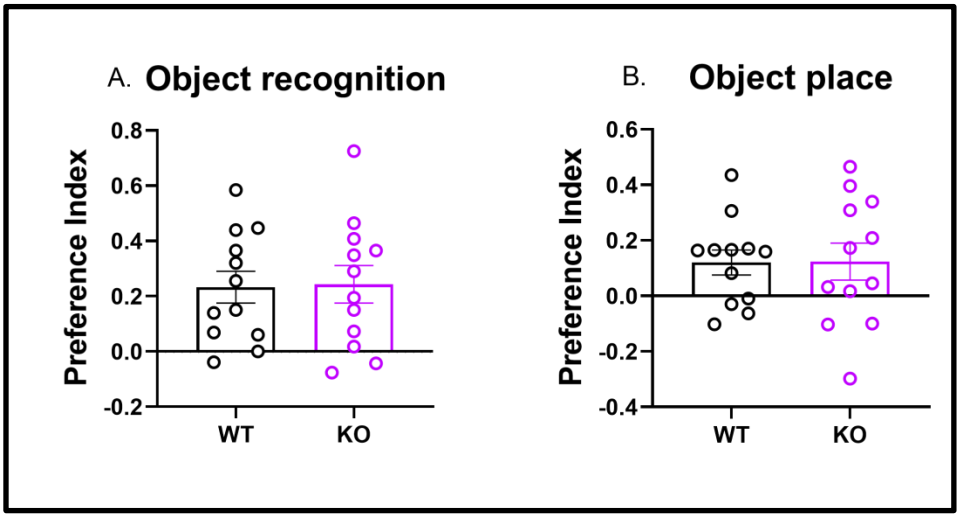


Figure.3.7. Short term object location memory is comparable between WT and *Nlgn3*$^{-/y}$ rats. Unpaired t-test, p=0.08.

3.8. Nlgn3$^{-/y}$ *rats show comparable short term memory in spontaneous alteration tasks*

To investigate further the effect of loss of NLGN3 protein on cognitive deficits I subjected these rats to a set of spontaneous recognition memory tests such as object recognition (OR), object place (OP), object context (OC) and object place context (OPC). Both WT and *Nlgn3*$^{-/y}$ rats explored novel over familiar object/place/context configurations in 3 versions (OR, OP & OC) of the spontaneous exploration tasks respectively (**Fig. 3.8.A, B & C**. OR: WT 0.23±0.05, *Nlgn3*$^{-/y}$ 0.24±0.06; OP: WT 0.12±0.04, *Nlgn3*$^{-/y}$ 0.12±0.06; OC: WT 0.14±0.05, *Nlgn3*$^{-/y}$ 0.09±0.07) Note, exploration of novel place and context was rather weak compared to object recognition task. However, in the complex OPC task which is hippocampus-dependent and involves the associative recognition of objects with respect to their spatial locations and context both WT and *Nlgn3*$^{-/y}$ rats did not show required exploration either of familiar or novel configurations (**Fig.3.8.D.**WT: 0.01±0.05, *Nlgn3*$^{-/y}$ -0.003± 0.05). This probably is due to the sameness and disinterest towards the procedure which involves back to back explorations over days.



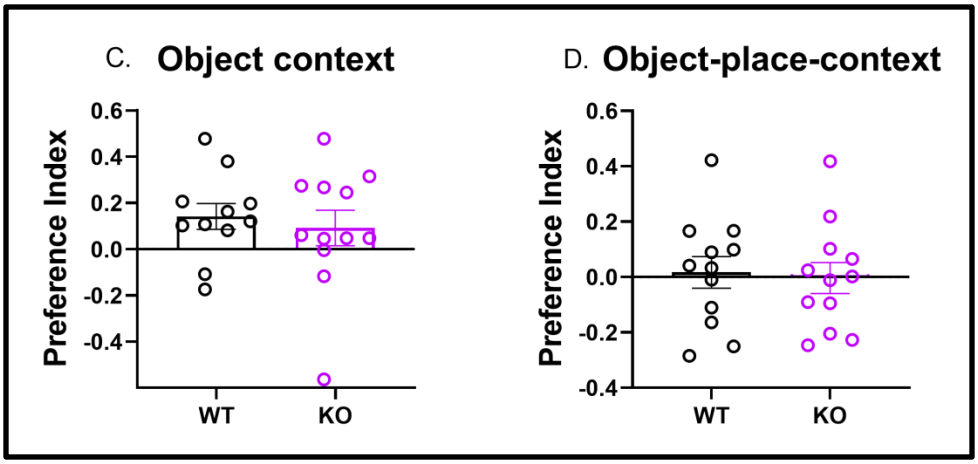


Figure.3.8. *Nlgn3*$^{-/y}$ rats show similar exploration to that of WT in A. Object recognition. B. Object place. C. Object context and D. Object-place-context tasks. Unpaired t-test p>0.05. WT, N=12; *Nlgn3*$^{-/y}$, N=12. Data represented as mean ±SEM.

3.9. Nlgn3$^{-/y}$ *rats show normal social interactions in three chambered social interaction task*

Social deficits are one of the core symptoms observed in individuals with ASD. To probe the social interactions in these rats I used a three-chamber social interaction paradigm in both the WT and *Nlgn3*$^{-/y}$ rats. After habituating the rats to the apparatus, I first measured sociability in these rats where they had a choice to explore a caged, same-sex non-familiar WT conspecific rat over a caged novel object. Time spent in sniffing a social stimulus and novel object was measured. Both WT and *Nlgn3*$^{-/y}$ rats spent significantly increased time exploring the social stimulus over a non-social novel object (**Fig.3.9.A**.WT: 117±12.96s; 117.25± 19.4s). Next, I assessed social preference in social novelty task where rats were given a choice between familiar conspecific rat and novel conspecific rat. Both WT and *Nlgn3*$^{-/y}$ rats spent equal time exploring novel animal (**Fig.3.9.B** WT: 69.09± 13.51s, *Nlgn3*$^{-/y}$: 64.51±14.80s). Hence, mutation in *Nlgn3* did not result in social interaction deficits in these rats. Interestingly, WT rats and *Nlgn3*$^{-/y}$ rats both showed novelty preference (WT-Familiar: 33.31±6.25s; Novel: 69.09±13.51s, *Nlgn3*$^{-/y}$ – Familiar: 38.36±9.92; Novel: 64.51±14.80s).

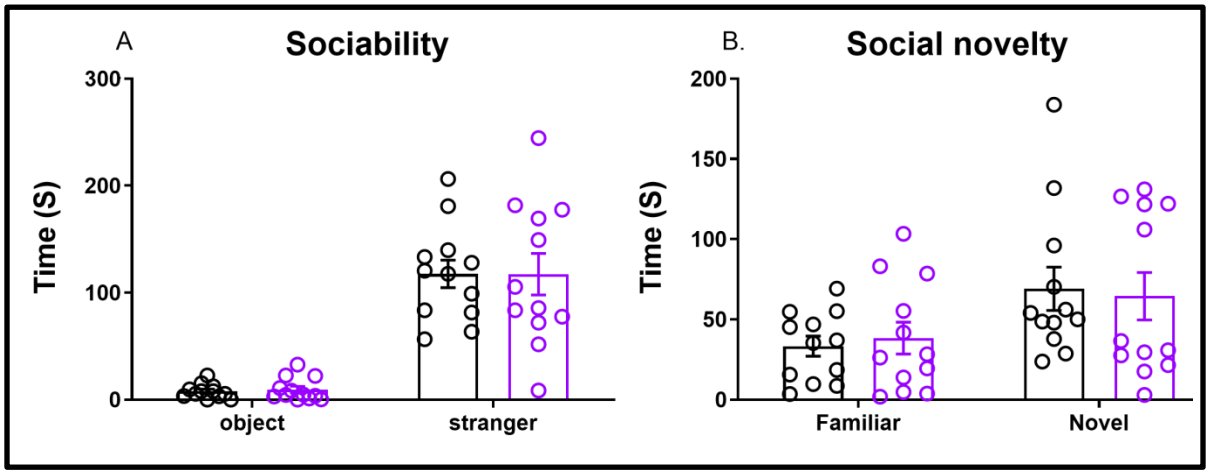
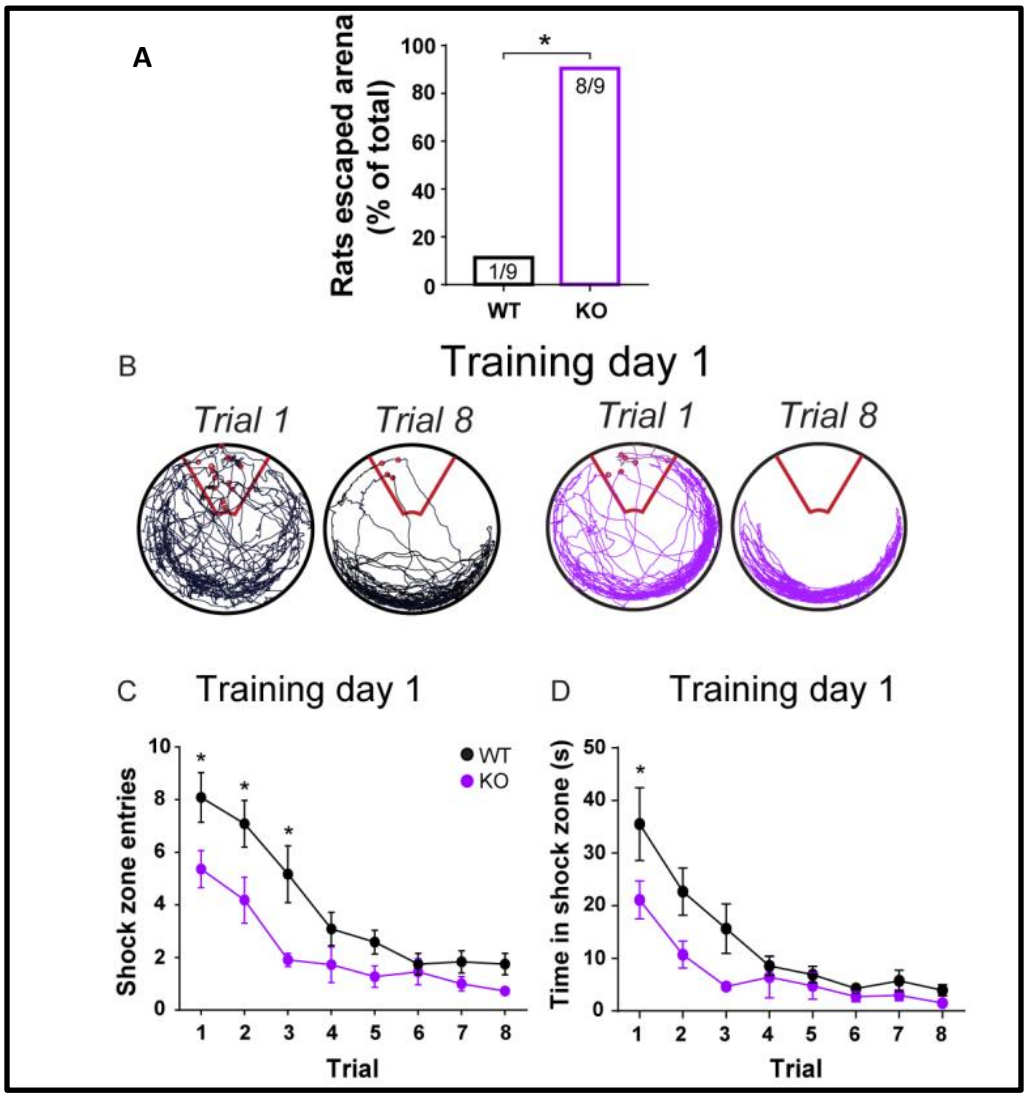


Figure.3.9. Social interaction is comparable between WT and *Nlgn3*$^{-/y}$ rats. A. Normal sociability in WT and *Nlgn3*$^{-/y}$ rats. Two way ANOVA. Genotype: $F_{(1,44)}$=0.003, p>0.05; Preference-Object vs Stranger rat: $F_{(1,44)}$ = 84.84, p<0.0001, Interaction: $F_{(1,44)}$=0.007, p>0.05.. Sidak's multiple comparison test, Stranger-WT vs Stranger-*Nlgn3*$^{-/y}$ rats, p>0.05. B. Social novelty is comparable between WT and *Nlgn3*$^{-/y}$ rats. Two way ANOVA followed by Sidak's multiple comparison test, Genotype: $F_{(1,44)}$=0.004, p>0.05; Interaction: $F_{(1,44)}$=0.17, p=0.68; Preference: $F_{(1,44)}$=7.11, p=0.01. Data represented as mean ± SEM. WT, N=12; *Nlgn3*$^{-/y}$, N=12.

3.10. Nlgn3$^{-/y}$ *rats show improved learning of the shock-zone in the active place avoidance task*

To further explore a potential role for NLGN3 in fear learning, I employed the active place avoidance (APA) task (See methods). During training, response to the low ampere foot-shock differed between genotypes. Over the course of the 8 trials, 8/9 *Nlgn3*$^{-/y}$ rats responding by jumping and escaping the arena altogether. Only 1/9 WT rats showed this behavior (**Fig.3.10.A**). Once an animal escaped the arena the trial had to be ended as it was not possible to measure the time to learn the location of the shock-zone. When testing was repeated in a modified arena with a lid to discourage jumping/escape behavior, naïve cohorts of both WT and *Nlgn3*$^{-/y}$ rats displayed no escape behavior in response to the foot-shock, in addition to learning the location of the shock-zone by the

end of the training sessions and could avoid it by actively remaining in the safe zone (**Fig. 3.10.B–G**). *Nlgn3*$^{-/y}$ rats displayed enhanced performance in this task; throughout training sessions 1 (TS1) and 2 (TS2) *Nlgn3*$^{-/y}$ rats entered into the shock-zone significantly fewer times across trials (**Fig. 3.10 B & C**, TS1: **Fig 3.10.E & F**, TS2), and spent significantly less time in this zone (**Fig. 3.10.D**, TS1; 9G, TS2) in comparison with WT rats. During the probe trial, *Nlgn3*$^{-/y}$ rats displayed significantly prolonged avoidance of previous shock-zone relative to WT animals, despite no shock being applied. *Nlgn3*$^{-/y}$ rats entered the previous shock-zone fewer times on average (**Fig. 3.10.H & I**), and spent less total time in this zone (**Fig.3.10.J**) in comparison with WTs. The ability of the *Nlgn3*$^{-/y}$ rats to successfully learn the location of the shock-zone indicates that spatial memory is unaffected by the loss of NLGN3. However, the exaggerated escape behavior of *Nlgn3*$^{-/y}$ rats seen in the unmodified arena, along with the increased avoidance during the probe trial, suggests NLGN3 loss results in altered fear expression to the shock.



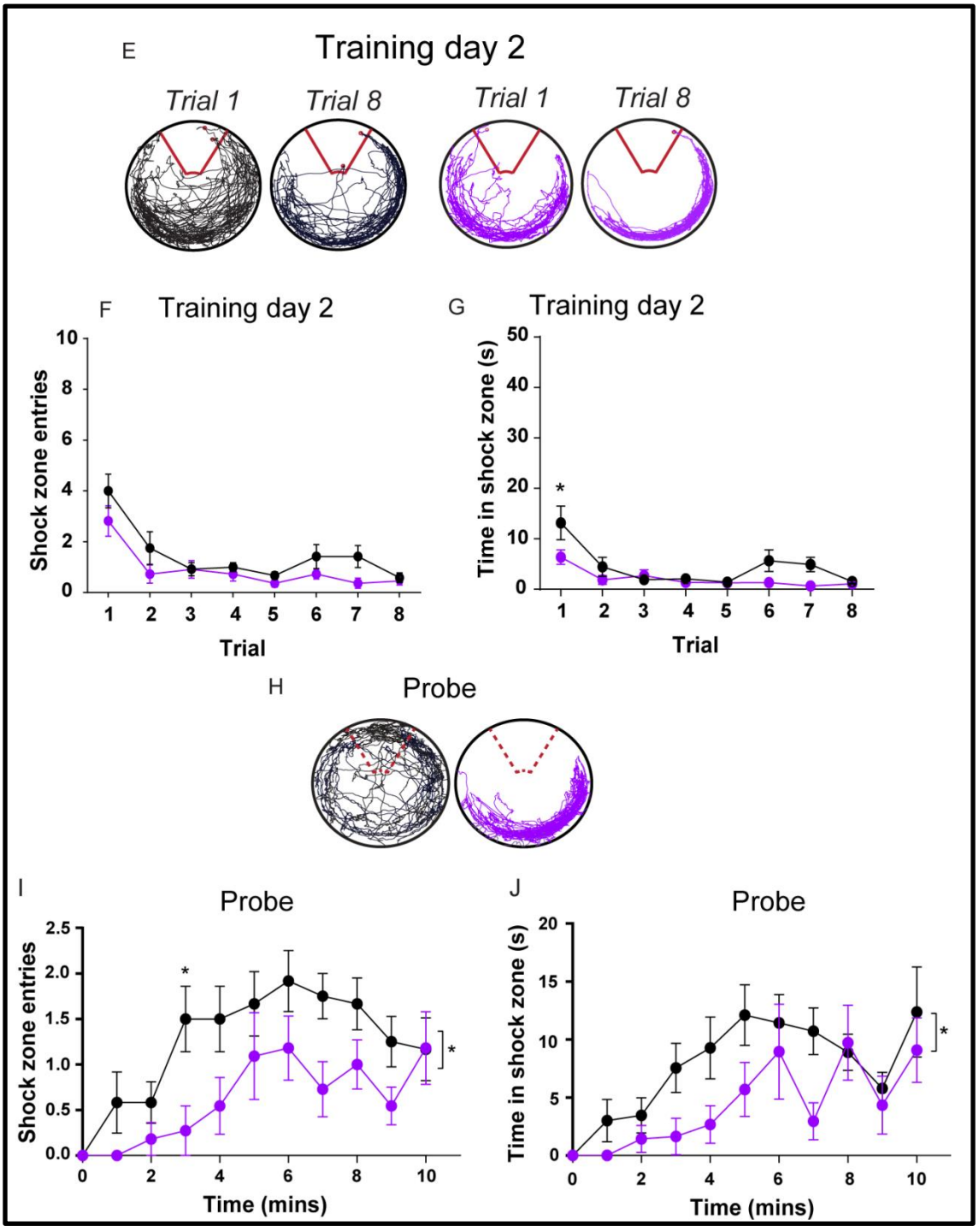


Figure.3.10. *Nlgn3*$^{-/y}$ rats show faster learning and prolonged avoidance of the shock-zone in an active place avoidance task. A. 88.9% *Nlgn3*$^{-/y}$ and 11.1% WT rats jumped

out of the arena following 0.2 mA foot-shocks given over the 8 training trials training ($p = 0.0034$, Fisher's exact test, WT n = 9, KO n = 9). B. Representative track plots for WT and *Nlgn3*$^{-/y}$ rats in trials 1 and 8 of training sessions 1 .C, D. *Nlgn3*$^{-/y}$ rats enter the shock-zone significantly fewer times during training session 1 ($p = 0.0045$, $F_{(1, 21)} = 10.09$, repeated measures two-way ANOVA) and spend significantly less time in the shock-zone ($p = 0.027$, $F_{(1, 21)} = 5.68$ repeated measures two-way ANOVA). E. Representative track plots for WT and *Nlgn3*$^{-/y}$ rats in trials 1 and 8 of training sessions 2. F, G. *Nlgn3*$^{-/y}$ rats enter the shock-zone significantly fewer times during training session 2 ($p = 0.044$, $F_{(1, 21)} = 4.60$, repeated measures two-way ANOVA), and spend significantly less time in the shock-zone ($p = 0.025$, $F_{(1, 21)} = 5.80$, repeated measures two-way ANOVA). H. Representative track plots for WT and *Nlgn3*$^{-/y}$ rats in the probe trial. I,J .*Nlgn3*$^{-/y}$ rats enter the shock-zone significantly fewer times during the probe trial ($p = 0.0039$, $F_{(1, 21)} = 10.51$, repeated measures two-way ANOVA) and spend significantly less time in the shock-zone ($p = 0.045$, $F_{(1,21)} = 4.53$, repeated measures two-way ANOVA. WT, N = 12; *Nlgn3*$^{-/y}$, N = 11). Data represented as mean ± SEM.

3.11. Nlgn3$^{-/y}$ *rats display increased jumping behavior during a shock-ramp test*

One possible explanation for the data described thus far is *Nlgn3*$^{-/y}$ rats are hypersensitive to electrical shocks, and this difference in sensitivity leads to atypical fear response behavior. To test this, I examined the response of naïve WT and *Nlgn3*$^{-/y}$ rats to increasing intensities of foot-shocks (0.06 to 1 mA). Backpedalling and paw withdrawal were the most common initial behaviors observed when an animal first responded to a foot-shock .The minimum shock required to elicit any response, or to elicit a backpedalling response, was not different between *Nlgn3*$^{-/y}$ and WT rats (**Fig.3.11.A&B**). This indicates *Nlgn3*$^{-/y}$ rats are not hypersensitive to foot-shocks. However, *Nlgn3*$^{-/y}$ rats exhibited significantly more jumping behavior than WT rats in response to the higher amplitude shocks (**Fig.3.11.C**), suggesting that *Nlgn3*$^{-/y}$ rats tend to exhibit flight behavior in response to foot-shocks. The greater incidence of jumping

behavior here in comparison with the fear conditioning paradigm shown in **Figure.3.11. C** is likely due to the repetitive, increasing nature of the foot-shocks given in the paradigm here. At the end of the ramp phase, the shock amplitude was reduced to assess sensitivity changes of the animals induced by the paradigm. Number of jumps to this lower shock intensity was not significantly different between WT and *Nlgn3*$^{-/y}$ animals (**Figure.3.11.D**). This data further suggests that the loss of NLGN3 leads to increased flight behavior in response to fearful stimuli.

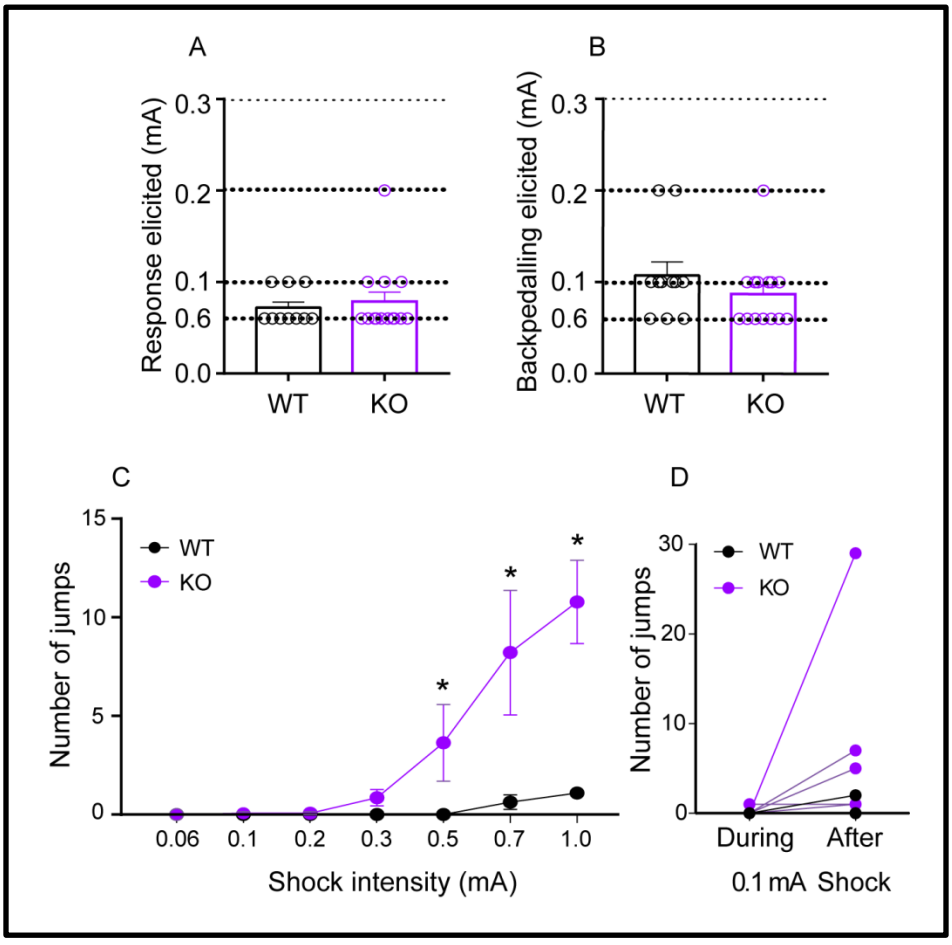


Figure.3.11. *Nlgn3*$^{-/y}$ rats display increased jumping behavior in response to electrical shocks. A. Lowest shock amplitude required to elicit a response of any kind was not different between WT and *Nlgn3*$^{-/y}$ rats (p = 0.13, unpaired t-test). B. Shock amplitude required to elicit backpedalling response was not different between WT and *Nlgn3*$^{-/y}$ rats (p = 0.26, unpaired t-test). C. *Nlgn3*$^{-/y}$ rats display significantly more jumps in

response to increasing intensity electrical foot-shocks (p = 0.0081, F (1, 23) = 8.39, repeated measures two-way ANOVA. WT, N = 11, *Nlgn3*$^{-/y}$, N = 14). Data represented as mean ± SEM, clear dots represent individual animals.

3.12. Nlgn3 $^{-/y}$ rats show deficits in fear memory consolidation, and transactivating protein synthesis using IGF (1-3) rescues freezing deficits

Seminal work by Nader and colleagues (Nader, Schafe and Le Doux, 2000) described the importance of protein synthesis at specific time points post conditioning in consolidation of retrievable memories. To refine whether this was a consolidation deficit or pure recall one, I performed recall protocols on conditioned animals 6 hours post conditioning. As shown in **Figure 3.12**, *Nlgn3*$^{-/y}$ rats showed significantly reduced levels of freezing levels at 6 hours (T1 33% p=0.039, T2 47% p<0.0001, T3 53% p<0.0001) post conditioning. This suggested that translation activation leading to consolidation was not being initiated for the *Nlgn3*$^{-/y}$ animals and that normally the presence of Neuroligin3 in the neuron-neuron or neuron-astrocyte synapses/contacts is critical to this process.

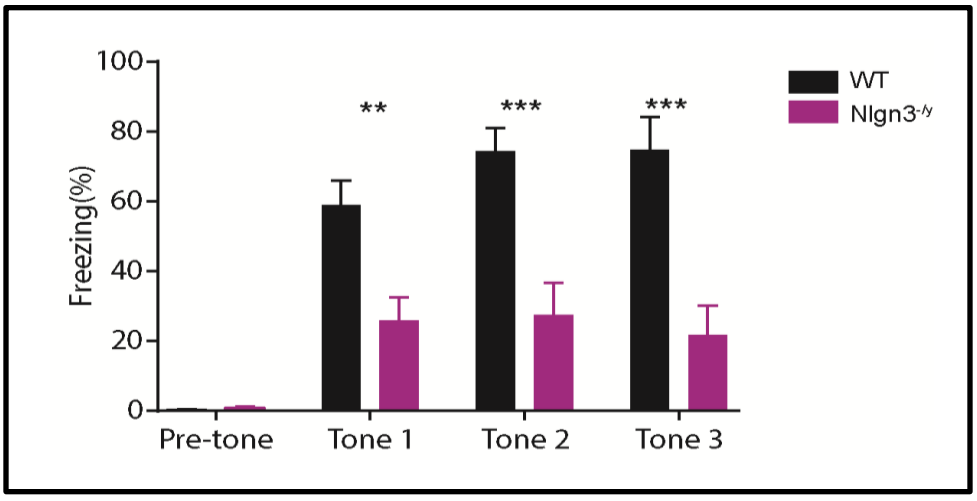


Figure.3.12. *Nlgn3*$^{-/Y}$ rats show fear recall deficits from 6h post conditioning. Two-way ANOVA, ** p<0.005, *** p<0.001 . WT, N=14; Nlgn 3$^{-/Y}$, N=14. Data represented as mean ±SEM.

The above result suggested that an approach to trans activate signaling that promotes translation coincidently with the presentation of recall tone may have the potential to circumvent the translational block seen and hence rescue the recall deficits seen. A reverse of this approach has been frequently used (Nader, Schafe and Le Doux, 2000; Gafford, Parsons and Helmstetter, 2011; Huynh, Santini and Klann, 2014) to disrupt memory consolidation and recall by having protein synthesis inhibitors on board when recall testing is done. Growth factor (GF) signaling via receptor tyrosine kinases like BDNF-TrkB, Insulin-like growth factor (IGF) - IGFR couple to pro-translation ERK and mTORC1 signaling pathways and promote translation in a variety of contexts. Furthermore, these receptors are present ubiquitously across neural cells. However to adapt to our experimental set up, we sought a GF variant that crossed the blood brain barrier rapidly and had a small half-life to only affect the translation at the time of recall. IGF-1 (1-3) is a Gly-Pro- Glu (GPE) tripeptide has been shown to influence astrocytic PI-3K/MAPK signaling. Which further induces production and secretion from neurons to impact the entire local circuit (Corvin *et al.*, 2012). IGF-1 (1-3) may also serve as a weak NMDA agonist in high doses (Vaaga, Tovar and Westbrook, 2014) and has been shown to preferentially partition into the brain vs the periphery in intraperitoneal (i.p.) injections (Baker *et al.*, 2005). Tangentially, IGF-1 (1-3) has been shown to have ameliorative effects on certain behaviors in mouse models of Rett's Syndrome and Fragile X syndrome (Tropea *et al.*, 2009; Deacon *et al.*, 2015).

Extending this, we tested the effect of IGF-1 (1-3) i.p. injection on *Nlgn3*$^{-/y}$ fear conditioned animals. A single i.p. injection given 40 mins before recall experiment (**Figure 3.13**), achieved a significant increase in recall-induced freezing in *Nlgn3*$^{-/Y}$ rats as compared to vehicle injected counterparts (mean difference of 39% for T3, p=0.0003, **Figure 3.13**). A similar experiment done in a cohort of WT animals did not show an appreciable change in freezing levels (Figure 12, $F_{(1.28)}$=0.5984, p=0.4457 for treatment).

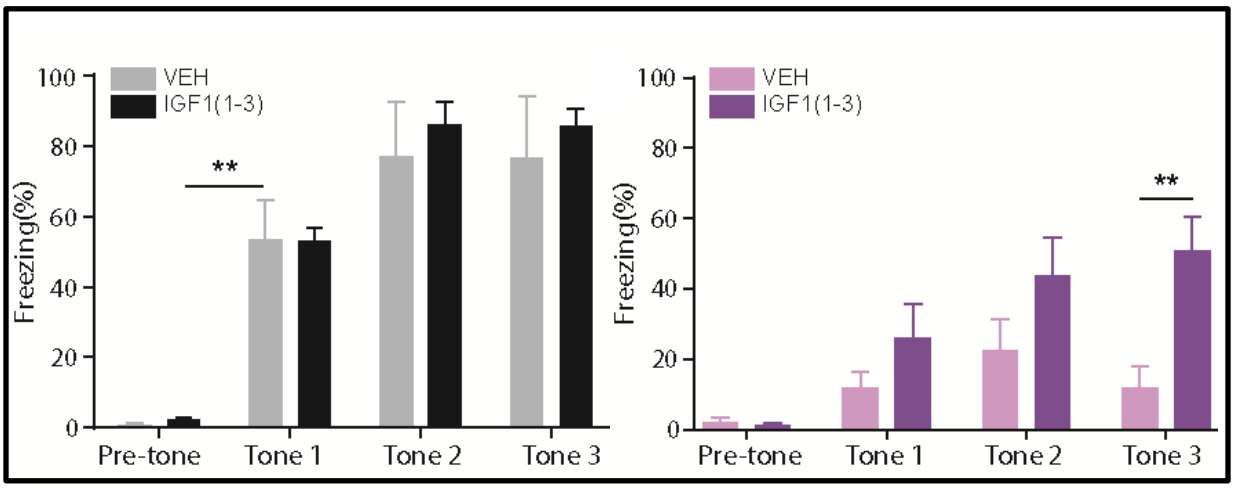


Figure.3.13. IGF (1-3) alleviates the recall deficit in *Nlgn 3*$^{-/Y}$ rats. Percentage freezing of Vehicle (lighter) and IGF (1-3) (darker) injected WT (black) and *Nlgn3*$^{-/Y}$ (purple) animals. ** $p<0.01$, two way ANOVA.N=8, ($F_{(1, 28)}$ =0.5984 for WT, $F_{(1, 22)}$ = 8.531, p=0.0079 for KO). WT, N=8; *Nlgn3*$^{-/Y}$, N=8. Data represented as mean ±SEM.

3.14. Discussion

The invention of new technologies to genetically modify rats to model human disease like ASD has provided a valuable resource to study the heterogeneous behavioral phenotypes observed in autism. This also provides cross species validation of the various phenotypes which helps in translating bench to bedside therapies. This study does not intend to anthropomorphize similarities in symptoms observed in humans to rodents. Instead, we question if the mutation in a specific gene would affect the rat specific behaviors which originates from common brain regions and neural circuitry in both humans and rats alike.

Neuroligins are cell adhesion molecules which are present on the post synaptic membrane(Missler, Sudhof and Biederer, 2012). Neuroligins binds to presynaptic neurexins and have role in synapse formation, maintenance and function (Dean and Dresbach, 2006; Cao and Tabuchi, 2017). There are four neuroligin genes i.e. neuroligin 1, neuroligin 2, neuroligin 3, neuroligin 4 and lastly a human variant neuroligin 4Y. Neuroligin 1 is exclusively found in excitatory neurons and is known to regulate of synaptic transmission and plasticity, neuroligin 2 is exclusively found in inhibitory neurons and facilitate inhibitory synaptic transmission. Neuroligin 3 & 4 both are found in excitatory and inhibitory neurons thus mediating the respective functions (Chanda *et al.*, 2017). Neuroligin 3 is also known as gliotactin and also found in some non-neuronal tissue (Sakers and Eroglu, 2019). Among these neuroligin 3 & 4 genes have been implicated as high risk gene for non-syndromic autism. In 2003 Stephane Jamain's group was first to identify missense mutations in *Nlgn*3 & 4 in Swedish siblings diagnosed with autism (Jamain *et al.*, 2003). Since then various groups have studied effect of loss of NLGN3 in mouse models and have reported multiple behavioral phenotypes (Tabuchi *et al.*, 2007; Etherton *et al.*, 2011; Baudouin *et al.*, 2012; Shipman and Nicoll, 2012; Földy, Malenka and Südhof, 2013; Rothwell *et al.*, 2014; Jaramillo *et al.*, 2018; Modi *et al.*, 2019). It is worth noting here that the genetic manipulation which was done in neuroligin 3 gene was variable with respect to their deletion of number of base pairs in

particular exon, different background strain of mice or specific knockin or knock down. The behavioral phenotypes observed were variable and hence clouds the unifying ethogram with respect to mouse behaviors affected in these ASD model.

In this chapter I have ascertained if loss of NLGN3 protein resulted in altered behavior in various domains like anxiety /repetitive behavior, spatial and emotional cognition, sensory-motor behaviors and social interaction. To begin with I assessed anxiety like or repetitive behaviors in *Nlgn3*$^{-/y}$ rats and WT rats in a marble interaction assay. This assay is predominantly used to assess anxiety like behavior in mice. More numbers of marbles buried could be due to increased anxiety or increased repetitive behavior in mice (Deacon, 2006). However, rats being more explorative compared to defensive mice and doesn't involve in intentional burying behavior. Instead, marbles buried by rats would be a consequence of the digging behavior. I wanted to investigate if these rats would indulge in compulsive digging or repetitive interactions with marbles. WT and *Nlgn 3*$^{-/y}$ rats both spent comparable time in digging and marble interaction. Further, they also display comparable number of marbles buried, frequency of marble interactions and latency to dig was similar to the WT rats. Few studies in *Nlgn 3*$^{-/y}$ mice show decrease in marble burying behavior (Bariselli *et al.*, 2018; Hörnberg *et al.*, 2020). Whereas studies from another group showed increased marbles buried (Kalbassi *et al.*, 2017). Studies in the same rats from another group have reported reduced anxiety in elevated zero maze where rats spent more time in open arm compared to WT rats (Hamilton *et al.*, 2014). The results observed here would be dependent on bedding material, criteria for marble burying, housing and genetic background. I report that the *Nlgn 3*$^{-/y}$ rats do not display any anxiety like or repetitive behaviors in marble interaction assay.

Next I wanted to assess the spatial learning and memory using object location recognition task in both WT and *Nlgn3*$^{-/y}$ rats. This task had two variants to measure long term and short term object location memory (see methods). Both WT and *Nlgn3*$^{-/y}$ rats displayed comparable preference for object placed for novel location in long term and short term object location recognition task. Previous research in *Nlgn3*$^{-/y}$ mice

showed comparable spatial learning and memory in Morris water maze task (Radyushkin *et al.*, 2009; Jaramillo *et al.*, 2018). Interestingly, Neuroligin-3 R451C KI mice showed enhanced spatial learning and memory in Morris water maze. This study for the first time in rats report that the mutation in neuroligin 3 does not result either in impairment or enhancement of spatial memory in object location recognition task.

Next I wanted to assess cognitive abilities in *Nlgn3*$^{-/y}$ and WT rats using behavioral methods which used spontaneous object exploration based novelty preference tasks. These tasks are based on the innate preferences of rats to explore a novel stimulus in an environment with the familiarity. We used 4 variants of these tasks namely object recognition, object place recognition, object context recognition and object place context recognition. In these behavioral experiments, object recognition is the non-associative memory test and rest others are the associative memory tests. These behaviors require different brain areas and cross talk between several brain areas are necessary to show intact memory in these paradigms (Asiminas *et al.*, 2019). *Nlgn3*$^{-/y}$ and WT rats showed similar preference for novelty for object/place and context. However, rats belonging to both the genotypes did not show any preference to either familiar or novel in the OPC task. This could be due to the occlusion effect or sameness induced disinterest in rats. Research in *Nlgn3*$^{-/y}$ mice had revealed that they have intact object recognition memory (Bariselli *et al.*, 2018). Rats with *Fmr1*$^{-/y}$ show intact memory for OR, OP, OC but deficits in OPC tasks (Till *et al.*, 2015; Asiminas *et al.*, 2019).

Emotional responses are heavily understudied in these models of ASD. So, I wanted to assess emotional learning and memory using auditory/ contextual fear conditioning and active place avoidance task in both the WT and *Nlgn3*$^{-/y}$ rats. Fear conditioning and recall are often used to assess emotional learning in ASD/ID models, using the quantification of freezing behavior as a proxy for the memory of the CS–US association. We find *Nlgn3*$^{-/y}$ rats display less freezing behavior (defined as no movement except for respiration) during both auditory and contextual fear recall than WT rats. Taken in isolation, these data could be interpreted as reduced fear learning and/or memory in

Nlgn3$^{-/y}$ rats. However, reanalysis of these data revealed that *Nlgn3*$^{-/y}$ rats stop exploratory behaviors following onset of the tone and respond by staying fixed in the same location within space but moving the head and neck. This type of fear behavior has been reported before in rats confronted with a snake (Andres, 2012; Calvo *et al.*, 2019), and suggests *Nlgn3*$^{-/y}$ rats do form an association between the CS and US, but are expressing their fear differently to WT rats. Two alternative explanations for this behavior are a change in exploratory activity due to altered anxiety levels, or an increase in repetitive, stereotypic behaviors. However, we found no change in locomotion during open field testing, or in tests believed to reflect stereotypic behavior (marble burying) in *Nlgn3*$^{-/y}$ rats. Hence, the most parsimonious explanation for this head movement is a change in flight-related fear responses. We did not observe escape behavior during this task, likely because the arena was fully enclosed with no possible escape route. A previous study (Radyushkin *et al.*, 2009) reported reduced freezing in the *Nlgn3*$^{-/y}$ mouse, however no further investigation was made into the fear responses of these mice, so it is not known whether these two models of Nlgn3 deficiency display converging phenotypes. Interestingly, a study on social interactions of Nlgn3 R451C mice (Hosie *et al*., 2018) reported increased jumping behavior of these mice, consistent with our findings. Further insight into the fear responses and learning of *Nlgn3*$^{-/y}$ rats was seen in direct response to electrical foot-shocks.

Active place avoidance (APA) and shock ramp paradigms revealed *Nlgn3*$^{-/y}$ rats exhibit escape behaviors in response to foot-shocks much more readily than WT controls. However, *Nlgn3*$^{-/y}$ rats were able to efficiently learn the location of a shock-zone in the APA task once escape routes were blocked. Moreover, shock sensitivity testing revealed that *Nlgn3*$^{-/y}$ rats are not hypersensitive to electrical shocks, but again show increased flight responses. These data further support the hypothesis that *Nlgn3*$^{-/y}$ rats do not display associative learning impairments, but preferentially exhibit flight over freezing behavior in response to fear. This hypothesis was further tested to find out the cellular correlates of freeze flight responses in our extended study (Anstey and Kapgal *et al.*, 2022). This study finds hyperactivity in dPAG neurons responsible for the imbalance in

freeze and flight responses in *Nlgn3*$^{-/y}$ rats. We observed a circuit specific alteration leading to the specific behavioral phenotypes in *Nlgn3*$^{-/y}$ rats. However, questions remain about the role of NLGN3 in other nodes of fear processing and if the re-expression of *Nlgn3* in adult rats would correct this imbalance.

Alternative to the hypothesis that *Nlgn3*$^{-/y}$ rats exhibit imbalance in freeze-flight responses is if these rats had problems with consolidating fear memory. So, I subjected these rats to one more variant of fear conditioning protocol where I tested their memory post 6 hours of conditioning. Interestingly, we found that *Nlgn3*$^{-/y}$ rats also freeze less during post 6 hours after conditioning which is attributed to reduced consolidation of the fear memory. Once it is established that they had trouble in consolidation of memory we employed a drug IGF (1-3) because IGF is known to be released in amygdala and known to increase active neurons encoding fear memory (Gisabella *et al.*, 2016). We hypothesized that the external IGF would boost the fear memory formation and thus would increase freezing.

It is very important to bear in mind that our use of Glypromate or IGF-1 (1-3) was not motivated by ongoing clinical trials in syndromic ASD conditions. The use of this reagent was purely as a proof of concept application because IGF-1 (1-3) is just one of the agents that increase general protein synthesis, but with an exceeding small half-life (Baker et al., 2015). Hence apart from a very “online” memory booster during rehabilitative training, this drug may not have a lasting effect. As mentioned earlier, there is a lack of specific activators of mTORC1-ERK pathways more so those that have shown graded effect on neurons and glia (Tropea *et al.*, 2009). Our data do not support that the restoration of fear recall is specific to IGF-1 signaling alone and in future it would be important to assess the restorative effects of dietary leucine or Brain-derived neurotrophic factor (BDNF) on recall memory in Nlgn3 mutants. Very recently, Nlgn 3 in cultures has been demonstrated to impact the Akt-mTORC1 axis (Xu *et al.*, 2019), which further reinforces the translational role of this cell adhesion molecule. Limitations of this this study is it could not ascertain if mutation in *Nlgn3* would anyway alter intrinsic IGF

production or release or receptor expression in the brain areas responsible for fear expression.

Motor deficits are the co-morbid symptoms which appear early in the childhood in majority of patients with ASD. 80-90% children with ASD present with some degree of motor impairments which includes issues with gait, balance, muscle tone, strength, fine and gross motor skills etc., (Hilton *et al.*, 2012). Previous research has shown strong correlation between motor impairments and social & cognitive deficits in children with ASD (Bruininks, 2005; Church et al. , 1999). I assessed the motor coordination in WT and *Nlgn3*$^{-/y}$ rats using rotarod apparatus over 5 days (see methods). Initially, during the baseline session both WT and *Nlgn3*$^{-/y}$ rats showed similar fall latency and thus reflected normal motor coordination. Interestingly, in the acceleration session *Nlgn3*$^{-/y}$ rats had longer fall latency and attained higher RPM compared WT rats. This was in agreement with previous studies in *Nlgn3*$^{-/y}$ mice which also displayed similar enhancement in performance. The researchers had attributed the enhanced motor capabilities to the selective circuit specific impairment in nucleus accumbens which facilitated these mice to acquire repetitive motor routines (Fuccillo, 2016). However, other studies did not see this enhancement in motor learning (Radyushkin *et al.*, 2009; Jaramillo *et al.*, 2018).

Social impairments are one of the defining features of ASD. Children with ASD exhibit have been shown to decreased attention to social stimuli (Dawson *et al.*, 2004). Furthermore, they have been shown to also show reduced responsiveness to novel visual stimuli and reduced habituation to faces (Webb *et al.*, 2011). I investigated the social behavior in WT and *Nlgn3* $^{-/y}$ rats using Crawley's three chambered social interaction task. Post habituation rats were tested for sociability and social preference /novelty (see methods). Both the WT and *Nlgn3* $^{-/y}$ rats explored stranger conspecific rat over a novel object during sociability phase of social interaction task. Further, during social preference test rats of both the genotype preferred interacting with a novel rat over a familiar rat. Previous studies in *Nlgn3* $^{-/y}$ mice also show similar sociability but were impaired in detecting social novelty (Etherton *et al.*, 2011; Hörnberg *et al.*, 2020).

The social novelty deficits observed in *Nlgn3* $^{-/y}$ mice is attributed to the role of NLGN3 in reward circuit neurons i.e. dopaminergic neurons of ventral tegmental area (VTA). Conditional knockout of *Nlgn3* in VTA mimics the social novelty deficits found in global knockouts of *Nlgn3* (Bariselli *et al.*, 2018). Another study showed that *Nlgn3* $^{-/y}$ mice are socially submissive to their wild-type littermates in the tube test which correlated with increased anxiety in marble burying task (Kalbassi *et al.*, 2017). Interestingly, in a previous rat study (Hamilton *et al.*, 2014) it was found that *Nlgn3* $^{-/y}$ rats show normal sociability which is in agreement with our results. This study further showed that *Nlgn3* $^{-/y}$ rats show deficits in juvenile play behavior. This could be interesting assuming implications of juvenile play, social hierarchies, motor stereotypies and ultrasonic vocalizations would shape social cognition in rats. Larger social domain relevant ethologically behaviors like empathy and social propinquity might shed more light on to these complex social behaviors.

3.15. Conclusion

This thesis for the first time reports comprehensive behavioral profiling of novel transgenic *Nlgn3* $^{-/y}$ rats. *Nlgn3* $^{-/y}$ rats show enhanced motor learning, reduced freezing behavior and exaggerated flight responses. It is interesting to note that they were not hyper sensitive to foot shocks but strangely were hypo-responsive to thermal pain stimuli. Further, freezing deficits in *Nlgn3* $^{-/y}$ was rescued by intra peritoneal injections of IGF (1-3). This indicates towards altered protein synthesis profile during learning and recall which needs to be assessed in this rat model. Further, convergent synaptic roles of *Nlgn3* could shed more light towards the pathophysiology of ASD. Evidence indicates molecular cross talk does occur between NLGN3, FMR1, PTEN, Cyfip1 etc., which might link with mTOR signaling and protein synthesis (Baudouin, 2014; Xu *et al.*, 2019; Sledziowska, Galloway and Baudouin, 2020). Other phenotypes like anxiety, learning - memory deficits and social deficits were not observed in *Nlgn3* $^{-/y}$ rats. Literature in mice is variable with respect to observed phenotypes. Novel rat models thus accrue great value with respect to screening for ethologically relevant rat behaviors. This study would

facilitate researchers to ask further questions with respect to mechanisms and selective circuit specific dysfunctions behind the observed phenotypes in *Nlgn3* $^{-/y}$ rats.

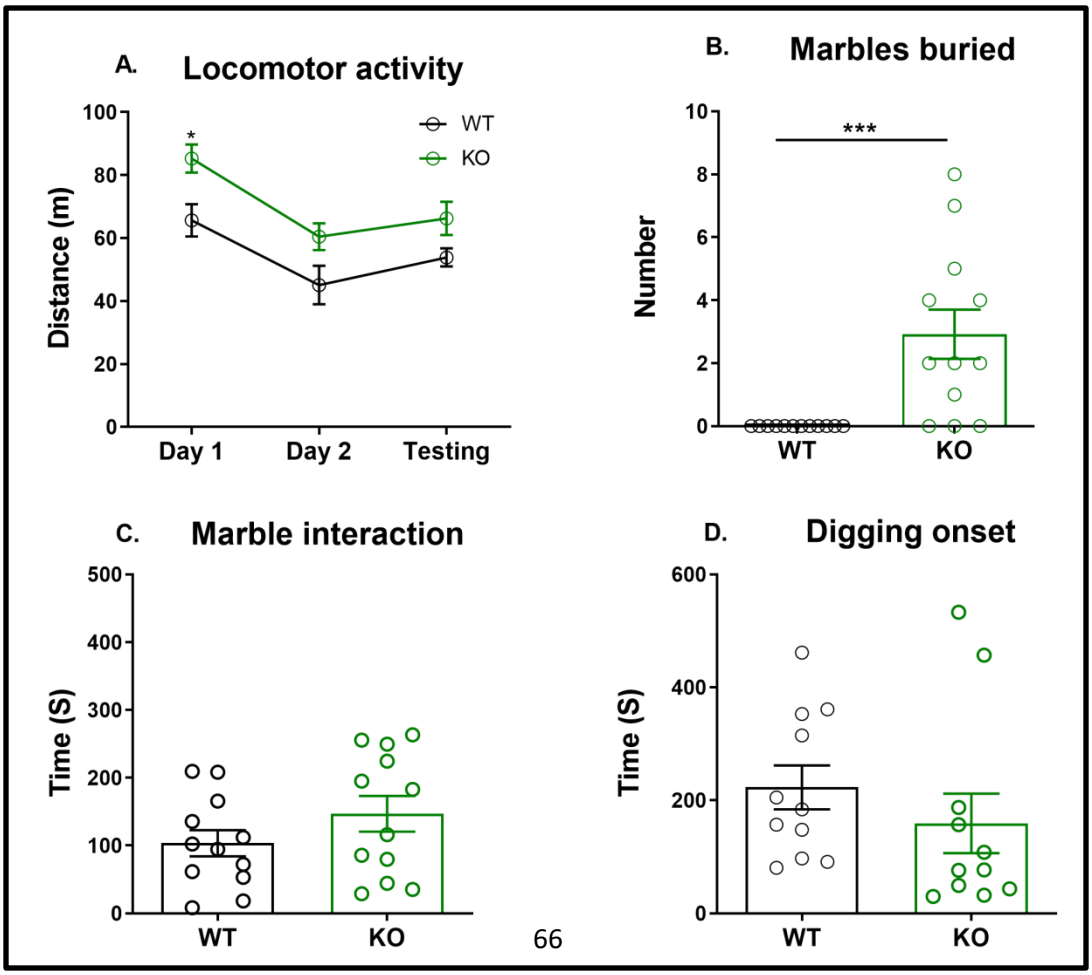
Chapter.4. *Cdkl5*$^{-/y}$ rats exhibit social, cognitive, motor, and anxiety-like behavioral deficits

Cyclin dependent kinase like-5 (CDKL5) also known as serine-threonine kinase 9 is a kinase predominantly expressed in fore brain regions (Montini *et al.*, 1998; Chen *et al.*, 2010). CDKL5 deficiency leads to early onset epilepsy, infantile spasms, developmental delay and motor impairments (Olson *et al.*, 2019). Many patients with CDKL5 deficiency also present with autistic features (Bahi-Buisson *et al.*, 2008; Szafranski *et al.*, 2015). CDKL5 deficiency disorder is predominantly found in females (ratio 4:1) with a heterozygous mutations and rare hemizygous mutations severe deficits have been found in males (Siri *et al.*, 2021). Established functions of the protein include cell proliferation, axonal outgrowth, dendritic arborization and synaptic formation, maintenance and function (Zhu *et al.*, 2013; Fuchs *et al.*, 2014; Zhu and Xiong, 2019). CDKL5 exerts these functions by acting on its substrates such as MeCP2, DNMT1, Amph1, NGL-1 and HDAC4 (Zhu and Xiong, 2019). Previous research substantiates CDKL5 role in stabilization of mature spines and expression of glutamate receptors (Della Sala *et al.*, 2016). CDKL5 regulates many signaling pathways which include BDNF-Rac1, AKT/mTOR, and ATK/GSK-3 (Fuchs *et al.*, 2014; Zhu and Xiong, 2019). CDKL5 is at pivotal position during development to exert its functions and lacking which leads to ASD. Multiple mouse models replicate the molecular, morphological and behavioral phenotypes observed in humans except epilepsy (Amendola *et al.*, 2014b; Jhang *et al.*, 2017; Tang *et al.*, 2017). This is the first study to the best of my knowledge that investigates a transgenic rat modelling *CDKL5* deficiency.

4.1. Cdkl5$^{-/y}$ *rats show hyperactivity and repetitive behavior in marble interaction task*

I began with assessing the anxiety-like/ repetitive behavior in *Cdkl5*$^{-/y}$ and WT rats. Rats were habituated to the arena and then exposed the arena laden with marbles (see methods). The following behaviors were measured i.e. loco-motor activity, number of

marbles buried, marble interaction time, latency to dig and total digging time. *Cdkl5*$^{-/y}$ rats show increased distance travelled compared to WT rats during first day of habituation of marble interaction task (**Fig 4.1.A.** WT: 65.64 ± 5.12m; *Cdkl5*$^{-/y}$: 85.25 ± 4.25m). However, they do show comparable loco-motor activity during subsequent phases of the task. Further, on the testing day *Cdkl5*$^{-/y}$ rats buried more number of marbles compared to WT rats (**Fig 4.1.B.** WT: 0 ± 0; *Cdkl5*$^{-/y}$: 2.9 ± 0.78). Although, marble interaction time (**Fig 4.1.C.**WT: 103.5 ± 19.24s; *Cdkl5*$^{-/y}$: 146.7 ± 26.36s) and digging onset (**Fig 4.1.D.** WT: 223 ± 38.92s; *Cdkl5*$^{-/y}$: 59.1 ± 52.56s) was found to be comparable in both *Cdkl5*$^{-/y}$ rats and WT rats. Interestingly, *Cdkl5*$^{-/y}$ rats spent more time in digging behavior (**Fig 4.1.E.** WT: 19.27 ± 5.53s; *Cdkl5*$^{-/y}$: 59.22 ± 14.76s) compared to the WT rats. Hence, increased marble burying was consequence of increased digging behavior in *Cdkl5*$^{-/y}$ rats. Moreover, *Cdkl5*$^{-/y}$ rats did not show compulsive or repetitive marble interaction. Hence, mutation in *Cdkl5* results in transient hyper activity and increased digging time leading to more marbles buried in these transgenic rats.



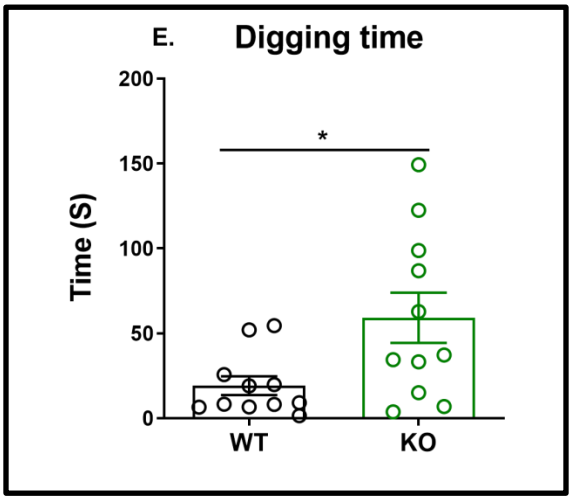


Figure.4.1. *Cdkl5*$^{-/y}$ rats are hyperactive and show anxiety like behavior in marble interaction task. A. *Cdkl5*$^{-/y}$ rats show increased distance travelled in habituation phase. Repeated measures two way ANOVA followed by Sidak's multiple comparisons test (Genotype: $F_{(1, 22)} = 7.644$, p=0.01*; Interaction: $F_{(2, 44)} = 0.6753$, p=0.51).B. *Cdkl5*$^{-/y}$ rats show increased number of marbles buried. (Mann Whitney test, p<0.0001***). C & D. *Cdkl5*$^{-/y}$ rats and WT rats spend equal time exploring marbles and have similar latency to dig. Unpaired t test (p=0.19, p=0.34). E. *Cdkl5*$^{-/y}$ rats show increased digging compared to WT rats during marble interaction test. Unpaired t test (p=0.01*).WT, N=12; *Cdkl5*$^{-/y}$, N=12. Data represented as mean ± SEM.

4.2. Cdkl5$^{-/y}$ *rats show normal spatial learning and memory*

Next I investigated the spatial learning and memory in *Cdkl5*$^{-/y}$ and WT rats. For this an object location recognition task was used where the rats were allowed to sample the location of two similar obliquely placed objects in the open field over 3 days. Post 3 days after the sampling phase rats were subjected to a probe trial where one of the objects was moved to a novel location and their preference to the novel location was measured. *Cdkl5*$^{-/y}$ rats showed increased in loco-motor activity compared to the WT rats on first habituation session (**Fig 4.2.A.** WT: 45.11 ± 2.99m; *Cdkl5*$^{-/y}$: 60.71 ± 3.18m). However, they gradually habituated to the arena over subsequent days (**Fig.4.2.B**). Further, both WT and *Cdkl5*$^{-/y}$ rats showed comparable exploration of objects during acquisition phase (**Fig.4.2.C**). Post retention phase, both WT and *Cdkl5*$^{-/y}$ rats show similar preference for the object displaced to the novel location during the probe trial (**Fig 4.2.D.** WT: 0.1800 ±

0.07; *Cdkl5*$^{-/y}$: 0.03 ± 0.07). Hence, *Cdkl5*$^{-/y}$ did not show any impairment in long term spatial learning and memory.

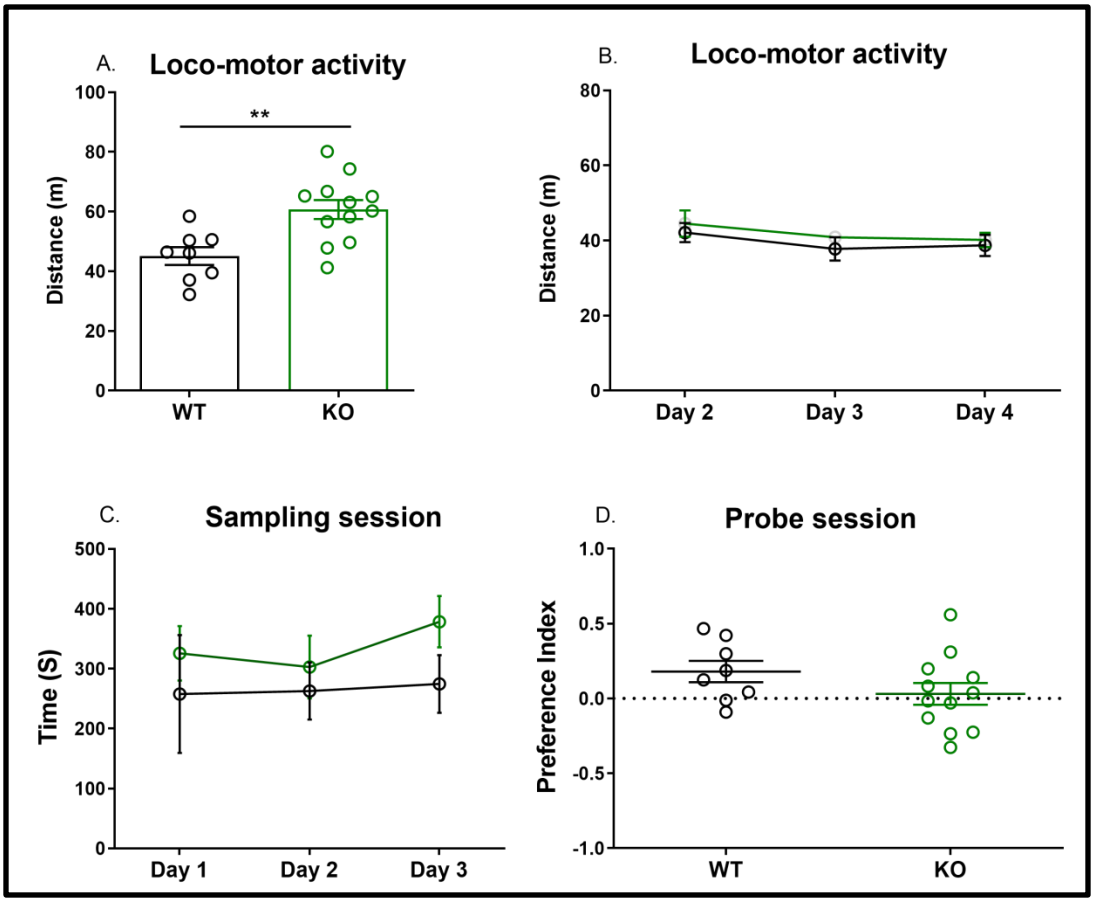


Figure.4.2. *Cdkl5*$^{-/y}$ rats show comparable spatial learning and memory in long term object location recognition task. A. *Cdkl5*$^{-/y}$ rats show hyperactivity during first habituation session (unpaired t test, p=0.003**).B. *Cdkl5*$^{-/y}$ rats show similar locomotion during rest of the habituation days (Repeated measures two way ANOVA) C. *Cdkl5*$^{-/y}$ rats show comparable exploration of objects during sampling/ acquisition phase. Repeated measures two way ANOVA (Genotype: $F_{(1, 10)} = 1.185$, p=0.3). C. *Cdkl5*$^{-/y}$ rats show comparable preference to object at novel location. Unpaired t test, p=0.17. WT, N=8; *Cdkl5*$^{-/y}$, N=12. Data represented as mean ± SEM.

4.3. Cdkl5$^{-/y}$ rats displayed impaired learning but normal fear recall

After assessing the spatial learning I investigated the emotional learning and memory in *Cdkl5*$^{-/y}$ and WT rats using auditory fear conditioning task. *Cdkl5*$^{-/y}$ rats show reduced freezing compared to WT rats during conditioning (**Fig.4.3.A**). However, they show comparable freezing behavior during both recall -extinction and extinction recall phases (**Fig.4.3.B&C**). Thus, genetic mutation in Cdkl5 did not alter their ability to retain the auditory cue memory during auditory fear conditioning task. Also, *Cdkl5*$^{-/y}$ rats showed similar extinction profile and extinction memory to that of WT rats.

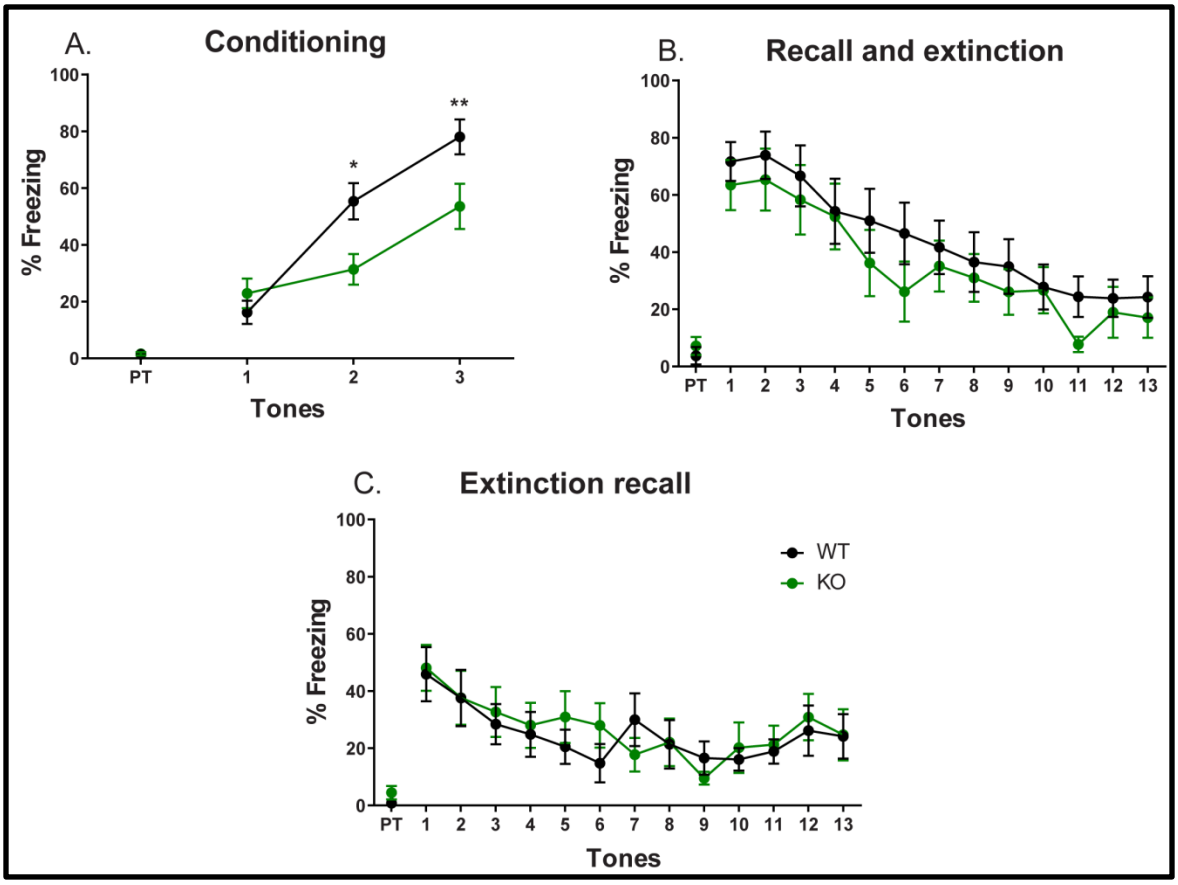


Figure 4.3. Associative fear learning and memory is unaltered in *Cdkl5*$^{-/y}$ rats. A. *Cdkl5*$^{-/y}$ rats show reduced freezing behavior compared to WT rats during conditioning. Repeated measures two way ANOVA (Genotype: $F_{(1, 22)} = 4.297$, p=0.05*; Interaction: $F_{(3, 66)} = 6.876$, p=0.0004***). Sidak's multiple comparison test : p=0.05*, p=0.001**. B.

Cdkl5$^{-/y}$ and WT rats show similar freezing during recall and extinction phase. Repeated measures two way ANOVA (Genotype: $F_{(1, 22)} = 0.73$, p=0.40; Interaction: $F_{(13, 286)} = 0.47$, p=0.93. C. *Cdkl5*$^{-/y}$ and WT rats show similar freezing during extinction recall phase. Repeated measures two way ANOVA (Genotype: $F_{(1, 22)} = 0.10$, p=0.75; Interaction: $F_{(13, 286)} = 0.56$, p=0.88. WT, N=12; *Cdkl5*$^{-/y}$, N=12. Data represented as mean ± SEM.

4.4. Cdkl5$^{-/y}$ *rats show motor learning deficits*

Patients with CDKL5 deficiency present with severe motor impairments, hypotonia and gait deficits. Mice models of CDKL5 deficiency have also showed motor impairments in rotarod task and hind limb clasping (Wang *et al.*, 2012; Sivilia *et al.*, 2016; Jhang *et al.*, 2017; Terzic *et al.*, 2021). I further investigated the motor coordination in these rats using rotarod task. *Cdkl5*$^{-/y}$ and WT rats were subjected to rotarod test over 5 days. *Cdkl5*$^{-/y}$ and WT rats both show comparable fall latencies during baseline phase of the task (**Fig.4.4.A**). Interestingly, *Cdkl5*$^{-/y}$ rats displayed impaired motor coordination during accelerating phase where the fall latency over days did not increase compared to the WT rats (**Fig.4.4.B**). Hence, a genetic perturbation resulted in impaired motor learning in *Cdkl5*$^{-/y}$ rats.

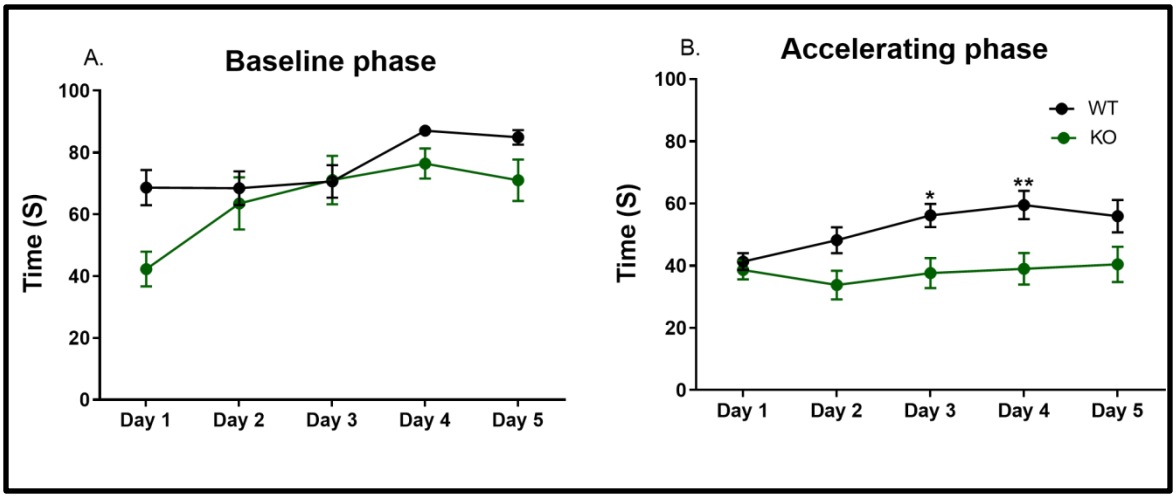


Figure 4.4. *Cdkl5*$^{-/y}$ rats show impaired motor coordination in rotarod test. A. Motor coordination in *Cdkl5*$^{-/y}$ and WT rats was comparable in baseline phase of rotarod test.(Repeated measures two-way ANOVA . Genotype: $F_{(1, 22)} = 3.862$), p=0.06;

Interaction: $F_{(4, 88)} = 2.418$, p=0.054. B. Impaired motor learning in accelerating phase of rotarod task. Repeated measures two-way ANOVA. Genotype: $F_{(1, 22)} = 6.721$, p=0.01*; Interaction: $F_{(4, 88)} = 4.273$, p=0.003**. Sidak's multiple comparison test: p<0.05*, p<0.001**. WT, N=12; *Cdkl5*$^{-/y}$, N=12. Data represented as mean ± SEM.

4.5. Cdkl5$^{-/y}$ *rats have normal acute pain sensitivity*

Next I assessed the acute pain sensitivity in *Cdkl5*$^{-/y}$ and WT rats. Rat's tail was subjected to thermal pain stimulus and latency to flick the tail was measured in WT and *Cdkl5*$^{-/y}$ rats. Average tail flick latency was comparable in both WT and *Cdkl5*$^{-/y}$ rats (**Fig 4.5.**WT: 3.19±0.12s; *Cdkl5*$^{-/y}$: 2.87±0.15s). Hence, CDKL5 deficiency did not result in hyper or hyposensitivity to thermal pain stimulus.

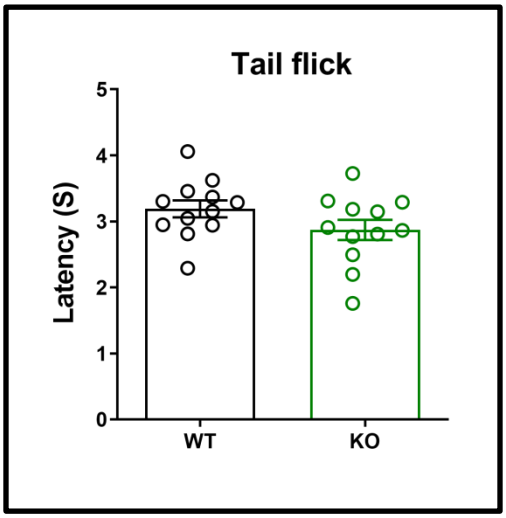


Figure.4.5. Acute pain sensitivity is normal in *Cdkl5*$^{-/y}$ rats. Unpaired t test p>0.05

4.6. Cdkl5$^{-/y}$ *rats show deficits in short term spatial memory*

A separate set of naïve rats went through another set of behavioral experiments. To begin with I first investigated the short term object location memory in both WT and *Cdkl5*$^{-/y}$ rats. The set up was similar to the long term version of the same task (see methods). Interestingly, I observed deficits in short term memory for object location in *Cdkl5*$^{-/y}$ rats compared to WT rats. WT rats show preference to object location and

Cdkl5$^{-/y}$ rats showed significantly less preference to object at novel location (**Fig 4.6.**WT: 0.25±0.09; *Cdkl5*$^{-/y}$ rats: -0.06±0.07).

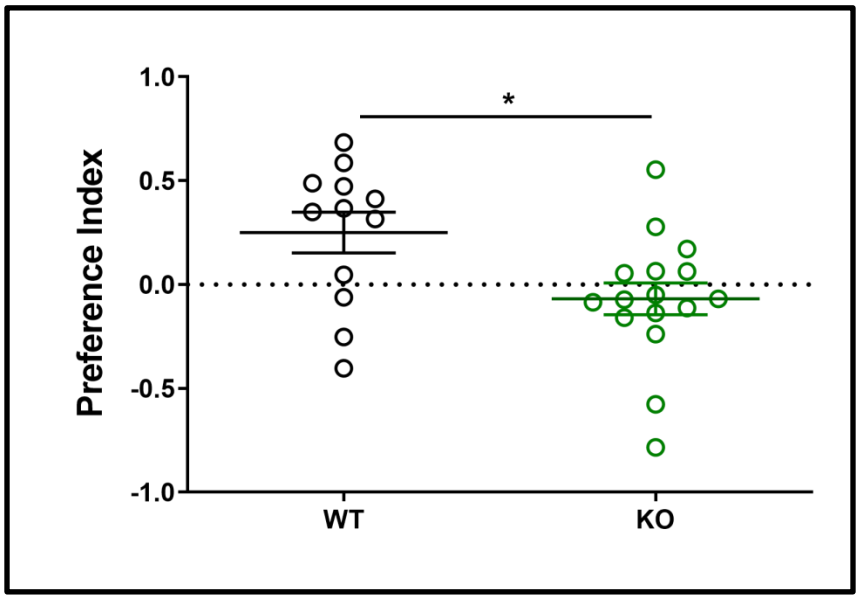


Figure.4.6. *Cdkl5*$^{-/y}$ rats show short term memory deficits in object location recognition task. Unpaired t test, p<0.05*. WT, N=12; *Cdkl5*$^{-/y}$, N=16. Data represented as mean ± SEM.

4.7. Cdkl5$^{-/y}$ *rats show normal spontaneous exploration except in object place (OP) task*

Next, to further investigate the effect of loss of CDKL5 protein on cognitive deficits I subjected these rats to a set of spontaneous recognition memory tests such as object recognition (OR), object place (OP), object context (OC) and object place context (OPC). A. Both WT and *Cdkl5*$^{-/y}$ rats explored novel over familiar object in the object recognition (OR) task (**Fig.4.7.A.**OR: WT 0.24 ± 0.07, *Cdkl5*$^{-/y}$ 0.20 ± 0.04).B. *Cdkl5*$^{-/y}$ rats display show less preference towards novel place in the object place (OP) task (**Fig.4.7.B.**WT 0.20 ± 0.06, *Cdkl5*$^{-/y}$ 0.03 ± 0.05). C&D. Both WT and *Cdkl5*$^{-/y}$ rats show no preference to novel object context and the complex object-place-context (OPC) task (**Fig.4.7.C&D.** WT: -0.005 ± 0.09, *Cdkl5*$^{-/y}$ 0.004 ± 0.05 & WT: -0.1 ± 0.08, *Cdkl5*$^{-/y}$: 0.02 ± 0.06). This probably is due to the sameness and disinterest towards the procedure which involves back to back explorations over days.

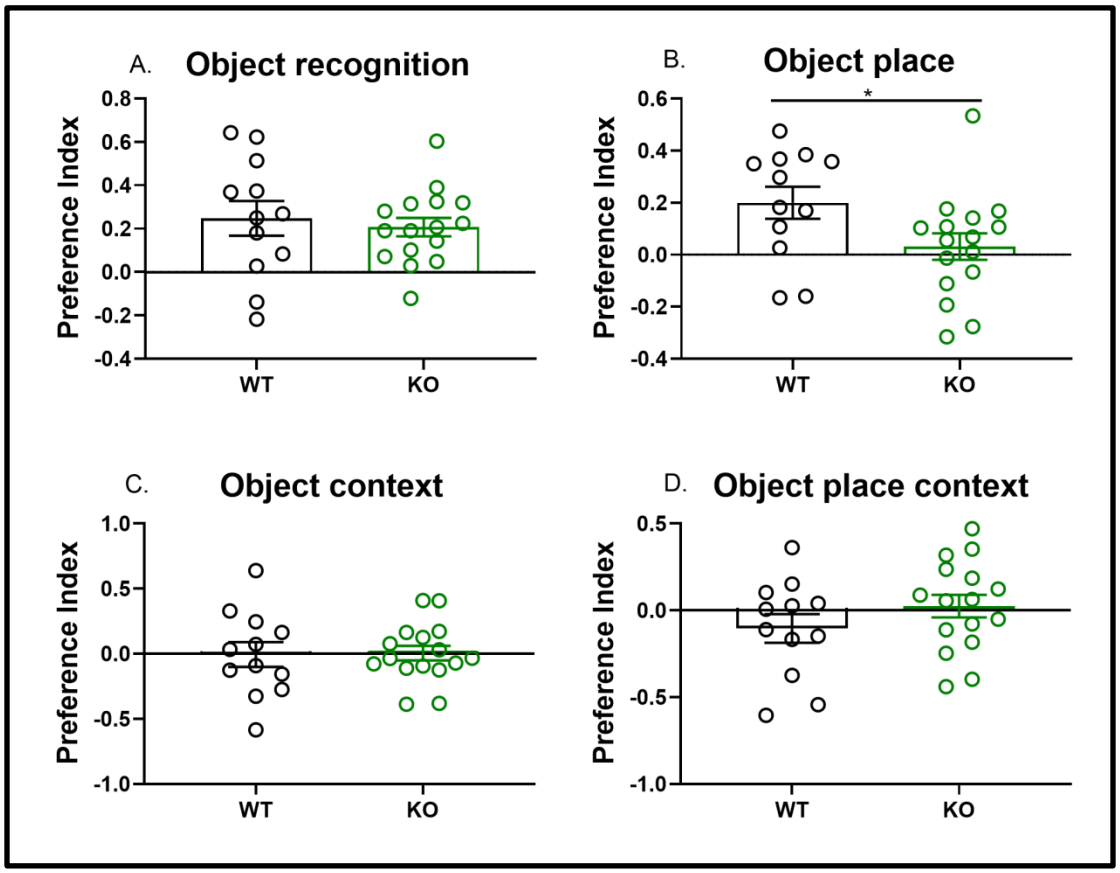


Figure.4.7. Spontaneous exploration tasks in *Cdkl5*$^{-/y}$ and WT rats. A. Similar object recognition in *Cdkl5*$^{-/y}$ and WT rats. B. *Cdkl5*$^{-/y}$ rats showed reduced preference to novel place compared to the WT rats. C& D. *Cdkl5*$^{-/y}$ and WT rats did not show any preference to novel context or novel object place context respectively. Unpaired t test, p=0.05*. WT, N=12; *Cdkl5*$^{-/y}$, N=12. Data represented as mean ±SEM.

4.8. Cdkl5$^{-/y}$ *rats display social interaction deficits*

After assessing their cognitive function I tested the social interaction in *Cdkl5*$^{-/y}$ and WT rats using three-chamber social interaction paradigm. Post habituation rats were first assessed for sociability where they had a choice to explore a caged, same-sex non-familiar WT conspecific rat over a novel object. Time spent in sniffing a social stimulus and novel object was measured. *Cdkl5*$^{-/y}$ rats spent significantly reduced time exploring the social stimulus compared to WT rats (**Fig.4.8.A**.WT: 189.58 ± 11.53s; *Cdkl5*$^{-/y}$: 90.95± 18.99s).

Furthermore, social preference was assessed in social novelty phase where rats were given a choice between familiar conspecific rat and novel conspecific rat. *Cdkl5*$^{-/y}$ rats spent less time exploring novel animal compared to WT rats (**Fig.4.8.B.** WT: 69.75 ± 11.04s, *Cdkl5*$^{-/y}$: 31.57 ± 7.30s) Hence, mutation in *Cdkl5* resulted in social interaction deficits in these rats.

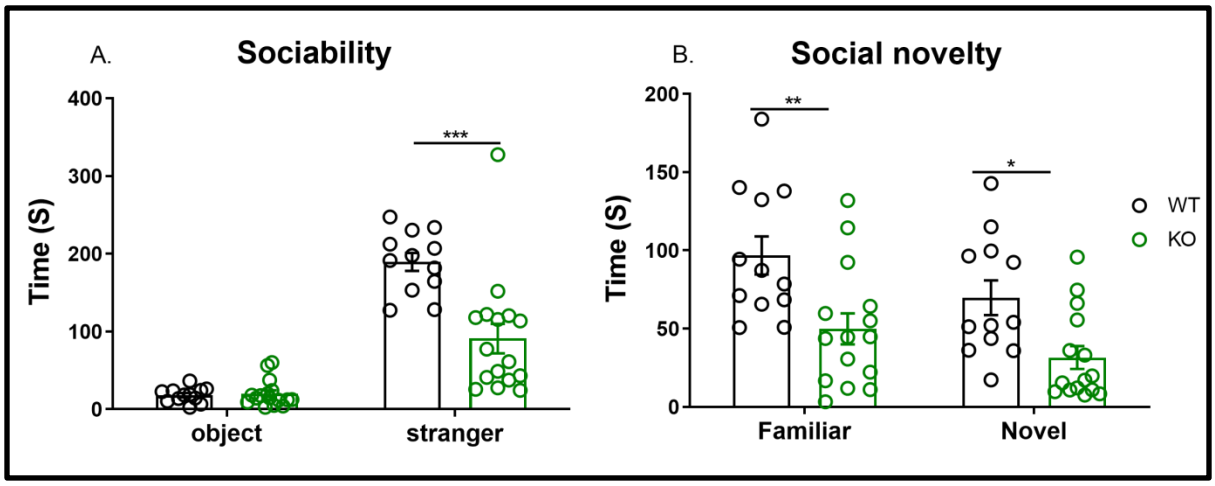
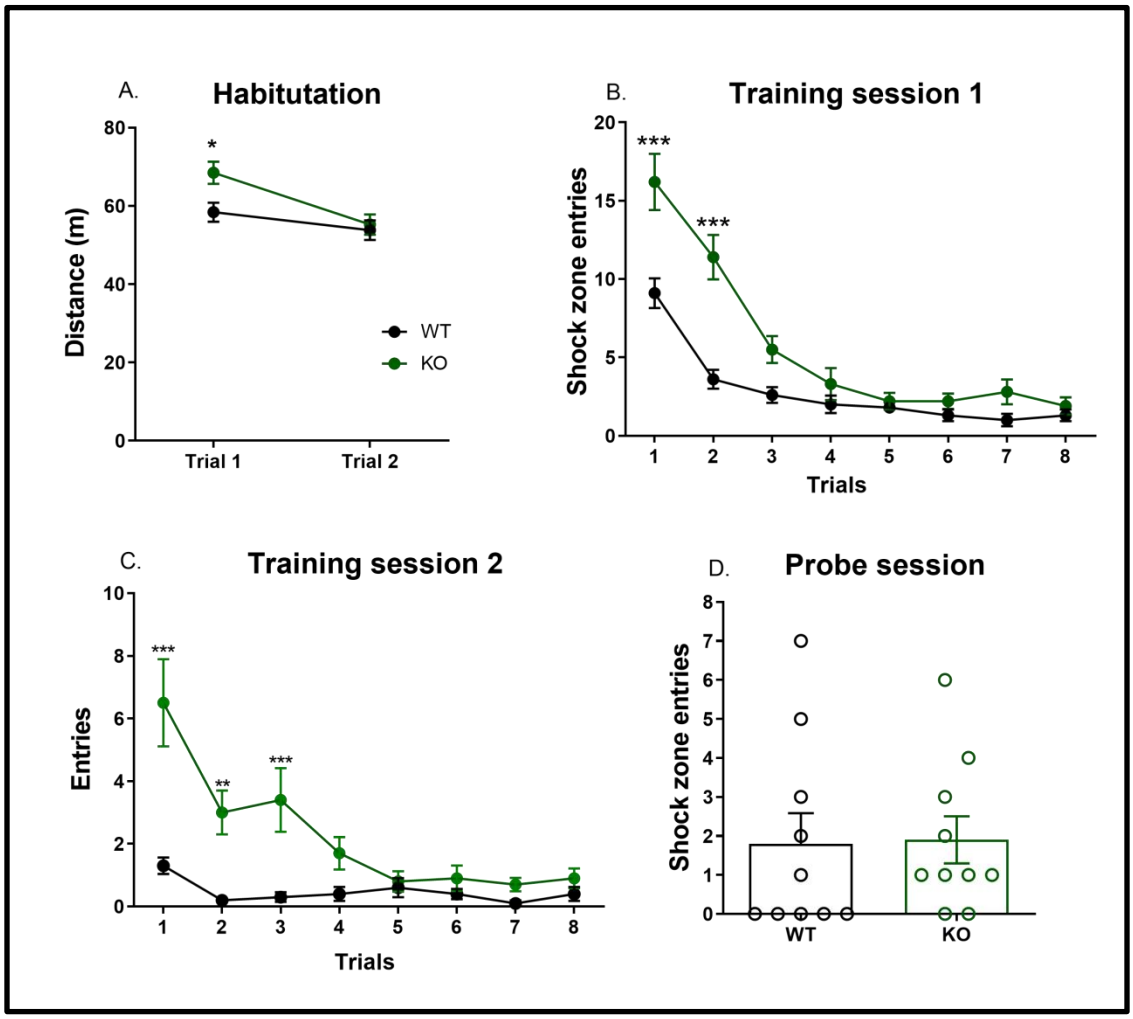


Figure.4.8. *Cdkl5*$^{-/y}$ rats show impaired social interaction in three chambered task. A. *Cdkl5*$^{-/y}$ rats spent less time interacting with social conspecific compared to the WT rats during sociability phase (Two way ANOVA: Genotype: $F_{(1, 52)}$ = 15.26, p=0.0003***; Interaction: $F_{(1, 52)}$ = 16.43, p=0.0002***; Post hoc : Sidak's multiple comparison test: p<0.0001***). B. *Cdkl5*$^{-/y}$ rats spent less time interacting with novel rat over familiar rat compared to the WT rats during social novelty phase (Genotype: F (1, 50) = 17.95), p=0.0001***; Interaction: F (1, 50) = 0.1922, p=0.66; Post hoc : Sidak's multiple comparison test: p<0.01**, p<0.05*). WT, N=12; *Cdkl5*$^{-/y}$, N=16. Data represented as mean ± SEM.

4.9. Cdkl5$^{-/y}$ *rats displayed altered avoidance learning and cognitive flexibility*

Lastly, I investigated the avoidance learning, memory and cognitive flexibility in *Cdkl5*$^{-/y}$ and WT rats in rotational platform (see methods). *Cdkl5*$^{-/y}$ showed increased loco-motor activity compared to WT rats during first trial of habituation. However, their activity was stabilized to that of WT rats in the second trial (**Fig.4.9.A**). Next, in the training session 1

and 2, *Cdkl5*$^{-/y}$ rats showed significant increase in number of entries into the shock zone over initial few trials. However, by the last trial in both training sessions their entry into the shock zone was similar to WT rats (**Fig.4.9.B&C**). Next I assessed the avoidance memory during a shock less probe trial. Surprisingly, during the probe trial both *Cdkl5*$^{-/y}$ and WT rats made comparable entries into the shock zone (**Fig.4.9.D**). Further, I tested reversal learning in a conflict session where we had flipped the shock zone location. *Cdkl5*$^{-/y}$ rats again displayed increased entries into the shock zone during initial trials compared to WT rats (**Fig.4.9.E**). Hence, *Cdkl5*$^{-/y}$ rats showed transient hyperactivity during habituation session. *Cdkl5*$^{-/y}$ rats displayed delayed avoidance learning in active place avoidance task both during training and reversal sessions of the task.



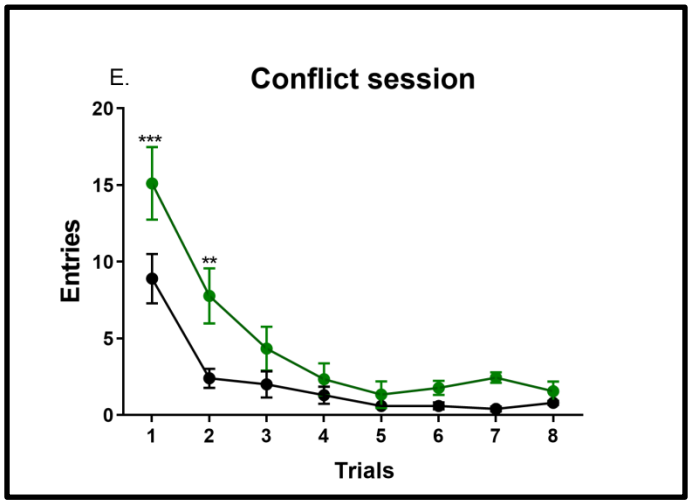


Figure.4.9. *Cdkl5*$^{-/y}$ rats show delayed avoidance learning and comparable avoidance memory in active place avoidance task. A. *Cdkl5*$^{-/y}$ rats show hyperactivity during habituation phase of active place avoidance task. Repeated measures two way ANOVA (Genotype: $F_{(1, 26)} = 4.258$, p=0.01*; Interaction: $F_{(1, 26)} = 2.762$, p=0.10; Trials: $F_{(1, 26)} = 11.81$, p=0.002**, post hoc test: Sidak's multiple comparisons test, p<0.05*). B. *Cdkl5*$^{-/y}$ rats' show delayed learning during training session 1. Repeated measures two-way ANOVA (Genotype: F (1, 18) = 22.66, p=0.0002***; Interaction: $F_{(7, 126)} = 7.663$, p=0.0001***; Trials: $F_{(7, 126)} = 54.11$, p=0.0001***, Post hoc test: Sidak's multiple comparison test, p<0.001**. C. *Cdkl5*$^{-/y}$ rats' show delayed learning during training session 2. Repeated measures two-way ANOVA (Genotype: $F_{(1, 18)} = 17.77$, p=0.0005***; Interaction: $F_{(7, 126)} = 7.155$, p<0.0001***; Trials: $F_{(7, 126)} = 12.05$, p<0.0001***, post hoc test: Sidak's multiple comparison test, p<0.0001***, p<0.001**. D. *Cdkl5*$^{-/y}$ and WT rats show comparable avoidance memory during probe trial (Unpaired t test, p=0.92). E. *Cdkl5*$^{-/y}$ rats' show delayed learning during conflict session. Repeated measures two-way ANOVA (Genotype: $F_{(1, 17)} = 8.077$, p=0.01*; Interaction: $F_{(7, 119)} = 3.040$, p=0.005**; Trials: $F_{(7, 119)} = 37.27$, p<0.0001***, post hoc test: Sidak's multiple comparison test, p<0.0001***, p<0.001**. WT, N=10; *Cdkl5*$^{-/y}$, N=9. Data represented as mean ±SEM.

4.9. Discussion

CDKL5 deficiency disorder (CDD) results from rare x-linked mutation which results in multitude of phenotypes like cognitive, language, social, motor, sleep and vision deficits. However, homozygous males show more grave clinical manifestations than females (Siri *et al.*, 2021). Children with CDKL5 disorder severely suffer from epilepsy and infantile spasms as early as three months in infancy. CDKL5 is implicated in dendritic arborization, spine stability and various other synaptic functions which make it a high risk protein for synaptopathy induced autism. CDKL5 is an upstream protein in the molecular cascade involving the MECP2 protein implicated in Rett's syndrome. Hence, it might be possible that these two proteins share disruption in the common neuronal circuits resulting in similar behavioral deficits. Many mice models of CDKL5 deficiency have reported similar phenotypes observed in humans (Amendola *et al.*, 2014b; Jhang *et al.*, 2017; Trazzi *et al.*, 2018; Terzic *et al.*, 2021). Further, the molecular functions during development have been well studied and have contributed tremendously in understanding the pathophysiology of the disease. Our study is a first attempt to study the CDKL5 deficiency induced synaptopathy in rats.

Cdkl5$^{-/y}$ rats displayed increased loco-motor activity during first session of habituation. Further, they buried more marbles and spent increased time in digging behavior during testing phase of marble interaction task. However, we did not observe repetitive interaction with marbles during this test. Previous study in mice also had shown hyperactivity in open field and stereotypic digging behavior (Amendola *et al.*, 2014b; Jhang *et al.*, 2017; Trazzi *et al.*, 2018; Terzic *et al.*, 2021). Further, one of these studies attributed stereotypic digging to increased dopamine release in striatum and medial prefrontal cortex in *Cdkl5*$^{-/y}$ mice (Jhang *et al.*, 2017). Our data from rats is in line with the previous findings, however mice showed persistent increase in loco-motor behavior and we found it to be subtle increase in locomotion on the first exposure to novel environment.

Cdkl5$^{-/y}$ rats again displayed increased locomotion during first session of the habituation. However, they showed comparable locomotion to their WT rats over subsequent days of habituation. *Cdkl5*$^{-/y}$ rats showed normal acquisition of object location and comparable object location memory during the long term version of object location recognition task. Surprisingly, *Cdkl5*$^{-/y}$ rats showed deficits in short term object location recognition memory. Studies in mice lacking CDKL5 showed impairment in spatial learning and memory in Morris water maze (Fuchs, 2015; Tang *et al.*, 2017; Trazzi *et al.*, 2018) and Barnes maze (Jhang *et al.*, 2017). Moreover, in Y- maze *Cdkl5*$^{-/y}$ mice showed reduced spontaneous alternations which indicated of the working memory deficits (Fuchs *et al.*, 2014; Tang *et al.*, 2017). Also, studies in *Cdkl5* KO mice demonstrate that they exhibited impairment in neurogenesis and reduced dendritic branching which has been correlated with deficits in hippocampal based spatial memory tasks (Fuchs *et al.*, 2014). One possible reason for the discrepancy between the observed phenotypes in mice and rats would be because of the behavioral task itself. Barnes maze and Morris water maze requires rodents to use their loco motor capabilities guided by their hippocampal function to achieve learning and memory. The *Cdkl5*$^{-/y}$ mice show persistent hyperactivity and motor impairments which might cloud our comprehension of results in learning and memory tasks which require intact motor abilities and locomotion. Therefore, spontaneous exploratory behaviors driven by novelty in a smaller arena might be more suitable to test the spatial learning and memory function in these models. We report comparable long term object location recognition memory and reduced short term object recognition memory in the *Cdkl5*$^{-/y}$ rats.

Cognitive functions of *Cdkl5*$^{-/y}$ rats were assessed in short term non associative and associative memory in other spontaneous alteration tasks like object recognition, object place, object context and object place context. These experiments were adapted from (Langston and Wood, 2009; Asiminas *et al.*, 2019). *Cdkl5*$^{-/y}$ rats show similar preference to novel object like the WT rats. Interestingly, they show reduced preference to object at novel place compared to WT rats. This is in line with our finding from short term version of object location recognition tasks. Furthermore, we did not observe novelty

preference to context and object place context versions in both WT and *Cdkl5*$^{-/y}$ rats. This may be due to the general disinterest in exploration of objects over consecutive days of testing. One study in Cdkl5 null mice had reported deficits in novel object recognition (Adhikari *et al.*, 2022).

Investigation of emotional learning and memory of *Cdkl5*$^{-/y}$ rats was carried out using two different tasks using auditory fear conditioning and active place avoidance task. The former, is an associative Pavlovian learning and memory paradigm and latter being an associative spatial escape learning and memory paradigm. Both paradigms use an aversive stimulus as an unconditional stimulus. *Cdkl5*$^{-/y}$ rats showed reduced freezing behavior during conditioning phase where the rats learn to associate innocuous tone (conditional stimulus, CS) with the aversive foot shock (unconditional stimulus, US). However, rats belonging to both the genotypes showed comparable freezing behavior during recall - extinction and extinction recall phases of fear conditioning task. Mouse models of CDKL5 deficiency show variable phenotypes with respect to fear learning and memory. Few study show reduced freezing in cued and contextual fear conditioning tasks (Wang *et al.*, 2012; Terzic *et al.*, 2021) and other study reported increased freezing during the same tasks (Okuda *et al.*, 2018). The studies showing reduced fear memory showed less anxiety and the study with enhanced fear memory showed increased anxiety. Though both these studies have used same genetic background (C57BL6) but their fear conditioning protocols were very different to each other. One group had used a protocol where they have used white noise (60 dB) as conditional stimulus and 0.5 mA foot shock as unconditional stimulus (Okuda et al., 2018). Whereas, another group used 85 dB pure tone of 2 KHz as conditional stimulus and 0.75 mA foot shock as unconditional stimulus (Wang *et al.*, 2012). White noise by itself can be used as an unconditional stimulus in rodents (LaBar and LeDoux, 1996). Hence, a dual effect of aversive stimulus would have led to increase in freezing response in these mice. However, in the studies with low freezing levels the mice were generally hyperactive and could probably reduce freezing responses in these mice. In this study as well, the freezing deficit was observed which could be either due to hyperactivity in the novel

arena or hypersensitivity towards foot shock which is not tested in this rat model. Hence, the phenotypes observed should be carefully interpreted and the conclusions drawn should be compared among various other studies.

Cdkl5$^{-/y}$ rats displayed delayed acquisition in active place avoidance task. However, by the end of respective training sessions they showed comparable entries into shock zones. Surprisingly, *Cdkl5*$^{-/y}$ rats and WT rats show similar avoidance memory for the shock zone during the probe trial. Furthermore, they show delayed reversal learning during a conflict session. Rats use cues present in arena, room and internal idiothetic cues arising from its movements at various joints to figure out escape strategies to avoid mild foot shocks from a fixed location (Fenton *et al.*, 1998; Bures and Fenton, 2000). Active place avoidance tests rodent's ability of cognitive control and cognitive flexibility which requires intact hippocampal functioning in rodents (O'Reilly *et al.*, 2014). Rats use different strategies to avoid an aversive outcome, it is possible that initially rats use hippocampal function to learn the shock location and in later trials they use interval timing using idiothetic cues to escape the shock zone. *Cdkl5*$^{-/y}$ mice show reduced passive avoidance in a dark-light shuttle box (Fuchs, 2015; Trazzi *et al.*, 2018). For the first time we report deficits in cognitive control and flexibility in novel rat model of CDKL5 deficiency. Therefore this paradigm and the results we have observed might offer useful framework for future investigations testing hippocampal functions in rodent models of neurodevelopmental disorders.

CDKL5 deficiency results in motor impairments which affects day to day functions like problems in fine motor skills like grasping pencil to write or using utensils to eat etc. In some severe cases patients display severe hypotonia and gait abnormalities (Szafranski *et al.*, 2015; Siri *et al.*, 2021). Multiple studies in mice lacking CDKL5 have reported motor and gait impairments (Wang *et al.*, 2012; Sivilia *et al.*, 2016; Jhang *et al.*, 2017; Terzic *et al.*, 2021). In this study I observed rats from both the genotypes show normal motor coordination during baseline phase of rotarod (steady speed) and impaired motor learning during accelerating phase of rotarod. Previous studies in *Cdkl5* null mice have

attributed motor impairments to reduction in inhibition in cerebellum which results in overall excitation. These observations were supported by morphological deficits in inhibitory neurons in cerebellum (Sivilia *et al.*, 2016). These findings in rats are in line with the previous studies but warrants further in detailed motor assessments using gait, grip strength and other motor parameters. Motor assessment in the running wheel, gait analysis using CatWalk (Walter *et al.*, 2020) to measure stride parameters and fine motor movement analysis using motion sequencing (Wiltschko *et al.*, 2020) along with assessing structural changes in cerebellum, striatum and motor cortical regions could bring out finer phenotypic characteristics in these rats.

After assessment of motor functions I subjected these rats to acute pain thermal pain sensitivity assay. It is worth noting that increased tolerance to pain is a consistent finding observed in children with CDKL5 disorder (Szafranski *et al.*, 2015). Conditional deletion of CDKL5 in dorsal root ganglionic sensory neurons impaired nociception (La Montanara *et al.*, 2020). We observe no change in pain sensitivity in *Cdkl5*$^{-/y}$ rats.

Finally, I investigated social behavior of WT and *Cdkl5*$^{-/y}$ rats in three chambered social interaction task. Patients with CDKL5 deficiency show autistic like phenotypes with reduced social interaction and eye fixation (Bahi-Buisson *et al.*, 2008). Analysis of the behavior indicated that the *Cdkl5*$^{-/y}$ rats show reduced exploration time for social stimulus compared to WT rats. In the subsequent phase of the task, *Cdkl5*$^{-/y}$ rats again displayed reduced time interacting with novel rat compared to WT rats. Several studies in mice also report reduced social interaction in both direct and three chambered social interaction tasks (Wang *et al.*, 2012; Jhang *et al.*, 2017; Terzic *et al.*, 2021). Contrary to this, forebrain conditional knockout of *Cdkl5* show unaltered social interaction (Tang *et al.*, 2017; Okuda *et al.*, 2018). Discrepancies observed with respect to social phenotypes could be due to the genetic background, order of experiments in the behavioral test battery, generalized anxiety profiles of mice etc.

4.10. Conclusion

Novel rat model of CDKL5 deficiency show altered behavioral phenotypes in most of the behavioral domains. Surprisingly, like mice models, rats as well do not show seizure phenotype which is the most debilitating symptom of CDKL5 deficiency in humans. Furthermore, novel rat model of CDKL5 deficiency offers great translational value and could help researchers further understand pathophysiology of the disease.

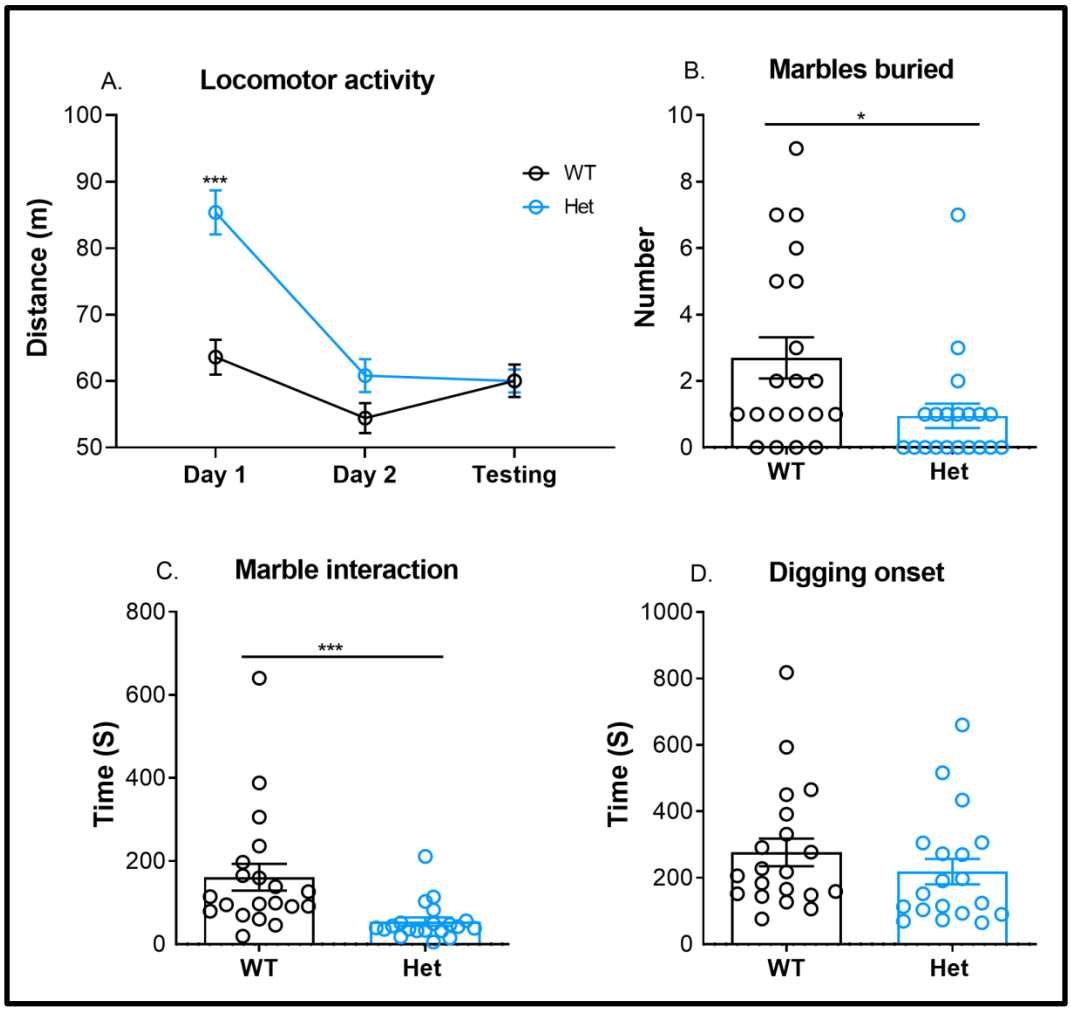
Chapter.5. SynGAP$^{-/+}$ rats exhibit deficits in social interaction, motor co-ordination, and repetitive behavior.

SYNGAP1 gene is high risk locus for loss of function mutations known to cause neurodevelopmental disorders (Hamdan *et al.*, 2009; Vlaskamp *et al.*, 2019). SynGAP haploinsufficiency results in severe mental retardation with symptoms like cognitive dysfunction, epilepsy and severe language deficits (Mignot *et al.*, 2016). Interestingly, haploinsufficiency of SynGAP results in synaptopathy which is often implicated in autism (Iossifov *et al.*, 2014; Holder, 2019; Vlaskamp *et al.*, 2019). Mice with homozygous deletion of SynGAP does not survive for long, suggesting SYNGAP plays a critical role in brain developmental (Komiyama *et al.*, 2002; Kim *et al.*, 2003). SYNGAP is postsynaptic protein localized with post synaptic density protein complex which regulates the *Ras* activation and AMPA receptor insertion on the postsynaptic membrane (Sheng and Kim, 2011). SYNGAP is also implicated in spine formation and maturation (Clement *et al.*, 2012). Dysregulated spine maturation during development results in various functional and behavioral phenotypes of ASD-associated neurodevelopmental disorders. Studies in heterozygous SynGAP mice have reported deficits in LTP in hippocampal brain slices (Ozkan *et al.*, 2014; Katsanevaki *et al.*, 2020). This study for the first time reports the behavioral phenotypes observed in novel transgenic *SynGAP* heterozygous rats.

5.1. SynGAP$^{+/-}$ rats do not show repetitive burying but show repetitive grooming in marble interaction task

Firstly, I investigated the basal anxiety state of both the *SynGAP$^{+/-}$* and WT rats in a marble interaction task. Rats were habituated to the arena over two days and on the third day they were tested in the same arena with marbles (see methods). Behavior was recorded for offline analysis and parameters like distance travelled, marbles buried, marble interaction time, digging onset, time spent in digging and total grooming time were measured. *SynGAP$^{+/-}$* rats displayed increased locomotor activity during first habituation phase of marble interaction task (**Fig.5.1.A.** WT: 63.60 ± 2.6m; *SynGAP$^{+/-}$*:

85.39 ± 3.31m). However, *SynGAP*$^{+/-}$rats did not display hyperactivity during subsequent sessions. Further, *SynGAP*$^{+/-}$ rats buried less marbles (**Fig.5.1.B.** WT: 2.7 ± 0.62; *SynGAP*$^{+/-}$: 0.95 ± 0.36) & showed reduced interaction time compared to their WT controls (**Fig.5.1.C**.WT: 161.1 ± 32.17s; *SynGAP*$^{+/-}$: 54.39 ± 10.19s). I did not notice genotype differences in time spent in digging (**Fig.5.1.D**. WT: 57.84 ± 15.6s; *SynGAP*$^{+/-}$: 47.86 ± 10.72s) and digging onset (Fig. 1E.WT: 276.5 ± 41.73s; *SynGAP*$^{+/-}$: 218.4 ± 38.21s). Interestingly, *SynGAP*$^{+/-}$ rats show significant increase in grooming time compared to WT rats (**Fig.5.1.F**.WT: 30.8 ± 9.31s; *SynGAP*$^{+/-}$: 118.2 ± 25.72s). Thus, heterozygous deletion of SYNGAP resulted in transient hyperactivity, less marble burying, reduced marble interaction time and increased repetitive grooming behaviors in rats.



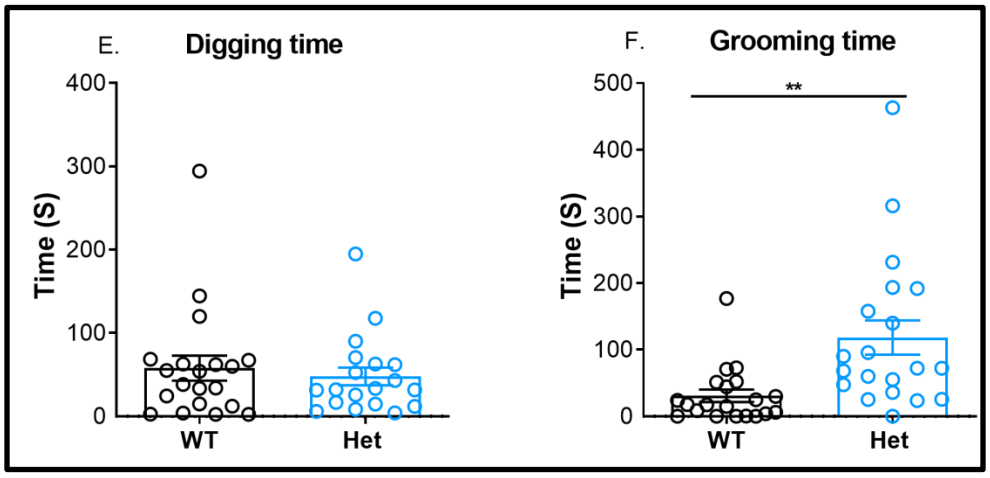


Figure.5.1. *SynGAP*$^{+/-}$ rats show reduced anxiety and increased repetitive grooming in marble interaction task. A. *SynGAP*$^{+/-}$ rats show increased distance travelled during first habituation session. Repeated measures two way ANOVA. (Genotype: $F_{(1, 38)} = 10.02$, p=0.003**; Interaction: $F_{(2, 76)} = 21.54$, p<0.0001***. Post hoc: Sidak's multiple comparison test, p<0.0001***). B. *SynGAP*$^{+/-}$ rats buried less marbles (Mann Whitney test ,p=0.01*). C. *SynGAP*$^{+/-}$ rats spent less time interacting with marbles (Unpaired t test, p=0.003**). D & E. *SynGAP*$^{+/-}$ rats and WT rats show similar onset of digging and time spent in digging(Unpaired t test, p=0.31& p=0.59 respectively). F. *SynGAP*$^{+/-}$ rats show repetitive grooming (Unpaired t test, p=0.002**). WT, N=20; *SynGAP*$^{+/-}$, N=20. Data represented as mean ± SEM.

5.2. SynGAP $^{+/-}$ rats show normal spatial learning and memory

Next, to test spatial learning and memory in both WT and *SynGAP*$^{+/-}$ rats I subjected these rats to object location recognition task. *SynGAP*$^{+/-}$ rats displayed hyperactivity on first session of habituation (**Fig. 5.2.A.**WT: 64.1 ± 2.93m; *SynGAP*$^{+/-}$:77.49 ± 3.15m). On the subsequent habituation sessions both WT and *SynGAP*$^{+/-}$ rats showed comparable locomotion (**Fig. 5.2.B**). Further, both WT and *SynGAP*$^{+/-}$ rats showed similar acquisition profile during sampling session (**Fig. 5.2.C**) and comparable preference to the object placed at novel location (**Fig.5.2.D.**WT: 0.12 ± 0.06; *SynGAP*$^{+/-}$: 0.004 ± 0.06). Hence,

heterozygosity in *SynGAP* did not result in spatial learning and memory deficits in these rats.

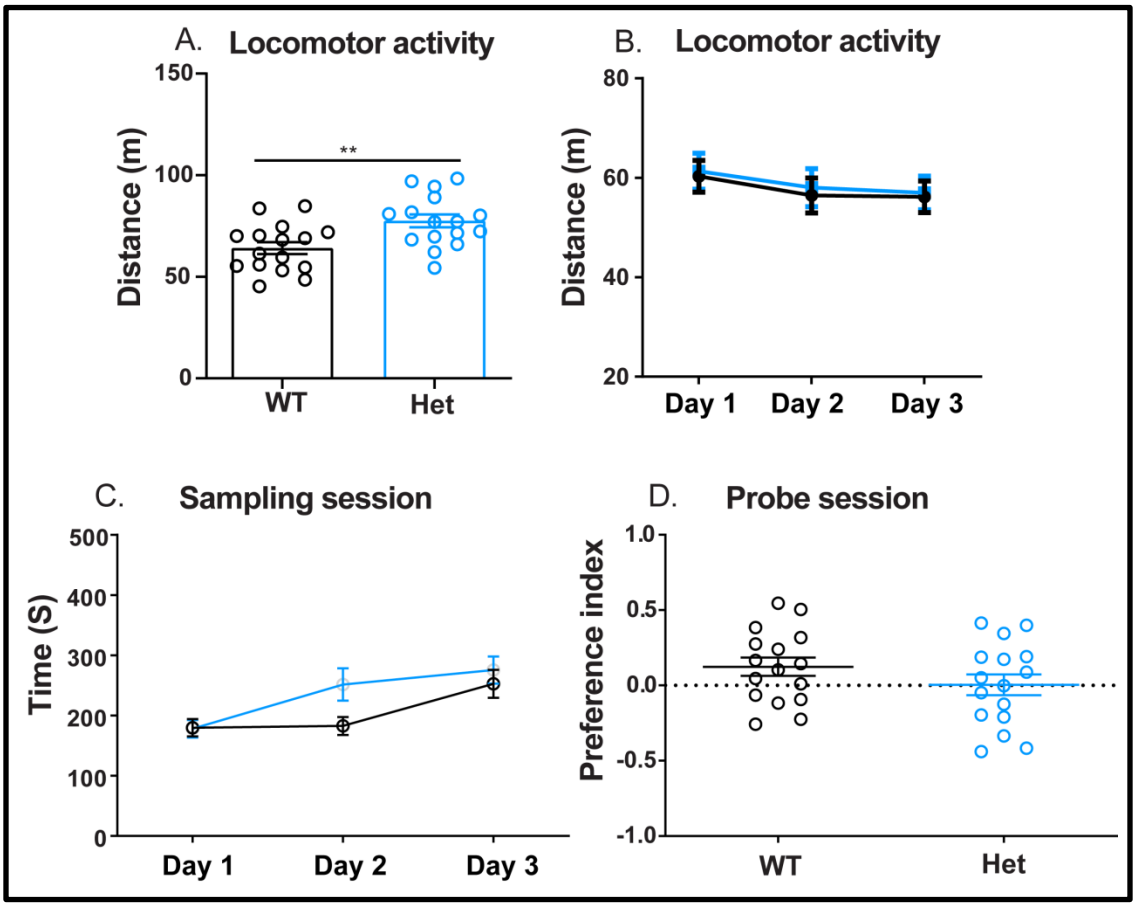


Figure.5.2. *SynGAP*$^{+/-}$ rats show transient hyperactivity and comparable spatial learning and memory. A. *SynGAP*$^{+/-}$ rats show hyperactivity during first session of habituation (Unpaired t test, p=0.004**). B. WT and *SynGAP*$^{+/-}$ rats show similar locomotion during rest of the habituation sessions (Repeated measures two way ANOVA, Genotype: $F_{(1, 30)}$ = 0.05860, p=0.81; Interaction: $F_{(2, 60)}$ = 0.04773, p=0.95).C. WT and *SynGAP*$^{+/-}$ rats show similar acquisition profiles during sampling sessions (Repeated measures two way ANOVA, Genotype: $F_{(1, 30)}$ = 2.19, p=0.14; Interaction: $F_{(2, 60)}$ = 2.18, p=0.12). D. WT and *SynGAP*$^{+/-}$ rats show similar preference to object placed at novel location (Unpaired t test, p=0.2). WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.3. SynGAP$^{+/-}$ rats show impaired acquisition and comparable fear recall, extinction and extinction recall during auditory fear conditioning task

Further I investigated the effects of the genetic manipulation in *SynGAP* on emotional learning and memory. To that end I employed a Pavlovian fear conditioning task to test emotional learning and memory in *SynGAP*$^{+/-}$ rats and their WT control littermates. Animals were habituated to a recall context (Context A) for two days followed by auditory fear conditioning in a separate context (Context B) on day 3. Recall for the fear memory and extinction was tested on day 4 followed by extinction recall on day 5 in the habituation context (Context A) (see Materials and Methods). *SynGAP*$^{+/-}$ rats showed reduced freezing compared to the WT rats during conditioning where the rats had to associate innocuous tone (conditional stimulus) with aversive foot shock (unconditional stimulus) (**Fig. 5.3.A**). Interestingly, they exhibited similar freezing profiles during recall-extinction and extinction recall phases of auditory fear conditioning task (**Fig.5.3.B&C**). Hence, genetic perturbation in *SynGAP* did not alter the ability to recall and extinguish fear memory.

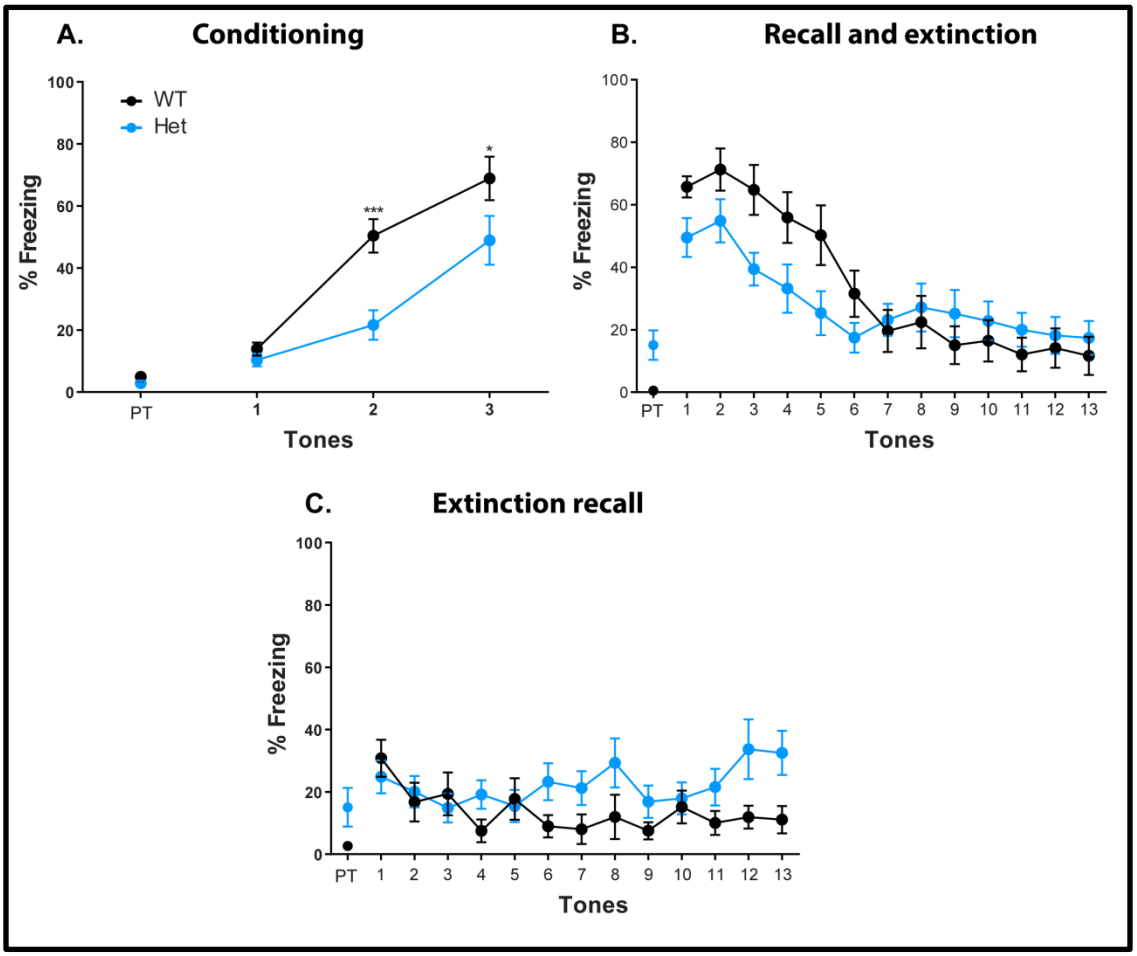


Figure.5.3. A. *SynGAP*$^{+/-}$ rats show reduced freezing during conditioning phase of auditory fear conditioning task. Repeated measure two way ANOVA, Genotype: $F_{(1, 30)}$ = 11.82, p=0.001**; Interaction: $F_{(3, 90)}$ = 4.513, p=0.005**, post hoc: Sidak's multiple comparisons test, p<0.0001***, p<0.05*. B. *SynGAP*$^{+/-}$ rats show comparable freezing levels during recall and extinction phase of auditory fear conditioning task. C. *SynGAP*$^{+/-}$ rats show comparable freezing levels during extinction recall phase of auditory fear conditioning task. Repeated measure two way ANOVA followed by Sidak's multiple comparisons test. WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.4. SynGAP$^{+/-}$ *rats show motor impairment in rotarod task*

To test for motor coordination and impairments in motor learning due to underlying transgenic mutation we performed a rotarod test on *SynGAP*$^{+/-}$ rats and compared their performance to WT controls. A baseline was first established for each animal followed by a testing phase where the task was made progressively harder with an accelerating rotarod (see Materials and Methods). We found the *SynGAP*$^{+/-}$ rats to perform comparably to the WT control population across all 5 days of the assay in the baseline trials (**Fig.5.4A**). However, *SynGAP*$^{+/-}$ rats show impaired motor performance during accelerating phase of rotarod task. (**Fig.5.4B**). Therefore the genetic perturbation resulted in impairment of motor learning in the *SynGAP*$^{+/-}$ rats.

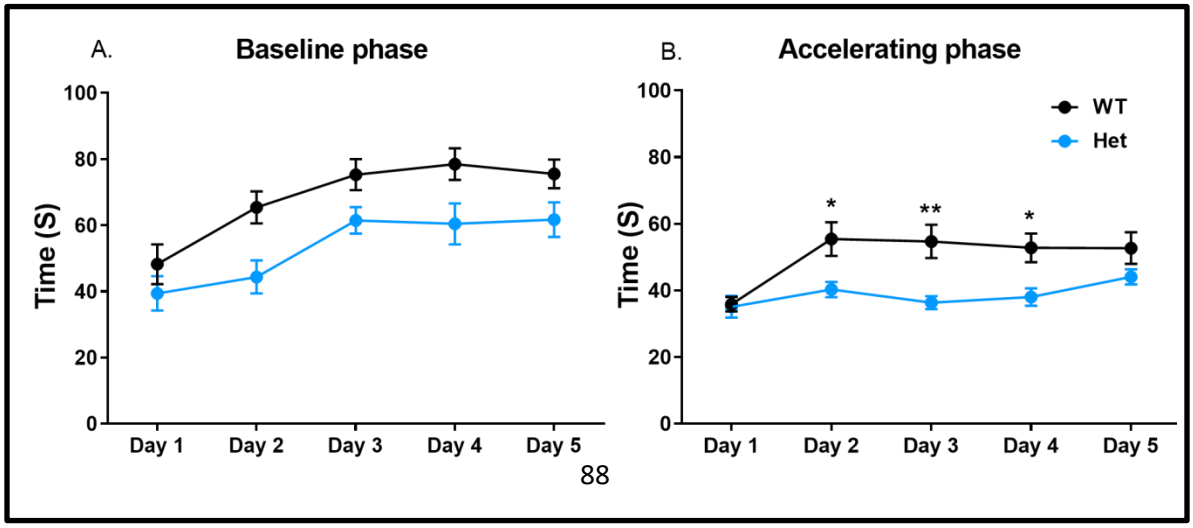


Figure.5.4. *SynGAP*$^{+/-}$ rats display motor impairment in rotarod task. A. Both WT and *SynGAP*$^{+/-}$ rats performed similarly over all 5 days during baseline phase of rotarod. Repeated measures two way ANOVA. Genotype: $F_{(1, 28)} = 12.17$, p=0.001**; Interaction: $F_{(4, 112)} = 0.5209$, p=0.72. B. *SynGAP*$^{+/-}$ rats show impaired motor learning to fall during accelerating phase of rotarod. Repeated measures two way ANOVA. Genotype: F (1, 28) = 13.64, p=0.0009***; Interaction: F (4, 112) = 2.737, p=0.03*. Post hoc test: Sidak's multiple comparison test, p<0.001**, p<0.05*. WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.5. Acute thermal pain sensitivity is unaffected in SynGAP$^{+/-}$ *rats*

In order to test for nociception using thermal stimuli, I utilized a tail-flick test in both *SynGAP*$^{+/-}$ and WT control rats. Rats were habituated to a restrainer for 3 days prior to the experiment and then tested for latency to flick their tail after the onset of a thermal stimulus (see Materials and Methods). We found the *SynGAP*$^{+/-}$ and WT rats to have a similar latency to flick their tail upon exposure to the thermal stimulus (**Fig 5.5.**WT: 2.73 ± 0.14s; *SynGAP*$^{+/-}$: 3.06 ± 0.14s).

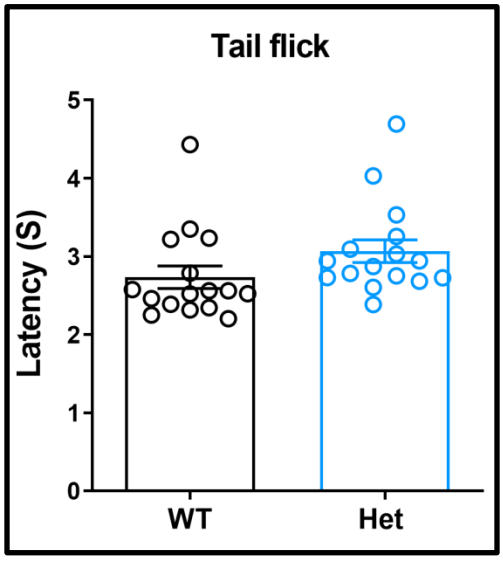


Figure.5.5. *SynGAP*$^{+/}$ and WT rats display similar tail flick latencies. Unpaired t test, p=0.11. WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.6. SynGAP$^{+/-}$ rats show intact short term object location recognition memory

To test the short term spatial memory I used object location recognition task which was done in similar setup used to test long term version described earlier (Fig. 2). Sampling and testing was done on the same day with a retention period of 30 minutes. Our investigation revealed no significant difference between WT and *SynGAP*$^{+/-}$ rats in preference towards object placed at novel location (Fig.6.WT: 0.19 ± 0.08; *SynGAP*$^{+/-}$: 0.12 ± 0.09). Hence, mutation in rat *SynGAP* had no influence over short term object location memory.

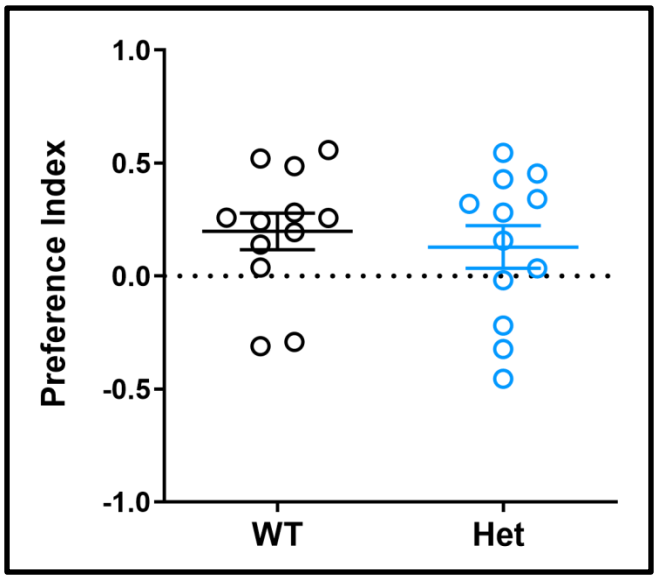


Figure 5.6. *SynGAP*$^{+/-}$ and WT rats show similar short term memory for object at novel location. Unpaired t test, p=0.58. WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.7.SynGAP$^{+/-}$ rats show unaltered non associative and associative short term memory in spontaneous alteration tasks.

Next I wanted to investigate novelty based associative and non-associative memory tasks in both WT and *SynGAP*$^{+/-}$ rats. For this, we employed novel object recognition and object place context tasks. These tests rely on innate exploratory nature of the rats towards environmental novelty. Both WT and *SynGAP*$^{+/-}$ rats show similar preference to novelty in both these tasks (**Fig.5.7A**.WT: 0.2 ± 0.07; *SynGAP*$^{+/-}$: 0.19 ± 0.07; **Fig.5.7B**.WT:

0.09 ± 0.06; *SynGAP*$^{+/-}$: -0.01 ± 0.05). Genetic perturbation in *SynGAP* did not result in short term memory deficits in these tasks.

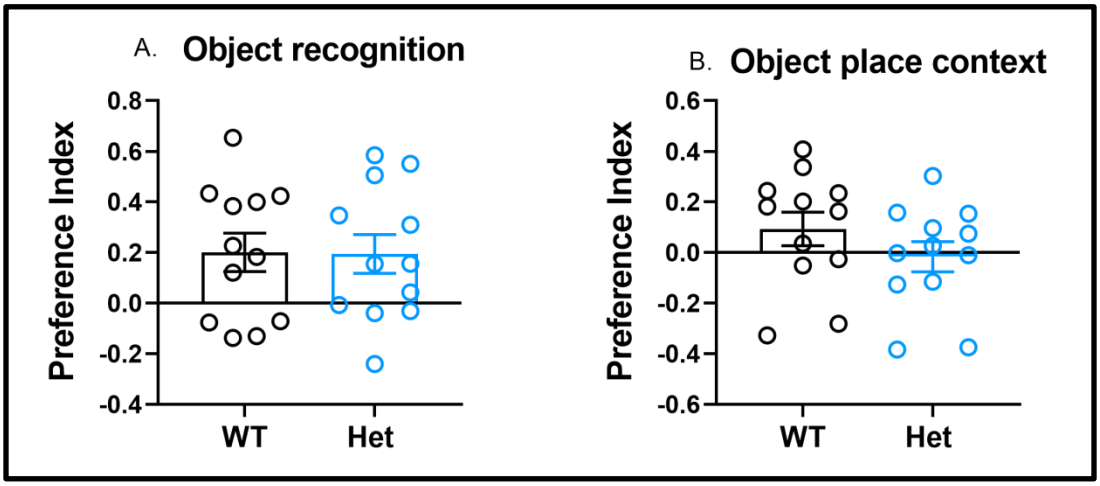


Figure.5.7. *SynGAP*$^{+/-}$ rats show comparable novelty based spontaneous exploration of objects. A. *SynGAP*$^{+/-}$ and WT rats show similar preference to novel object (Unpaired t test, p=0.95). B. Both groups show similar preference to object place context dependent novelty (Unpaired t test, p=0.23). WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.8. SynGAP$^{+/-}$ *rats show social deficits compared to WT rats in three chambered social interaction task*

Further, I investigated the social interaction in both WT and *SynGAP*$^{+/-}$ rats using three chambered apparatus. Post habituation rats were tested for sociability where rats had a choice between a novel object and stranger conspecific rat. In the subsequent phase of the task the rats were tested for social novelty where they had an option to interact either with familiar rat or a novel conspecific rat (see methods). Behavior was recorded with an overhead camera and offline analysis of the videos was carried out to measure time spent in interaction for both the phases of the experiment. *SynGAP*$^{+/-}$ rats displayed reduced interaction time with the stranger animal during sociability phase compared to WT rats (**Fig. 5.8.A.**WT: 154.12 ± 15.37s; *SynGAP*$^{+/-}$: 49.61 ± 10.98s). Additionally, *SynGAP*$^{+/-}$ rats also displayed overall reduction in interaction with both familiar (**Fig.5.8.**

B. WT: 84.25 ± 18.75s; *SynGAP*$^{+/-}$: 5.83 ± 1.12 s) and novel conspecific rats (**Fig.5.8. B.** WT: 105.12 ± 14.75s; *SynGAP*$^{+/-}$: 10.66 ± 2.03s) compared to control rats during social novelty phase of the task. Hence, *SynGAP*$^{+/-}$ rats show deficits in social interaction in three chambered social interaction task which is in line with the previous mouse literature. However, it is important to ascertain if the deficits observed is consistent with respect to other social behavioral tests like social propinquity, juvenile play and conspecific empathy.

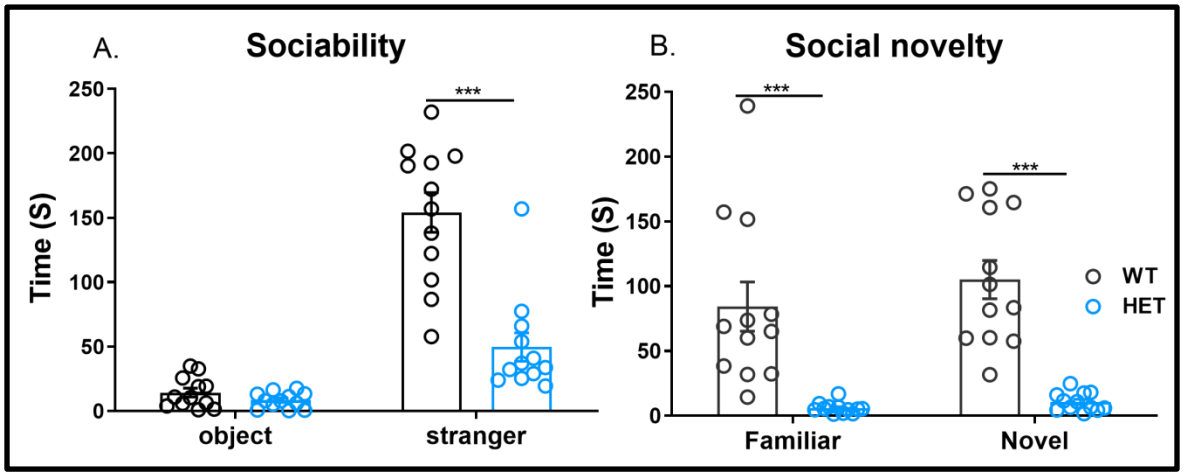
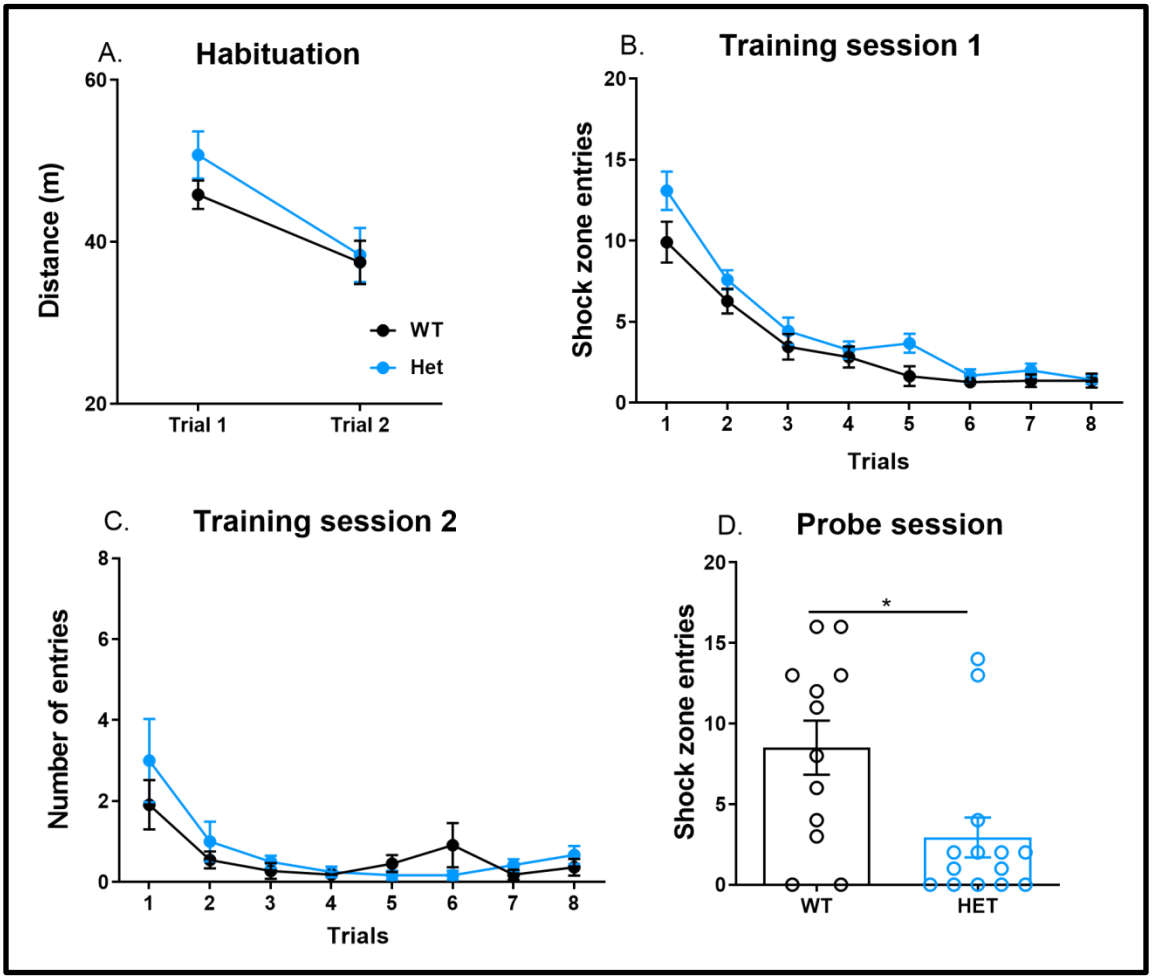


Figure.5.8. *SynGAP*$^{+/-}$ rats displayed impaired social interaction. A. *SynGAP*$^{+/-}$ rats show reduced exploration of social stimulus during sociability phase. Two way ANOVA, Genotype: $F_{(1, 44)} = 32.76$, p<0.0001***, Interaction: $F_{(1, 44)} = 26.16$, p<0.0001***.Post hoc: Sidak's multiple comparison test, p<0.0001***. B. *SynGAP*$^{+/-}$ rats show impaired social novelty exploration. Two way ANOVA, Genotype: $F_{(1, 44)} = 51.17$, p<0.0001***; Interaction: $F_{(1, 44)} = 0.4404$, p=0.51. Post hoc: Sidak's multiple comparison test, p<0.0001***. WT, N=16; *SynGAP*$^{+/-}$, N=16. Data represented as mean ± SEM.

5.9. SynGAP$^{+/-}$ *rats delayed acquisition, enhanced avoidance memory and impaired extinction in reversal session of active place avoidance task*

Post assessment of social behavior, I investigated active place avoidance in WT and *SynGAP*$^{+/-}$ rats using a rotational platform. After habituation rats were trained over two days to avoid a shock zone on a slowly rotating circular arena. On the subsequent day,

rats were tested for avoidance memory in a shock less probe trial immediately followed by reversal learning session (see methods). Both WT and *SynGAP*$^{+/-}$ rats showed similar locomotion profiles during habituation (**Fig. 5.9A**). *SynGAP*$^{+/-}$ rats show initial increase in shock zone entries during the first training session compared to WT rats (**Fig. 5.9.B**). However, they showed comparable number of shock zone entries during second training session (**Fig. 5.9.C**). Interestingly, *SynGAP*$^{+/-}$ rats made significantly less entries into shock zone compared to WT rats during probe session (**Fig.5.9.D.**WT: 8.5 ± 1.68; *SynGAP*$^{+/-}$: 2.9 ± 1.23). Furthermore, *SynGAP*$^{+/-}$ rats made similar number of entries into shock zone compared to WT rats during the reversal session. Further minute wise analysis of time spent in old shock zone during first trial of reversal learning revealed impaired extinction memory in *SynGAP*$^{+/-}$ rats (**Fig. 5.9.F.**WT: 84.34 ± 19.1s; *SynGAP*$^{+/-}$: 16.06 ± 6.11s). Hence, *SynGAP* heterozygosity resulted in altered active avoidance learning and memory in these rats.



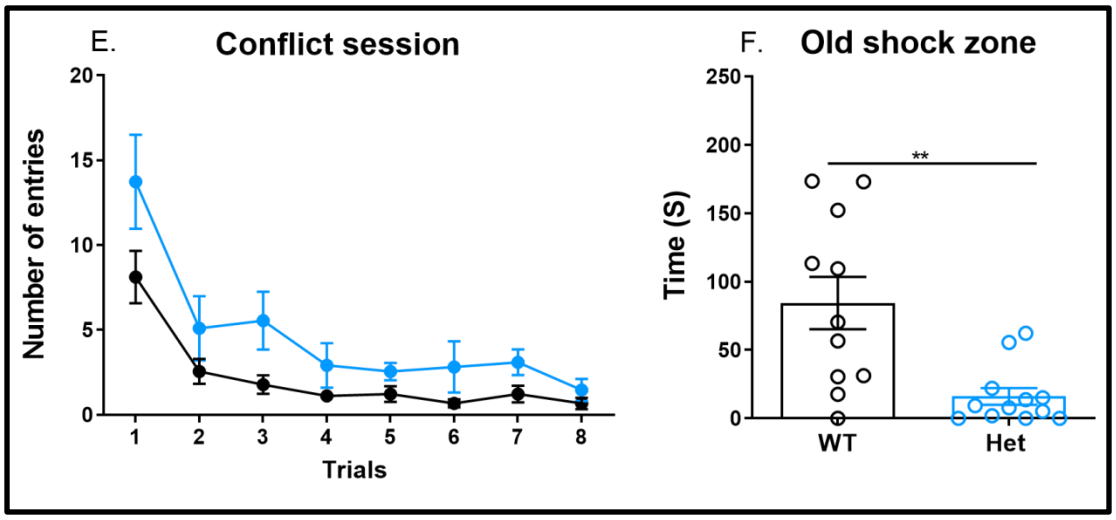


Figure.5.9. *SynGAP*$^{+/-}$ rats showed comparable acquisition, robust avoidance memory and impaired extinction memory for shock zone in active place avoidance task. A. *SynGAP*$^{+/-}$ and WT rats displayed similar locomotion during habituation session. Repeated measures two way ANOVA, Genotype: $F_{(1, 19)} = 0.6681$, p=0.42; Interaction: $F_{(1, 19)} = 1.486$, p=0.23. B. *SynGAP*$^{+/-}$ rats display delayed avoidance learning compared to the WT rats. Repeated measures two way ANOVA, Genotype: $F_{(1, 21)} = 5.589$, p=0.02*; Interaction: $F_{(7, 147)} = 1.325$, p=0.24. C. *SynGAP*$^{+/-}$ rats display comparable avoidance during second training session compared to the WT rats. Repeated measures two way ANOVA, Genotype: $F_{(1, 21)} = 0.4372$, p=0.51; Interaction: $F_{(7, 147)} = 1.086$, p=0.37. D. *SynGAP*$^{+/-}$ rats display enhanced avoidance during probe session. Unpaired t test, p=0.01*. E. *SynGAP*$^{+/-}$ rats display similar avoidance compared to WT rats during conflict session. Repeated measures two way ANOVA, Genotype: $F_{(1, 18)} = 3.706$, p=0.07; Interaction: $F_{(7, 126)} = 1.198$, p=0.3. F. *SynGAP*$^{+/-}$ rats spend less time in old shock zone during first trial of conflict session.

5.10. Discussion

Synaptic dysfunction is often identified as underlying mechanism of ID and ASD (Zoghbi and Bear, 2012). SYNGAP is a synaptic protein which is critical for synaptic development, structure, function and plasticity. SYNGAP is a cytosolic protein which negative regulates small GTPases (e.g., Ras, Rap) and controls α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) insertion to the postsynaptic membrane

(Sheng and Kim, 2011). SynGAP is associated with postsynaptic density (PSD) proteins and is an important constituent of NMDA receptor signaling complex (Chen *et al.*, 1998; Kim *et al.*, 1998; Bayés *et al.*, 2011). SynGAP interacts with various other synaptic proteins to form macromolecular complexes within dendritic spines (Walkup *et al.*, 2015; Zeng *et al.*, 2016). Furthermore, SynGAP also has role in regulating mRNA translation in excitatory neurons (Barnes *et al.*, 2015). Above mentioned roles of SYNGAP makes it an ideal candidate in pathogenesis of ASD. Children with heterozygous de novo mutations in SynGAP present with moderate to severe intellectual disability, epilepsy, high pain threshold, hypotonia, gait abnormalities, autism like phenotypes including repetitive behaviors, reduced social interactions and language delay(Hamdan *et al.*, 2009; Berryer *et al.*, 2013; Vlaskamp *et al.*, 2019). In this study I have attempted to behaviorally characterize the novel *SynGAP*$^{+/-}$ rat. To do so, I have employed multiple behavioral experiments (see methods).

To begin with I observed increased locomotion during first habituation session after which their locomotion profile in subsequent days were similar to the WT rats. During testing phase I observed reduced number of marbles buried and reduced interaction time with marbles. Additionally, there was no difference in digging onset and total time spent in digging. Interestingly, I observed significant increase in repetitive grooming in *SynGAP*$^{+/-}$ rats. Studies in *SynGAP*$^{+/-}$ mice also have reported hyperactivity, increased time in center in open field and increased open arm time in elevated plus maze which are indicative are less anxiety (Muhia *et al.*, 2010; Ozkan *et al.*, 2014). Recent study in SynGAP heterozygous rats with loss of enzymatic function (GAP deletion) did not show hyper activity in open field and anxiety like behavior in elevated plus maze tasks (Katsanevaki *et al.*, 2020). Grooming behavior requires complex repetitive, self-directed and sequential motor patterns and thus might be of translational value considering behavioral perseveration observed in patients with ASD (Kalueff *et al.*, 2016). Hence, the variability of phenotypes observed in both mouse and rat models of *SynGAP* heterozygosity depends upon the mutations in different functional isoforms of SYNGAP and the genetic background of the rodent.

SynGAP $^{+/-}$ rats were subjected to cognitive assessment using object location recognition task. Locomotion profile was similar to the previous task where rats showed transient increase in first habituation session only. *SynGAP*$^{+/-}$ rats show comparable exploration to that of WT rats during acquisition phase and exhibited similar preference to object at novel location. Previous studies in *SynGAP* heterozygous mice show variable results with spatial learning. Komiyama and colleagues reported deficits in spatial learning and memory in Morris water maze test (Komiyama *et al.*, 2002). Whereas, Muhia and colleagues have reported intact reference memory in water maze task but deficits in working memory in radial arm maze (Muhia *et al.*, 2010). Further, Guo and colleagues also reported spatial working memory deficits in radial arm maze (Guo *et al.*, 2009). Another study in mice reported deficits in spatial reference memory in Barnes maze and working memory in T- maze and Y-maze tests (Nakajima *et al.*, 2019). A recent study in SynGAP heterozygous rat with GAP deletion did not show spatial learning and memory deficits in Morris water maze test (Katsanevaki *et al.*, 2020). It is imperative to assume that the discrepancies observed in spatial learning and memory deficits is task dependent and requires different neural substrates for normal behavior. Lastly, most of the mouse studies and a recent rat study had reported decreased seizure threshold and absence seizures. The onset of absence seizures could confound the behavioral results in these tasks. However, we need to ascertain if our rat model displays the seizure phenotypes observed in other studies.

Further I explored spontaneous alternation in these rats. I employed novel object recognition and object place context tasks. I report similar preference for novel object recognition in both WT and *SynGAP*$^{+/-}$ rats. Previous studies in mice also showed comparable novel object recognition in *SynGAP*$^{+/-}$ mice (Muhia *et al.*, 2010; Yokoi *et al.*, 2017). Another study which was a modified version of novel object recognition which utilized novelty of object texture found that *SynGAP*$^{+/-}$ mice had deficits in whisker dependent senorsy processing (Michaelson *et al.*, 2018). Thus, the data from this study is in line with the previous published reports. Next, I tested these rats for object place context memory and found them to have a similar memory for novelty in this task

compared to WT rats. This is in line with a recent study in SnyGAP rats lacking enzymatic function due to deletion of C2/GAP domain of *SynGAP,* which also report similar results in these tests (Katsanevaki *et al.*, 2020). Hence, heterozygous mutation in *SynGAP*$^{+/-}$ rats doesn't alter working memory in spontaneous alternation tasks.

SynGAP$^{+/-}$ rats were tested for emotional learning and memory in Pavlovian fear conditioning task. Animals were subjected to auditory fear conditioning to assess the acquisition, recall, extinction learning and extinction recall. I observed reduced freezing in *SynGAP*$^{+/-}$ rats compared to WT rats only during conditioning. Freezing was found to be normal during all other phases of the fear conditioning task. One reason for reduced freezing responses during the conditioning phase would be sensitivity towards the foot shock which we have not measured in this study. A recent study in multiple mice and rat models of ASD reported problems in touch and pain processing (Orefice, 2020). Second reason we presume would be the hyperactivity in novel contexts which was consistently observed in previous experiments with these rats could affect freezing levels upon exposure to foot shocks during conditioning. Previous research in conditional forebrain *SynGAP*$^{+/-}$ mice showed reduced freezing in contextual fear conditioning (Guo *et al.*, 2009; Ozkan *et al.*, 2014). Another group have reported reduced freezing during recall in cued fear conditioning task (Guo *et al.*, 2009). Contrastingly, another study reported unaltered freezing response in contextual and cued fear conditioning tasks (Yokoi *et al.*, 2017). Furthermore, ethologically relevant amygdala dependent defensive behaviors are required to ascertain the role of SYNGAP in associative or non – associative fear behaviors.

I went on to assess another form of defensive behavior using active place avoidance task in these rats. I observed similar avoidance learning in both WT and *SynGAP*$^{+/-}$ rats in both the training sessions. Interestingly, we found *SynGAP*$^{+/-}$ rats to possess stronger avoidance memory for shock zone compared to WT rats in the probe trial. WT rats learnt the absence of foot shock early and ventured multiple times into the shock zone whereas *SynGAP*$^{+/-}$ rats had stronger avoidance memory hence did not show extinction.

This was further confirmed during reversal learning in conflict session where *SynGAP*$^{+/-}$ rats showed cognitive inflexibility. On the first trial of conflict session the *SynGAP*$^{+/-}$ rats spent less time in old shock zone compared to WT rats which means WT rats showed extinction during probe trial and *SynGAP*$^{+/-}$ rats did not. It is known in both humans and rodents that mPFC is involved in cognitive flexibility (Shin and Liberzon, 2010; Ko, 2017) and altered mPFC function has been described in Syngap$^{+/-}$ mice (Clement *et al.*, 2013; Ozkan *et al.*, 2014).

SynGAP$^{+/-}$ rats show motor learning impairment in rotarod task. As described earlier patients with *SynGAP* mutations show motor abnormalities including gait, balance, fine motor control (Parker *et al.*, 2015; Mignot *et al.*, 2016). We used rotarod test assess motor function in *SynGAP*$^{+/-}$ rats. We do not observe normal gross motor coordination deficits during steady phase of rotarod test in *SynGAP*$^{+/-}$ rats. Interestingly, *SynGAP*$^{+/-}$ rats showed reduced fall latency compared to WT rats during accelerating phase of rotarod test. Studies in SynGAP heterozygous mice have reported similar deficits in motor performance (Nakajima *et al.*, 2019). Surprisingly, another study in rats lacking enzymatic function due to deletion of C2/GAP domain of *SynGAP* did not show motor deficits in rotarod (Katsanevaki *et al.*, 2020). More studies utilizing are required to determine if mutations in different isoforms of *SynGAP* would lead to different behavioral deficits in rats.

SynGAP$^{+/-}$ rats do not show any impairment in acute thermal nociception in tail flick assay. Both WT and *SynGAP*$^{+/-}$ rats displayed similar latency to flick tail to thermal stimulus. Reports in human patients with *SYNGAP* mutation show there is increased pain threshold (Parker *et al.*, 2015; Vlaskamp *et al.*, 2019). Previous studies also reported unaltered mechanical and thermal pain sensitivity in *SynGAP* heterozygous mice (Muhia *et al.*, 2010; Duarte *et al.*, 2011). However, one study in *SynGAP* heterozygous mice show hyposensitivity to pain in hot plate pain sensitivity test (Nakajima *et al.*, 2019). Findings in this study are in line with previous studies.

SynGAP$^{+/-}$ rats show social deficits in three chambered social interaction task. Social deficits are consistently observed in patients lacking functional SYNGAP protein (Hamdan *et al.*, 2009; Berryer *et al.*, 2013; Vlaskamp *et al.*, 2019). In this study I observed both WT and *SynGAP*$^{+/-}$ rats significantly chose to interact with social stimulus over a novel object during sociability test. However, the *SynGAP*$^{+/-}$ rats explored significantly lesser compared to WT rats. Strangely, during the social novelty phase both the WT rats and *SynGAP*$^{+/-}$ rats did not show significant difference between familiar and novel social stimuli. There was strong genotype effect between WT and *SynGAP*$^{+/-}$ rats but lacked interaction effect. This could be due to the significant reduction in exploration of social stimulus (both familiar and social) by *SynGAP*$^{+/-}$ rats during the social novelty phase compared to WT rats. A previous study in mice showed similar deficits in social in sociability but not social novelty (Nakajima *et al.*, 2019). Another study in mice reported deficits in social novelty in *SynGAP*$^{+/-}$ mice (Berryer *et al.*, 2016). Further, a recent study in rats lacking enzymatic function due to deletion of C2/GAP domain of *SynGAP* show social deficits in three chambered social interaction task (Katsanevaki *et al.*, 2020). The reduced social interaction can be attributed to the reduced exploratory drive rather than social deficits in these rats. Interestingly, previous research in *SynGAP*$^{+/-}$ mice reported abnormal tactile processing which also conforms to the clinical studies with impairments in sensory processing in patients with *SynGAP* mutations (Michaelson *et al.*, 2018; Weldon *et al.*, 2018). These studies indicate impaired social deficits could be due to altered sensory processing while exploring social stimuli.

5.11. Conclusion

synaptopathy resulting from *SynGAP* heterozygosity has complex behavioral outcomes and hence further studies are warranted to understand the pathophysiology. Further, different isoforms of SYNGAP mediates different functions (Kilinc *et al.*, 2022). Thus, one should be careful while comprehending the phenotypes observed in the transgenic models of *SynGAP* haploinsuffiency. This study from a new rat model with altered

scaffolding function of SYNGAP protein highlights unique behavioral phenotypes in regards to the *SYNGAP* haploinsufficency.

Chapter.6. Pten$^{-/+}$ rats show impairment in social and cognitive behaviors

Around 10 % patients with increased brain size (macrocephaly) and intellectual disability were associated with ASD (Rademacher and Eickholt, 2019). Germline mutations in Phosphatase and tensin homolog (PTEN) are found in patients with four PTEN hamartoma syndromes: Bannayan- Riley-Ruvalcaba syndrome (BRRS), Cowden syndrome (CS), Proteus syndrome, and Proteus-like conditions (Butler, 2005). Recent data indicates around 25% of patients with PTEN mutations may fall in the category of ASD (Cummings, 2022). Children with PTEN mutations displayed ASD-like phenotypes which include lower global IQ, working memory impairment, attention deficits, reduced motor skills, perseverative behavior and strong social interaction deficits (Frazier, 2019). Lack of PTEN, results in hyper-activation of PI3K/Akt/mTOR pathway leading to altered cycle progression, induction of cell death, transcription, translation, stimulation of angiogenesis, and stem cell self-renewal (Lugo *et al.*, 2014; Milella *et al.*, 2015). Loss of PTEN results in disordered laminar neuronal arrangement in neo-cortex, increased cell size and various autism related behavioral deficits in *Pten* cKO mice (Kwon *et al.*, 2006). Several mouse models of *Pten* haploinsufficency replicate clinical findings in humans and have contributed for the understanding of molecular functions of PTEN (Clipperton-Allen and Page, 2014; Chen, 2019; Huang, Chen and Page, 2019; Clipperton-Allen *et al.*, 2021). In this thesis I have behaviorally characterized novel transgenic rat model of *Pten* haplo-insufficiency.

6.1. Pten $^{+/-}$ rats do not show anxiety or repetitive behavior in marble interaction task

To begin with, I investigated if *Pten*$^{+/-}$ rats showed any baseline anxiety like behavior or repetitive behavior in the marble interaction assay. This investigation revealed no significant difference in total number of marbles buried (**Fig.6.1.A.** WT: 1.92±0.48;

Pten$^{+/-}$: 0.92 ± 0.24), marble interaction time (**Fig.6.1.B.** WT: 123.6±20.29s; *Pten*$^{+/-}$: 115.1 ± 16.37 s), digging onset (**Fig.6.1.C**. WT: 144.4 ± 30.07s; *Pten*$^{+/-}$: 186.4 ± 67.50s) and time spent in grooming (**Fig.6.1.E.**WT: 47.97 ± 11.79; *Pten*$^{+/-}$: 44.96 ± 11.16s). Interestingly, *Pten*$^{+/-}$ rats spent significantly less time in digging compared to WT rats (**Fig.6.1.D.** WT: 50.73±14.99s; *Pten*$^{+/-}$: 14.37 ± 5.43). With all the parameters observed the *Pten*$^{+/-}$ rats did not show repetitive interaction with marbles / compulsive burying compared to control rats.

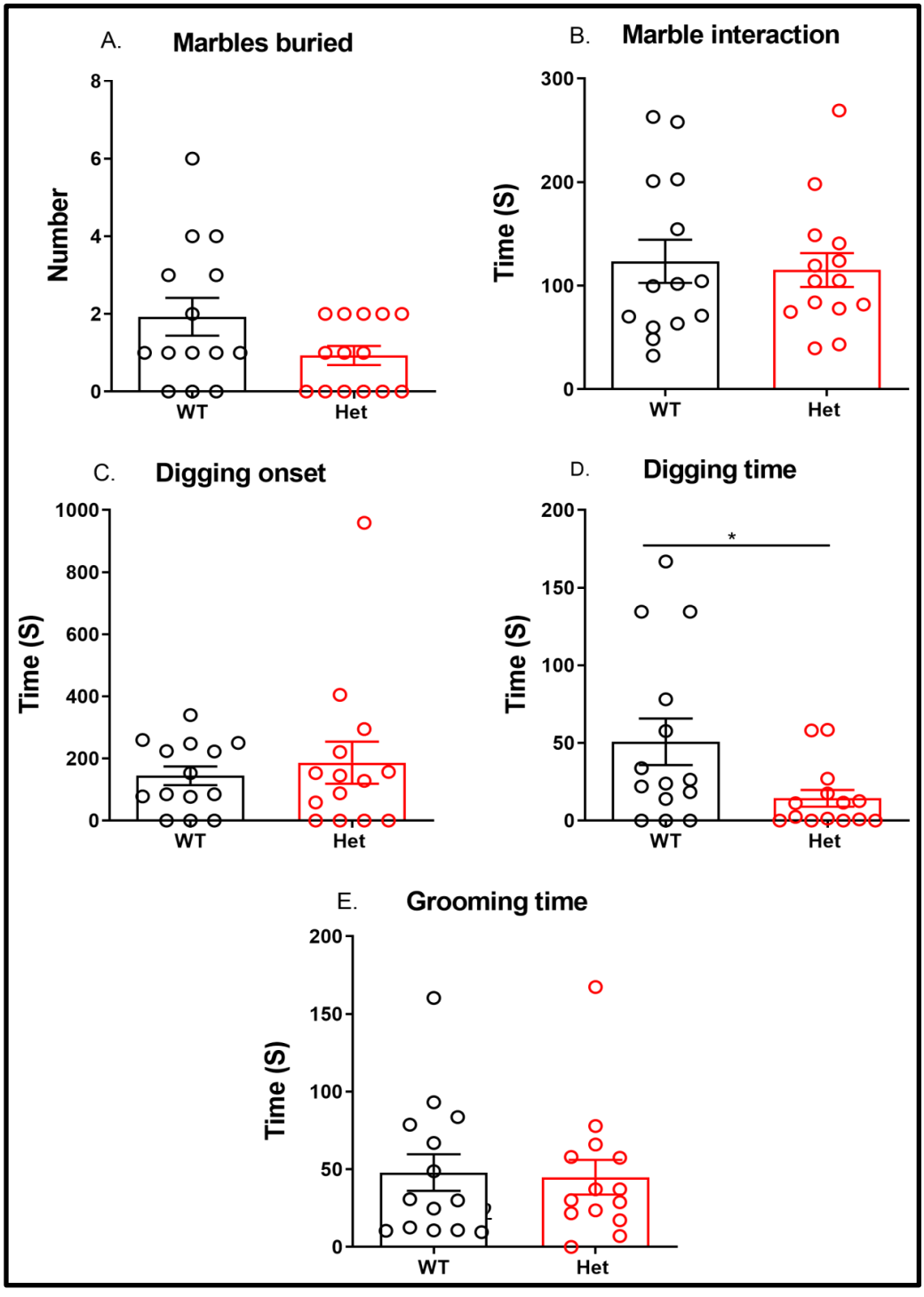
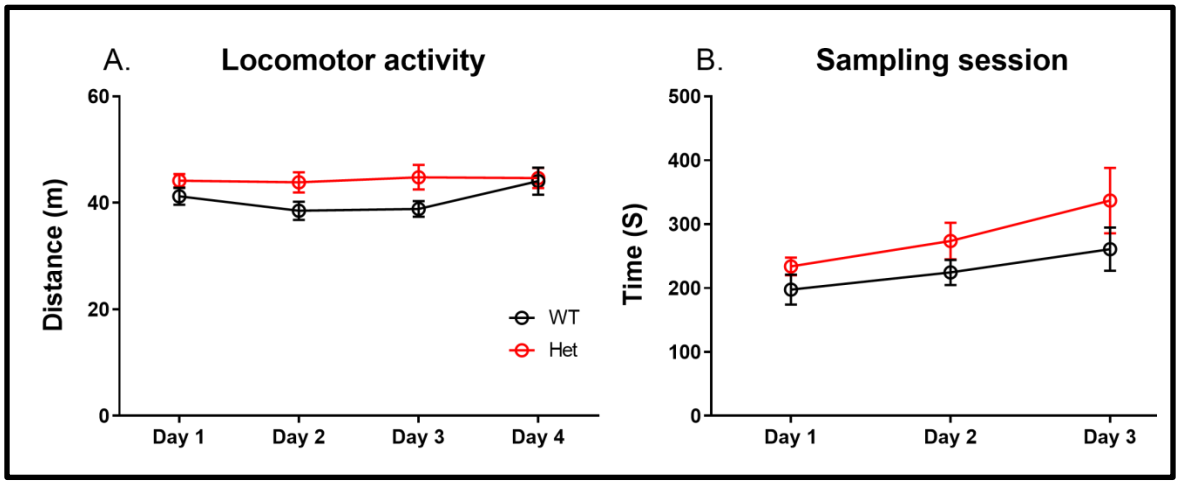


Figure.6.1. A. *Pten*$^{+/-}$ and WT rats display similar marble burying behavior. Mann Whitney test, p=0.15. B. *Pten*$^{+/-}$ and WT rats display comparable marble interaction time. Unpaired t test: p=0.74. C. *Pten*$^{+/-}$ and WT rats show similar latency to dig. Unpaired t test: p=0.57. D. *Pten*$^{+/-}$ dig significantly less compared to WT rats. Unpaired t test: p=0.03*. E. *Pten*$^{+/-}$ and WT rats spend similar time in grooming. Unpaired t test: p=0.85. WT, N=14; *Pten*$^{+/-}$, N=14. Data represented as mean ± SEM.

6.2. Pten $^{+/-}$ rats show deficit in spatial memory in long term object location recognition task

Next, I investigated if genetic mutation in *Pten* resulted in deficits in spatial learning and memory. To do so I employed long term object location recognition task. The behavioral analyses revealed both WT and *Pten*$^{+/-}$ rats show comparable loco-motor activity during habituation session (**Fig.6.2.A**). This was followed by sampling session where time taken to acquire 60 seconds overall object exploration of the objects was measured. Both WT and *Pten*$^{+/-}$ rats took similar exploration time during sampling session of objection location recognition task (**Fig.6.2.B**). Interestingly, during the probe session the *Pten*$^{+/-}$ rats show reduced preference to the object at novel location compared to WT rats (**Fig.6.2.C.** WT: 0.27 ± 0.03;*Pten*$^{+/-}$:-0.10 ± 0.06). Therefore, *Pten*$^{+/-}$ rats show normal spatial learning but impaired memory in long-term object location recognition task.



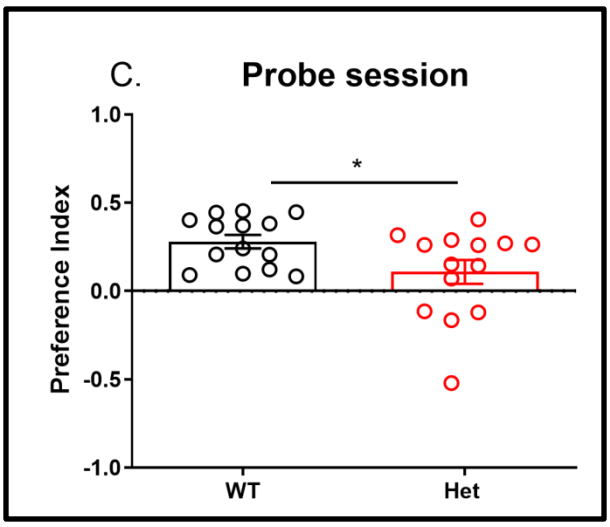


Figure.6.2. *Pten*$^{+/-}$ rats showed impaired spatial memory in object location recognition task. A. *Pten*$^{+/-}$ and WT rats show similar loco motor activity during habituation session. Repeated measures two way ANOVA (Genotype: $F_{(1, 26)} = 3.398$, p=0.07; Interaction: $F_{(3, 78)} = 1.524$, p=0.21.B. *Pten*$^{+/-}$ and WT rats show similar acquisition profile during sampling session. Repeated measures two way ANOVA (Genotype: F (1, 18) = 2.717, p=0.11; Interaction: F (2, 36) = 0.3235, p=0.72. C. *Pten*$^{+/-}$ rats show reduced preference to object placed at novel location compared to WT rats. Unpaired t test: p<0.03*. WT, N=14; *Pten*$^{+/-}$, N=14. Data represented as mean ± SEM.

6.3. Pten $^{+/-}$ rats show normal associative fear learning and memory in auditory fear conditioning task

To investigate the effect of genetic perturbation in *Pten* on emotional learning and memory I employed an auditory fear conditioning task. Both the genotypes showed similar pre-tone freezing during all the phases of fear conditioning task. *Pten*$^{+/-}$ rat's show similar freezing compared to WT rats during conditioning (**Fig.6.3.A**). Similarly, they show comparable freezing behavior during both recall -extinction and extinction recall phases (**Fig.6.3.B & C**). Thus, genetic mutation in *Pten* did not alter their ability to learn, retain and extinguish the cued fear memory during auditory fear conditioning task.

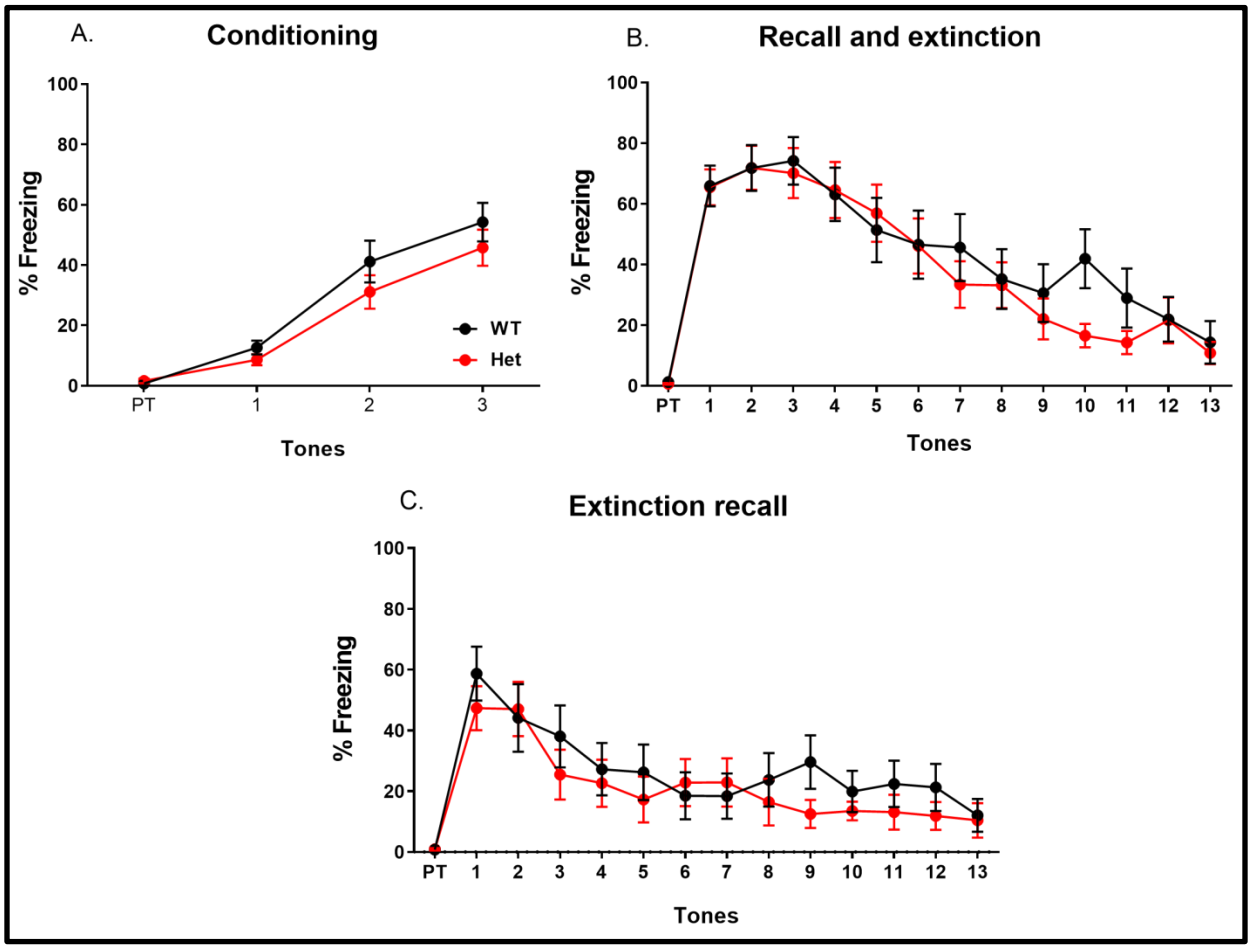


Figure.6.3. *Pten*$^{+/-}$ rats show unaltered emotional freezing responses duringauditory fear conditioning task. A. *Pten*$^{+/-}$ and WT rats show comparable freezing during conditioning. Repeated measures two way ANOVA (Genotype: $F_{(1, 26)} = 2.85$, p=0.10; Interaction: $F_{(3, 78)} = 0.59$, p=0.62. B. *Pten*$^{+/-}$ and WT rats show similar freezing during recall and extinction phase. Repeated measures two way ANOVA (Genotype: $F_{(1, 26)} = 0.32$, p=0.57; Interaction: $F_{(13, 338)} = 1.03$, p=0.41. C. *Pten*$^{+/-}$ and WT rats show similar freezing during extinction recall phase. Repeated measures two way ANOVA (Genotype: $F_{(1, 26)} = 0.59$, p=0.44; Interaction: $F_{(13, 338)} = 0.609$, p=0.76. WT, N=14; *Pten*$^{+/-}$, N=14. Data represented as mean ± SEM.

6.4. Pten $^{+/-}$ rats show normal motor coordination in rotarod task

Patients with *Pten* haploinsufficiency present with severe motor impairments, hypotonia and gait deficits (Cummings, 2022). To screen for motor deficits in *Pten* haploinsufficient rats I employed the rotarod task. *Pten*$^{+/-}$ and WT rats both show comparable fall latencies during both baseline phase and accelerating phase of the task (**Fig. 6.4.A & B**). Hence, a genetic perturbation in *Pten* did not result in impaired motor learning in *Pten*$^{+/-}$ rats.

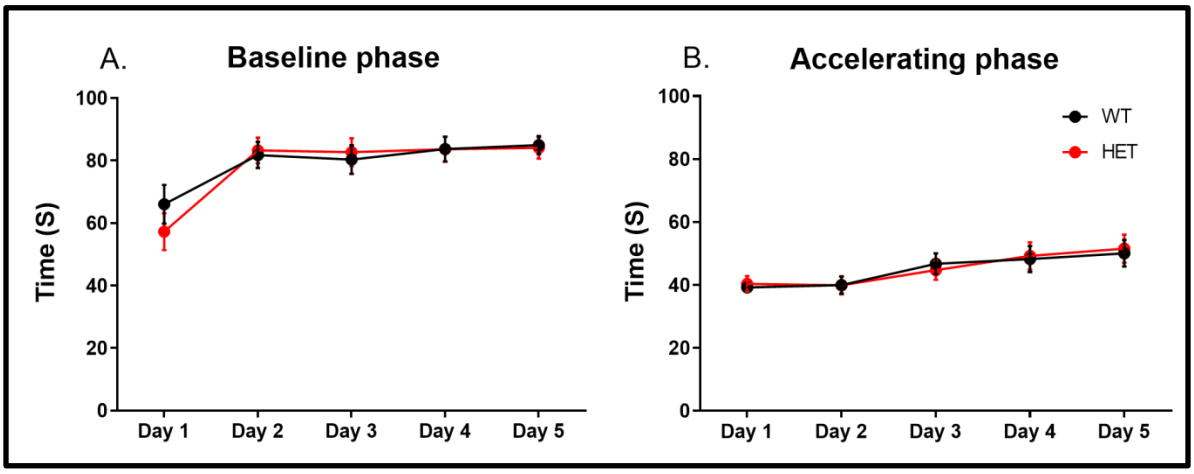


Figure.6.4.A. *Pten*$^{+/-}$rats showed unaltered motor coordination in rotarod test. A. Motor coordination in *Pten*$^{+/-}$and WT rats was comparable in baseline phase of rotarod test.(Repeated measures two-way ANOVA . Genotype: $F_{(1,\ 26)} = 0.05$), p=0.81; Interaction: $F_{(4,\ 104)} = 0.98$, p=0.42. B. *Pten*$^{+/-}$and WT rats show comparable motor learning in accelerating phase of rotarod task. Repeated measures two-way ANOVA. Genotype: $F_{(1,\ 26)} = 0.005$, p=0.94; Interaction: $F_{(4,\ 104)} = 0.22$, p=0.92. WT, N=14; *Pten*$^{+/-}$, N=14. Data represented as mean ± SEM.

6.5. Pten $^{+/-}$ rats show unaltered pain sensitivity in tail flick assay

Further I investigated if the *Pten* haploinsuffciency in rats resulted in hypo or hyper sensitivity for acute pain stimulus. To measure the pain responsiveness in both WT and *Pten*$^{+/-}$ rats were first habituated the restrainers. On the test day tail flick latency was

measured over 5 trials. The average tail flick latency was found to be comparable between WT and *Pten*$^{+/-}$ rats (**Fig.6.5.** WT:3.23 ± 0.1s; *Pten*$^{+/-}$:3.37 ± 0.13s).

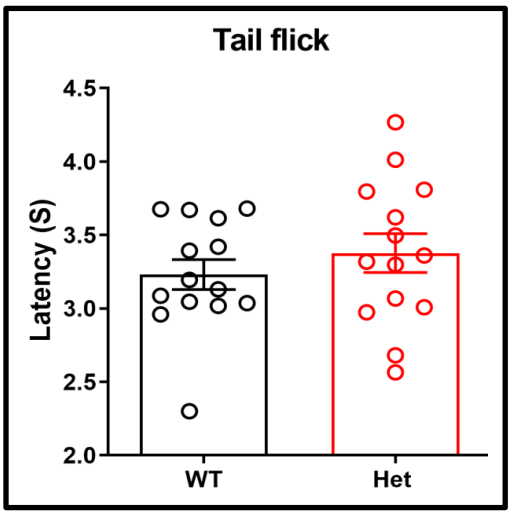


Figure.6.5. *Pten*$^{+/-}$ rats show unaltered pain sensitivity in tail flick assay. Unpaired t test, p=0.38. WT, N=14; *Pten*$^{+/-}$, N=14. Data represented as mean ± SEM.

6.6. Pten $^{+/-}$ rats show altered social novelty preference in three chambered social interaction task

Social deficits are defining features of ASD. Therefore next I investigated the social behavior of WT and *Pten*$^{+/-}$ rats in three chambered apparatus. Post habituation rats were subjected to social interactions tests during which sociability and social novelty were measured (see methods). Both WT and *Pten*$^{+/-}$ rats showed similar interests in exploring social stimulus (conspecific, non-littermate stranger rat) compared to a novel object (**Fig.6.6.A.**WT: 153.25 ± 16.84s; *Pten*$^{+/-}$: 133.91 ± 17.79s). Further, during the social novelty phase, WT rats and *Pten*$^{+/-}$ rats equally explored the novel social stimulus (**Fig.6.6.B.**WT: 111.92 ± 21.86s; *Pten*$^{+/-}$: 58.7 ± 11.02s). Detail analysis of preference index in both WT and *Pten*$^{+/-}$ rats revealed significant reduction in preference towards novelty in *Pten*$^{+/-}$ rats, although there was only the genotype effect present and lacked any interaction effect (**Fig.6.6.C**). Thus, *Pten*$^{+/-}$ rats display deficits in short term social

recognition memory. Additionally, unpublished data from our lab in the same rats revealed more complex social phenotypes like increased interaction latency during direct juvenile social interaction test and reduced co-occupancy in social propinquity test.

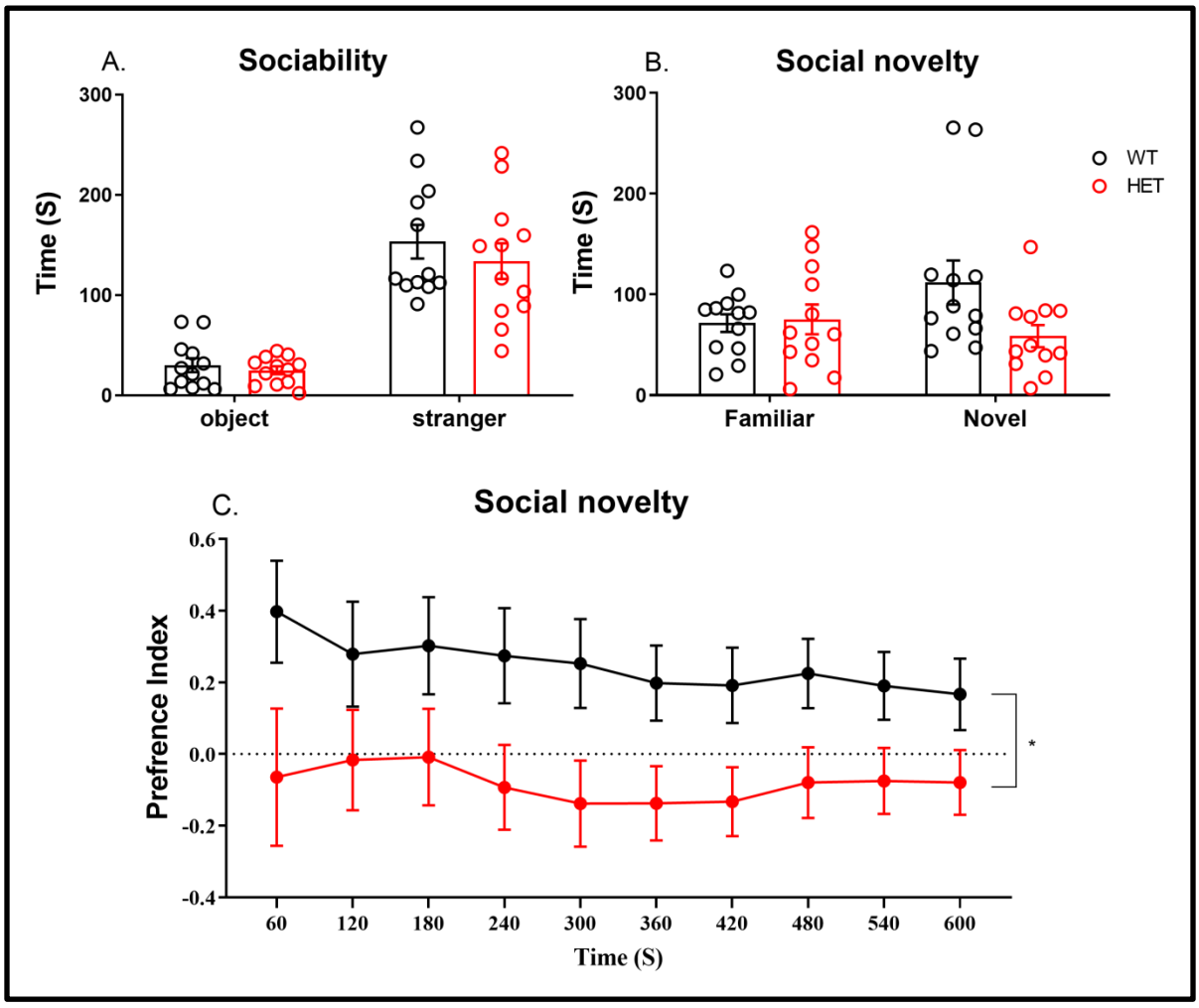


Figure.6.6. *Pten*$^{+/-}$ rats show altered preference in social novelty phase of three chambered task. A. Both WT and *Pten*$^{+/-}$ rats show comparable sociability. Two way ANOVA (Genotype: $F_{(1, 44)} = 0.8963$, p=0.34; Interaction: $F_{(1, 44)} = 0.3064$, p=0.58). B. Both WT and *Pten*$^{+/-}$ rats show comparable social novelty. Two way ANOVA. (Genotype: $F_{(1, 44)} = 2.758$, p=0.10; Interaction: $F_{(1, 44)} = 3.624$, p=0.06.C. *Pten*$^{+/-}$ rats show reduced preference for social novelty. Repeated measures two way ANOVA (Genotype: $F_{(1, 22)}$ =

5.525, p=0.02*; Interaction: $F_{(9, 198)}$ = 0.3785, p=0.94).WT, N=12; *Pten*$^{+/-}$, N=12. Data represented as mean ± SEM.

6.7. Discussion

The *Pten* gene is identified as a risk gene for autism with macrocephaly (Buxbaum *et al.*, 2007). PTEN is a negative regulator of the PI3K-AKT-mTOR pathway (Song, Salmena and Pandolfi, 2012). PTEN has a role in cell cycle progression, cell migration, cell death, transcription and translation (Chen, 2019; Rademacher and Eickholt, 2019). PTEN also regulates other synaptic proteins like mGluR, FMRP, S6K etc which are also implicated in autism (Lugo *et al.*, 2014). Patients with *Pten* mutation display macrocephaly and autistic features like social deficits, repetitive or restrictive behaviors, motor deficits, language delay and cognitive deficits (Busch *et al.*, 2019; Frazier, 2019; Cummings, 2022). Many mice models have been generated over years which replicate the clinical findings in humans with *Pten* haploinsuffiency (Clipperton-Allen *et al.*, 2019). In this thesis I have used novel heterozygous transgenic rat model *Pten* to identify autistic like behavioral traits.

Children with *Pten* haploinsufficency show variable emotional symptoms. Few studies have reported generalized anxiety in children with *Pten* mutation (Hansen-Kiss *et al.*, 2017; Balci *et al.*, 2018). In this study I examined the anxiety like or repetitive behavior in *Pten*$^{+/-}$ and WT rats using marble interaction assay. Various measures like number of marbles buried, marble interaction time, onset of digging, grooming time were comparable between *Pten*$^{+/-}$ and WT rats. *Pten*$^{+/-}$ rat's in fact showed significant reduction in digging behavior. These findings suggest that *Pten*$^{+/-}$ do not show any anxiety-like behavior in marble interaction task. A recent study using the same rat model had shown similar baseline anxiety like behavior in an open field test (Dey and Chattarji, 2022). On the contrary *Pten* heterozygous mice displayed increased repetitive marble burying behavior (Clipperton-Allen and Page, 2014; Clipperton-Allen *et al.*, 2021). Another study had reported Neuron specific conditional deletion of *Pten*

increased anxiety like behavior in open field and light dark anxiety tests (Kwon *et al.*, 2006). Marble burying per se in rats is a consequence of increased digging behavior and we see reduction in digging behavior hence these results suggest *Pten*$^{+/-}$ rats do not display stereotypic repetitive behaviors in marble interaction task. The discrepancy observed between two models would be due to species specific alterations in behavior and thus would need multiple different behavioral assays to screen anxiety-like behavior in these rats.

Pten-ASD patients display severe cognitive deficits presenting with reduced verbal or non-verbal intelligence and reduced attention (Busch *et al.*, 2019; Frazier, 2019; Cummings, 2022). In this study I have tested long term spatial learning and memory of *Pten*$^{+/-}$ rats using object location recognition test. I did not observe any hyperactivity during habituation session in both the genotypes. Indeed both the genotypes showed comparable learning during sampling phase. Interestingly, *Pten*$^{+/-}$ rats show reduced preference to object placed at novel location during probe session. *Pten*$^{+/-}$ rats show impaired spatial memory in object location recognition task. Previous studies in conditional KO *Pten* mice showed learning and memory deficits in Lashley maze (Hodges *et al.*, 2018). Several more studies have observed normal spatial learning and memory in Morris water maze task (Smith, White and Lugo, 2016; Wang *et al.*, 2017). Surprisingly, there are very few studies which have looked into spatial learning and memory in *Pten*$^{+/-}$ mice. More, studies are required to assess the cognitive function in *Pten*$^{+/-}$ rats.

PTEN-ASD patients showed frontal deficits which resulted in moderate to severe impairments in cognitive functioning (Frazier, 2019; Cummings, 2022). Further, they show anxiety, aggression as a comorbid symptom (Balci *et al.*, 2018). To test emotional learning in these rats I employed an auditory fear conditioning task. I did not observe any genotype specific difference between the *Pten*$^{+/-}$ rats and their WT controls. Both the genotypes showed similar learning curves in the conditioning phase of the experiment where they successfully associated an unconditioned stimulus (foot shock)

to a previously innocuous stimuli (tone). The following day, recall of fear memory was also identical between the groups followed by an identical extinction profile. Indeed recall of extinction memory also showed no difference between the genotypes across 13 iterations of CS presented without US. This finding led us to the inference that *Pten*$^{+/-}$ rats do not show any impairment in this form of emotional learning due to the genetic perturbation. Previous research in PTEN deficient mice showed deficits in contextual fear memory where they correlated PTEN's role in activation of CAMKII which regulating long-term potentiation, fear-conditioned memory and spatial learning (Wang *et al.*, 2017). However, they used specific isoform PTENα knockout mice rather than Pten heterozygous mice, which may explain differences between the results. Similar results were reported in study with neuron specific conditional knockout of Pten in mice which showed deficits in contextual fear memory (Lugo *et al.*, 2013). Further, another study reported comparable freezing profile in trace fear conditioning paradigm (Smith, White and Lugo, 2016). Thus, fear learning and recall in various *Pten* deficient mice showed variable results which could be due to different genetic backgrounds and the fear conditioning protocols used. This study is the first to my knowledge that evaluated fear learning and memory in PTEN haploinsufficient rats.

Following AFC, both groups of animals were subjected to a rotarod test to check for neuromuscular coordination. Patients with Pten ASD consistently display sever motor impairments (Busch *et al.*, 2019; Frazier, 2019; Cummings, 2022). Results of this study indicate similar motor performance in the *Pten*$^{+/-}$ and WT control rats during baseline phase and training phase of the rotarod task. Rats from both groups maintained themselves on the rotating spindle with comparable fall latencies and exhibit normal motor coordination. Therefore by these results, the *Pten*$^{+/-}$ mutation did not impact neuromuscular coordination adversely. Findings from this study are in line with previous research in mice which also showed similar motor coordination in rotarod test (Clipperton-Allen and Page, 2014; Tilot *et al.*, 2014). It appears to be species specific alteration which presents in humans but not in rodents. Moreover, it appears the

heterozygous mutation is not sufficient to cause gross motor deficits in rodents and homozygous mutations does show motor deficits in *Pten* deficient mice 2014).

After determining their motor performance *Pten*$^{+/-}$ rats were subjected to thermal pain sensitivity assay. As patients with ASD show altered sensory processing both at the peripheral and cortical levels (Tomchek and Dunn, 2007; Orefice, 2020). Results in this test indicated normal pain sensitivity in both the genotypes. Hence, genetic perturbation in *Pten* did not result in altered pain sensitivity in these rats. Previous research in mice showed similar pain sensitivity in *Pten* heterozygous mice using hot plate test (Clipperton-Allen and Page, 2014; Tilot *et al.*, 2014). It would be interesting to assess dorsal root ganglionic neurons, morphology and electrophysiological activity to assess the sensory processing in these rodents (Orefice, 2020).

Lastly, I investigated social behavior in both WT and *Pten* heterozygous rats using three chambered social interaction task. Rats were tested for their sociability and social novelty in this task. *Pten*$^{+/-}$ rats and WT rats spent comparable time in interacting with the stranger rat during sociability phase. However, WT rats showed a trend towards choosing novel rat over familiar rat whereas *Pten*$^{+/-}$ rats had shown no significant exploration of novel rat over familiar rat (WT: 111.92 ± 21.86s; *Pten*$^{+/-}$: 58.7 ± 11.02s). On detailed analysis of their preference indices revealed WT rats indeed show significant increase in novelty preference compared to *Pten*$^{+/-}$ rats. Previous research in Pten conditional knock-out mice has shown similar deficits in social novelty (Chai *et al.*, 2021). Another study used a neuron specific *Pten* KO which showed deficits in both sociability and social novelty exploration (Lugo *et al.*, 2014). Furthermore, studies in heterozygous *Pten* mice also show similar social deficits in three chambered social interaction mice (Clipperton-Allen and Page, 2014). Findings from this study partially are in line with the previous studies in mice. Unpublished data from my colleague (Dr. Suryanarayan Biswal) have also observed deficits in direct social interaction and social hesitancy in *Pten* heterozygous rats. Additionally, *Pten*$^{+/-}$ rats showed reduced social

propinquity in tube co-occupancy test. Taken together *Pten*$^{+/-}$ rats show diverse social deficits and further experiments are warranted to assess molecular and electrophysiological phenotypes in this rat model.

Chapter.7. Discussion

Synaptic dysfunction or synaptopathies are increasingly being implicated in many neurological and neuropsychiatric conditions including ASD in humans(Pinto *et al.*, 2010). Synapses being the fundamental structures of the brain comprise 1000's of proteins which provide molecular complexity with myriad essential functions(Grant, 2012). Proteomic profiling had revealed several genes with mutation resulting in cognitive, affective and motor phenotypes in many of the neurological and neuropsychiatric disorders(Bayés *et al.*, 2011). Further, same study had revealed strong protein sequence conservation in mammalian lineages including mice, rats and non-human primates making them ideal organisms for translational research. Other studies on human brain transcriptome have revealed that genes associated with synaptic proteins are expressed as early as 3-6 months of gestation (Huttenlocher and Dabholkar, 1997; Kang *et al.*, 2011). However, impacts of these mutations don't show up until one to three years post-gestational period. It is very likely that the heterogenic etiology of ASD results from interactions between several synaptic proteins, with the influence of environmental and epigenetic variables. Majority of high risk or rare variants of mutations are observed in genes coding for synaptic proteins like cell adhesion molecules, scaffolding proteins, protein synthesis regulators, degradation, synapse formation, elimination, synaptic transmission and plasticity etc. (see (https://gene.sfari.org/database/gene-scoring/)(Delorme *et al.*, 2013; Chen *et al.*, 2014b; Guang *et al.*, 2018). This suggests that synaptic disruption is one of the major factors in the etiology of ASD. In this thesis I have evaluated behavioral profiles of novel transgenic rat models with synaptopathies known in ASD. Novel rat models lacking Neuroligin 3 (NLGN3), cyclin dependent kinase like 5 (CDKL5), synaptic ras GTPase (SYNGAP) and phosphatase and tensin homolog (PTEN) were subjected to specific behavioral experiments to identify deficits in core and auxiliary domains of ASD. Previous literature, predominantly from the mice models of ASD have added to our understanding of cellular, molecular and behavioral phenotypes (Kumar *et al.*, 2011; Banerjee, Riordan and Bhat, 2014; Roubertoux and Bartoli, 2015; de la Torre-Ubieta *et*

al., 2016; Ueoka *et al.*, 2019). However, it is difficult to use these models to test the novel therapeutic candidate drugs for translational purpose due to phenotypic discrepancies observed across mouse lines from different research groups. This could be attributed to the variability in mutation loci, genetic background, use of inbred strains, behavioral protocols, age group etc. in the mouse models of ASD. Translatability between mammalian species is a requirement in forwarding the quest towards understanding the pathobiology of ASD. Rats offer unique advantage over mice in domains such as pharmacodynamics, metabolism, cognitive abilities, social complexity(Ellenbroek and Youn, 2016). And show evolutionary proximity towards humans compared to mice. Furthermore, although rats might not be the best alternative model compared to non-human primates, which have their own set of benefits and drawbacks, they do represent the next step in the translation of bench to bedside.

7.1. Altered behavioral profile of Nlgn3$^{-/y}$ rats

Nlgn3$^{-/y}$ rats did not show any repetitive or compulsive behavior in marble interaction assay. Also, in cognitive tasks like novel object location recognition (short term and long term) and spontaneous alteration tasks (OR, OP, OC AND OPC) *Nlgn3*$^{-/y}$ rats displayed comparable novelty preference. Interestingly, emotional responses were altered in *Nlgn3*$^{-/y}$ rats as displayed by reduced freezing responses during fear conditioning tasks (Contextual and auditory) and faster avoidance profile along with robust avoidance memory in active place avoidance task. Further, initial response to foot shock was found to be comparable in *Nlgn3*$^{-/y}$ rats which changed towards jumping when presented with higher intensity of current. These findings led to the first hypothesis that *Nlgn3*$^{-/y}$ rats choose flight over freezing. This hypothesis was further tested from our group to find the cellular correlates of imbalance in freeze flight responses in *Nlgn3*$^{-/y}$ rats (Anstey and Kapgal *et al.*, 2022). An alternative hypothesis was to find out if there were any memory consolidation deficits in*Nlgn3*$^{-/y}$ rats. This was ascertained using a variant of auditory fear conditioning task which tested consolidation of fear memory post 6 hours of

conditioning. Freezing deficits were indeed observed in this experiment. This led to use IGF (1-3) or Glypromate drug to facilitate increased freezing during fear recall in *Nlgn3*$^{-/y}$ rats.

Furthermore, on the contrary to response towards foot shock, response towards acute thermal pain stimulus was reduced in *Nlgn3*$^{-/y}$ rats. Interestingly, *Nlgn3*$^{-/y}$ rats displayed improved motor performance compared to their WT littermates during accelerating phase of rotarod assay. Lastly, *Nlgn3*$^{-/y}$ rats do not show deficits in sociability or social novelty in three chambered social interaction task.

7.2. Altered behavioral profile of Cdkl5$^{-/y}$ *rats*

Cdkl5$^{-/y}$ rats displayed increased locomotor activity, increased marble burying and increased digging time during marble interaction assay. Also, in cognitive tasks like object location recognition task, *Cdkl5*$^{-/y}$ rats show deficit in short term but not long term version of the task. Further, in spontaneous novelty exploration tasks (OR, OP, OC and OPC) *Cdkl5*$^{-/y}$ rats show deficits only in object place task. *Cdkl5*$^{-/y}$ rats did not show any freezing deficits during conditioning, recall and extinction and extinction recall phases of auditory fear conditioning task. However, *Cdkl5*$^{-/y}$ rats displayed delayed avoidance learning in active place avoidance task with comparable avoidance memory to the WT littermates. *Cdkl5*$^{-/y}$ rats display comparable acute thermal pain sensitivity in the tail flick assay. Interestingly, *Cdkl5*$^{-/y}$ rats displayed impaired motor coordination during accelerating phase of rotarod assay. Furthermore, *Cdkl5*$^{-/y}$ rats show deficits in sociability and social novelty during three chambered social interaction test.

7.3. Altered behavioral profile of SynGAP$^{-/+}$ *rats*

SynGAP$^{-/+}$ rats display increased locomotor activity, buried less marbles reduced marble interaction time and increased grooming time during marble interaction assay. Also, in cognitive tasks like object location recognition and spontaneous novelty exploration tasks (OR and OPC), *SynGAP*$^{-/+}$ rats show comparable short term, long term memory and comparable novelty preference respectively. *SynGAP*$^{-/+}$ rats did not show any freezing

deficits during conditioning, recall and extinction, and extinction recall phases of auditory fear conditioning task. Furthermore, *SynGAP*$^{-/+}$ rats displayed similar avoidance learning and robust avoidance memory compared to their WT littermates in active place avoidance task. Interestingly, during the reversal phase or conflict session *SynGAP*$^{-/+}$ rats were still avoiding old shock zone which shows their cognitive inflexibility. Next, *SynGAP*$^{-/+}$ rats also show deficits in motor coordination during accelerating phase of rotarod task. Additionally, *SynGAP*$^{-/+}$ rats show similar acute thermal pain sensitivity in tail flick assay. Interestingly, *SynGAP*$^{-/+}$ rats displayed reduced sociability and social novelty during three chambered social interaction test. However, it is worth noting that the deficits observed appeared to be due to overall lack of interest in exploring the stimulus in *SynGAP*$^{-/+}$ rats.

7.4. Altered behavioral profile of Pten$^{-/+}$ *rats*

Pten$^{-/+}$ rats show comparable number of marble burying, similar marble interaction time and digging time during marble interaction assay. Interestingly, *Pten*$^{-/+}$ rats show reduced preference to object placed at novel location compared to their WT littermates. Furthermore, *Pten*$^{-/+}$ rats did not show any freezing deficits during conditioning, recall and extinction and extinction recall phases of auditory fear conditioning task. Additionally, *Pten*$^{-/+}$ rats did not show any motor or sensory deficits during rotarod or tail flick pain sensitivity assay.

7.5. Altered locomotion and restricted or repetitive behaviors in novel transgenic rat models of ASD

Children with ASD present with hyperactivity and repetitive or restrictive behaviors (Bodfish *et al.*, 2002; Rodgers *et al.*, 2012; Jiujias, Kelley and Hall, 2017). Various brain regions including the cortex, amygdala, cerebellum, basal ganglia and dopamine signaling pathways have been implicated in motor stereotypes or repetitive behaviors (Chmielewski and Beste, 2015; Fuccillo, 2016; Kosillo and Bateup, 2021).Same has been replicated in various mouse models of ASD, where hyperactivity, marble burying,

jumping, circling, grooming etc. were quantified as repetitive or restrictive behavioral patterns (Ey, Leblond and Bourgeron, 2011; Kalueff *et al.*, 2016; Kim, Lim and Kaang, 2016; Ueoka *et al.*, 2019). A Phenobase has also been developed to catalogue behavioral phenotypes in different genetic mouse models of ASD (Kumar *et al.*, 2011). NLGN3 deficient mice show hyperactivity and reduced repetitive marble burying (Radyushkin *et al.*, 2009; Kalbassi *et al.*, 2017; Bariselli *et al.*, 2018; Hörnberg *et al.*, 2020). A recent study used *Nlgn3*$^{-/y}$ rats which displayed perseverative/repetitive chewing behavior (Hamilton *et al.*, 2014). Further studies in CDKL5 deficient mice have reported hyperactivity in open field and repetitive jumping behavior, increased marble burying behaviors and motor stereotypies measured by hind limb clasping (Fuchs *et al.*, 2018; Trazzi *et al.*, 2018; Tassinari *et al.*, 2022). Studies in SYNGAP deficient mice have reported hyperactivity and stereotypic motor patterns in open field test (Guo *et al.*, 2009; Ozkan *et al.*, 2014; Berryer *et al.*, 2016; Nakajima *et al.*, 2019). Studies in PTEN deficient mice have reported hyperactivity in open field, reduced circling and self-grooming, reduced repetitive marble burying and hole poking (Kwon *et al.*, 2006; Lugo *et al.*, 2014). On the contrary another study did not show hyperactivity in open field, increased repetitive marbles burying, digging and self-grooming in *Pten* heterozygous mice (Clipperton-Allen and Page, 2014; Smith, White and Lugo, 2016). In this study I have evaluated hyperactivity and repetitive or stereotypic behaviors in novel transgenic rats lacking NLGN3, CDKL5, SYNGAP and PTEN using marble interaction assay. Upon assessment of behavior I observed hyperactivity in *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats but not in *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$ rats. It is interesting to note that the increased locomotor activity was only on first exposure to the novel arena and was not present on the subsequent day of habituation. Further analysis revealed, increased marble buried and digging time in only *Cdkl5*$^{-/y}$ rats. Interestingly, *SynGAP*$^{-/+}$ rats show reduced marble burying and reduced marble interaction time. Both, *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$ rats did not show any repetitive or stereotypic behaviors in marble interaction assay. The phenotypes observed in this task could be mutation and strain specific behavioral manifestation. *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats are bred on LongEvans background and *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$

rats are bred on Sprague Dawley background. This study reports similar behavioral phenotypes of hyperactivity and stereotypic or repetitive behaviors observed in *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ mice models of ASD. However, contrasting behavioral profiles have been observed in *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$ rats. These rats do not display any changes in loco-motor activity and stereotypic or repetitive behaviors in marble interaction assay. Persistent hyperactivity could contaminate the other behavioral phenotypes as behavior is motion dependent observation and hence should be carefully interpreted. The heterogeneity observed in humans is reflected in these rats with synaptopathies showing gene specific behavioral alterations resulting in variable behavioral readout. Further experiments measuring motion sequence analysis and task specific exploratory profiles of these rats would offer more understanding of subtle behavioral phenotypes.

7.6. Cognitive deficits in novel transgenic rat models of ASD

Cognitive deficits are comorbid symptoms observed in patients with ASD (Williams, Goldstein and Minshew, 2006; Pugliese *et al.*, 2015; Velikonja, Fett and Velthorst, 2019; Hajri *et al.*, 2022). Cognitive deficits in rodents models of ASD have been assessed using various behavioral assays such as Morris water maze, radial arm maze, Barnes maze, novel object recognition etc., (Bey and Jiang, 2014). Studies in NLGN3 deficient mice display intact spatial learning and memory in Morris water maze.; their reversal learning was better compared to their control WT mice (Tabuchi *et al.*, 2007; Radyushkin *et al.*, 2009; Jaramillo *et al.*, 2018). Studies in CDKL5 deficient mice reported impaired spatial learning and memory in Morris water maze task, Barnes maze and working memory deficits in T- maze / Y-maze (Jhang *et al.*, 2017; Tang *et al.*, 2017; Fuchs *et al.*, 2018; Okuda *et al.*, 2018). Studies in SYNGAP deficient mice have reported impaired spatial memory in Morris water maze task, radial arm maze, working memory deficits in T - maze(Komiyama *et al.*, 2002; Guo *et al.*, 2009; Muhia *et al.*, 2010; Ozkan *et al.*, 2014; Berryer *et al.*, 2016). Studies in PTEN deficient mice have reported impairments in novel object recognition, spatial learning in Lashley maze, Morris water maze and intact working memory in T-maze task (Smith, White and Lugo, 2016; Hodges

et al., 2018; Huang, Chen and Page, 2019). In this study I have used object location recognition tasks to measure short term and long term spatial memory of the novel transgenic rat models of ASD. This specific task was chosen over radial arm maze or water maze because this assay does not demand food restriction or physical activity such as swimming. This task takes advantage of innate exploratory drive of the rodent and is predominantly dependent on hippocampal formation. The interpretation of behavioral phenotypes may be impacted by using other tasks which are dependent on multiple brain regions and have too many variables, such as food deprivation, motor deficits may affect swimming ability, normal vision (to identify cues), etc.I observed long term memory deficit in only *Pten*$^{-/+}$ rats and not in *Nlgn3*$^{-/y}$, *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats. Furthermore, short term memory deficit was observed only in *Cdkl5*$^{-/y}$ rats and not in rest of the rat lines. It is interesting to note that behavioral phenotypes observed in *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$ rats are in line with previous studies in *Nlgn3*$^{-/y}$ and *Pten*$^{-/+}$ mice respectively. Surprisingly, *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats do not show any long term spatial memory deficits whereas previous studies in respective mice reported spatial learning and memory deficits. Discrepancies observed in these studies may be due to variables such as task, strain, age, co-morbidities like epilepsy, motor deficits or altered reward signaling etc., To further test the cognitive abilities such as associative and non-associative working memory in these rats I employed novelty based spontaneous exploration tasks such as object recognition (OR), object place (OP), object context (OC) and object-place-context (OPC). Surprisingly, *Cdkl5*$^{-/y}$ rats showed deficits in object place (OP) task and no deficits were observed in OR, OC and OPC in the *Nlgn3*$^{-/y}$, *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats. Note, *Pten*$^{-/+}$ rats did not undergo these behavioral experiments. Previous studies in*Cdkl5*$^{-/y}$, *SynGAP*$^{-/+}$ and *Pten*$^{-/+}$ mice had reported deficits in working memory in T-maze or Y-maze (rewarded or spontaneous alternation) tasks. Both un-rewarded and rewarded versions of T-maze and Y-maze are confounded by side preference of rodents (left or right) and results are chance dependent. Hence, spontaneous novelty based exploration tasks could be better alternative behavioral experiments to test working memory in rodents. Having said that, one drawback of

using object based exploration tasks is the rodents use whiskers for exploring textures shapes and sizes. Recent studies have implicated faulty sensory processing in ASD models (Michaelson *et al.*, 2018; Orefice, 2020). Sensory processing deficits should be identified prior to establishing cognitive phenotypes.

7.7. Altered emotional learning and memory in novel transgenic rat models of ASD

Emotional processing is disrupted in individuals with ASD and is regarded as a comorbid symptom of ASD(Matson and Cervantes, 2014; Chandler *et al.*, 2016; Teh, Yap and Rickard Liow, 2018).Disrupted, or maladaptive, emotional regulation has been implicated in various neuropsychiatric disorders and in ASD (Mazefsky and White, 2014). Emotional processing in rodent models is widely studied using Pavlovian fear conditioning task and passive avoidance tests. Studies in *Nlgn3*$^{-/y}$ mice have reported variable behavioral phenotypes. A previous study reported deficits in fear learning and memory in both contextual and cue fear conditioning tasks (Radyushkin *et al.*, 2009). Whereas, another study from different research group had reported no such difference in freezing between control animals and *Nlgn3*$^{-/y}$ mice (Jaramillo *et al.*, 2018). Additionally, a recent study in the *Nlgn3*$^{-/y}$ rats which is also used in current study had reported comparable freezing to their WT control rats during contextual and cued fear recall (Hamilton *et al.*, 2014). Study in *Cdkl5*$^{-/y}$ mice had showed reduced freezing during conditioning, comparable freezing during contextual recall and increased freezing during cued recall (Okuda *et al.*, 2018). On the contrary, another study in *Cdkl5*$^{-/y}$ mice showed comparable freezing during conditioning and reduced freezing during contextual and cued fear recall (Wang *et al.*, 2012). Study in *SynGAP*$^{-/+}$mice showed comparable freezing during contextual recall and reduced freezing during cued fear recall (Guo *et al.*, 2009; Nakajima *et al.*, 2019). On the contrary ,a recent study reported reduced freezing during contextual fear recall in *SynGAP*$^{-/+}$mice (Kilinc *et al.*, 2022). Previous research in *Pten*$^{-/+}$mice hadreported similar freezing during testing phase of trace fear conditioning task (Smith, White and Lugo, 2016). On the contrary, a recent

study with forebrain specific conditional knockout of *Pten* hasreported reduced freezing during contextual fear memory test (Chen, 2019; Lugo et al.,2013). In the present study I have comprehensively assessed emotional learning and memory using two different behavioral tasks i.e. auditory fear conditioning and active place avoidance tasks which tests freezing and flight responses to aversive stimuli respectively. Further auditory fear conditioning protocol was designed to include acquisition, recall, extinction and extinction recall of fear. Analyzing freezing profiles in each transgenic rat models revealed reduced freezing in all phases of fear conditioning in *Nlgn3*$^{-/y}$ rats. Whereas, reduced freezing was observed only in acquisition phase in *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats but not in recall, extinction and extinction recall phases. *Pten*$^{-/+}$ rats showed comparable freezing profile during all the phases of auditory fear conditioning task. As I consistently observed reduced freezing profile in *Nlgn3*$^{-/y}$ rats, I further subjected separate set of naïve rats to contextual fear conditioning and short term fear recall. These experiments also showed reduced freezing profile in contextual fear conditioning and short term fear recall. To ascertain if this overall reduction in freezing response was due to altered sensitivity to foot shock, I employed a shock sensitivity test which revealed that *Nlgn3*$^{-/y}$ rats were not hypersensitive to foot shock at lower foot shock intensities. However, they showed increased jumping response towards higher foot shock intensities. This indicates that *Nlgn3*$^{-/y}$ rats express their fear differently compared to their WT littermate rats. Vast literature in ASD mice models which are listed above have used different fear conditioning protocols with varied CS and US parameters. Further majority of them have used white noise as conditional stimulus which itself is aversive (LaBar and LeDoux., 1996). Hence, the phenotypes reported must be carefully interpreted considering experimental rigor, genetic background, housing condition, age of testing etc. This study I have comprehensively studied fear learning, recall, extinction and extinction recall profiles of novel transgenic rat models of ASD.

Active place avoidance was employed to test the rat's ability to escape from the impending aversive stimulus from a stationary shock zone in a rotating arena (Bures and Fenton, 2000). The rat's uses local(arena)cues, distal (room)cues, and idiothetic cues

generated from its self-motion to avoid shock zone in a rotating arena (Fenton *et al.*, 1998). This task requires intact dorsal hippocampus to successfully avoid the shock zone and also measures the cognitive control in rats (Cimadevilla, Fenton and Bures, 2000; O'Reilly *et al.*, 2014). Hence, this task is one of sensitive task to detect hippocampal dysfunction in rodents. This task has never been used before in the context of ASD and this is the first study to screen for cognitive control in novel transgenic rat models of ASD. This study is first to report altered avoidance responses in novel transgenic rat models of ASD. *Nlgn3*$^{-/y}$ rats showed quicker avoidance learning and robust avoidance memory. *Cdkl5*$^{-/y}$rats showed delayed avoidance learning and comparable avoidance memory during probe trial. *SynGAP*$^{-/+}$ rats showed similar avoidance learning and robust avoidance memory. Furthermore, *SynGAP*$^{-/+}$ rats showed delayed extinction during the reversal learning. See appendix for reversal learning in *Nlgn3*$^{-/y}$, *Cdkl5*$^{-/y}$ and *Pten*$^{-/+}$ rats. *Nlgn3*$^{-/y}$ and *SynGAP*$^{-/+}$ rats showed similar avoidance memory. *Nlgn3*$^{-/y}$ and*Cdkl5*$^{-/y}$ showed contrasting phenotypes during acquisition phase of the task. Further species specific ethological studies like USV induced avoidance or approach behavior(Shukla and Chattarji, 2022) and avoidance of looming stimuli (De Franceschi *et al.*, 2016) etc., might add to our understanding of the underlying neural circuitry and their implication in the case of genetic perturbation in ASD.

7.8. Altered motor coordination in novel transgenic rat models of ASD

Motor deficits are increasingly been considered as cardinal symptoms of ASD (Fournier *et al.*, 2010). Evidence indicates that 80-90 % of children detected with ASD show some degree of motor impairment which is also highly correlated with autistic severity and IQ (Hilton *et al.*, 2012). Previous research both in humans and mice have implicated striatal and cerebellar dysfunction resulting in motor impairmentin ASD (Fuccillo, 2016; Kelly, Escamilla and Tsai, 2021). Studies in *Nlgn3*^{R451C} and *Nlgn3*$^{-/y}$mice have reported enhanced performance in rotarod due to theirincreased ability to acquire repetitive motor routines which is facilitated by specific impairment in striatal synaptic function (Radyushkin *et al.*, 2009; Etherton *et al.*, 2011; Rothwell *et al.*, 2014). On the contrary,

studies in *Cdkl5*$^{-/y}$mice show reduced motor coordination in rotarod test (Jhang *et al.*, 2017). Additionally, other studies had reported mild gait impairment in Cat walk tests (Wang *et al.*, 2012; Sivilia *et al.*, 2016; Okuda *et al.*, 2018). Further, studies in another mice model of ASD with heterozygous mutation in *SynGAP*reported impairment in motor coordination in rotarod. However, their grip strength was unaffected (Muhia *et al.*, 2010; Nakajima *et al.*, 2019). Evidence from another ASD mice model with heterozygous mutations in *Pten*does not present with deficits in motor coordination in rotarod assay (Clipperton-Allen and Page, 2014; Tilot *et al.*, 2014). Hence, multiple mice model of ASD present with varied motor performance indicating heterogeneity with respect to observed phenotypes. In this study motor co-ordination was assessed using rotarod. Our findings are in line with the previous literature in respective mice models. All the four novel transgenic rat lines display comparable motor coordination during baseline (steady-fixed speed rotation) phase of rotarod task. Interestingly, *Nlgn3*$^{-/y}$rats showed increased fall latencies during accelerating phase of rotarod, suggesting enhanced motor learning due to their acquired repetitive motor routines. Further,*Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats displayed decreased fall latencies during accelerating phase of rotarod suggesting impairment in motor learning. Furthermore, motor coordination was unaffected in *Pten*$^{-/+}$ rats compared to their WT littermates. It is interesting to note that the phenotypes observed in all the four novel rat transgenic lines replicated the findings from the respective mice lines. This can be attributed to the fact that all these studies used rotarod test with very similar protocols. However, there are emerging techniques like cat walk (Walter *et al.*, 2020) and MoSeq (Wiltschko *et al.*, 2020) which can further be used to assess subtle, granular motor impairments in similar rat models of ASD.

7.9. Altered acute pain sensitivity in novel transgenic rat models of ASD

Patients with ASD present with atypical pain perception, expression which makes it difficult to assess the extent of pain (Bogdanova *et al.*, 2022). Research in mice models of ASD indicates towards altered processing of sensory stimulus at multiple levels of neural organization (Orefice, 2020). Studies in *Nlgn3*$^{-/y}$ mice had reported no sensory

abnormalities in processing pain using hot plate test and tail flick assay (Chadman *et al.*, 2008). Recentstudy in *Cdkl5*$^{-/y}$ mice and patients with CDKL5 deficiency disorder reported reduced pain sensitivity (La Montanara *et al.*, 2020). On the contrary, another previous study in *Cdkl5*$^{-/y}$ mice reported normal pain sensitivity in hotplate test (Okuda *et al.*, 2018). Studies in *SynGAP*$^{-/+}$ mice reported contrasting phenotypes in pain sensitivity. One study reports no change in pain sensitivity (Muhia *et al.*, 2010; Duarte *et al.*, 2011)and other reports less sensitivity to pain in hotplate test (Nakajima *et al.*, 2019). Studies in *Pten*$^{-/+}$mice also had reported comparable pain sensitivity (Clipperton-Allen and Page, 2014). In this current study pain sensitivity was assessed in tail flick assay.Other withdrawal tests which are used in rodents include Von Frey filament test and Hargreaves test (La Montanara *et al.*, 2020).*Cdkl5*$^{-/y}$, *SynGAP*$^{-/+}$ and *Pten*$^{-/+}$ rats show comparable pain sensitivity and *Nlgn3*$^{-/y}$ rats were found to be less sensitive towards acute thermal pain stimulus. The variability in the observed phenotypes makes it difficult to comprehend the pain sensitivity in these ASD models. However, acute pain sensitivity assessments using protocols such as tail flick assay and hot plate assay which are basically withdrawal reflexes might not highlight the root cause of the sensory processing deficits in ASD models. Peripheral sensory neuron dysfunction is increasingly been studied in the context of ASD and may contribute to subsets of behavioral phenotypes in rodents (Orefice, 2020).

7.10. Altered social interaction in novel transgenic rat models of ASD

Social behaviors are defining features of ASD which typically found to be impaired very early in childhood and are among the first signs reported by parents(Fodstad *et al.*, 2009; Morris and Vollmer, 2021). Multiple studies in mice have used behavioral assays like reciprocal social interaction, social recognition memory, social novelty assessment, juvenile play, conditioned social place preference etc., to screen for social deficits in mice models for ASD (Crawley, 2007; Moy *et al.*, 2009). Studies in *Nlgn3*$^{-/y}$ and *Nlgn3*^{R451C}Knock in mice had reported variable behavioral readouts in social interaction assays. Tabuchi and colleagues had reported deficits in sociability in *Nlgn3*^{R451C} Knock in

mice (Tabuchi *et al.*, 2007). But, Radyushkin and colleagues had reported comparable sociability and impaired social novelty in *Nlgn3*$^{-/y}$mice (Radyushkin *et al.*, 2009). Further, subsequent studies from Tabuchi's group in different background strain of mice with the same mutation did not find any social deficits (Jaramillo *et al.*, 2018). Furthermore, strangely, Bariselli and colleagues showed social interaction deficits in *Nlgn3* $^{-/y}$ mice with same genetic background as used by Jaramillo and colleagues (Bariselli *et al.*, 2018). Interestingly, a study in *Nlgn3* $^{-/y}$ mice showed the negative influence of housing KO mice on the WT mice behavior (Kalbassi *et al.*, 2017). Multiple studies in *Cdkl5*$^{-/y}$ mice have also shown variability in social deficits observed in three chambered social interaction task. Wang and colleagues had reported that *Cdkl5*$^{-/y}$mice showed reduced direct social interaction and showed increased interaction over social stimulus in three chambered social interaction task (Wang *et al.*, 2012).Jhang and colleagues show deficits in sociability and social novelty deficits in three chambered apparatus (Jhang *et al.*, 2017). Interestingly, Okuda and colleagues did not find genotype difference in social interaction parameters in three chambered social interaction task compared to their control mice (Okuda *et al.*, 2018). Another study by Tang and colleagues reported forebrain specific ablation of CDKL5 in glutamatergic neurons did not result in alterated sociability (Tang *et al.*, 2017). Contrastingly, multiples studies in *SynGAP*$^{-/+}$ mice have shown consistent social interaction deficits. Guo and colleagues had reported that the*SynGAP*$^{-/+}$ mice showed impaired social recognition memory and showed social isolation (Guo *et al.*, 2009). Berryer and collegues reported impaired social novelty in three chambered task in *SynGAP*$^{-/+}$ mice (Berryer *et al.*, 2016). Further, Nakajima and colleagues also reported reduced reciprocal social interaction in novel environment, reduced sociability and social novelty in three chambered task (Nakajima *et al.*, 2019). Additionally, studies in *Pten*$^{-/+}$mice reported social novelty recognition deficits in three chambered social interaction task (Page *et al.*, 2009). Further, same research group did not replicate similar findings in sociability and social novelty. But reported habituation deficit in the *Pten*$^{-/+}$mice during social recognition memory test (Clipperton-Allen and Page, 2014). Moreover, another study in mice with neuron specific conditional knockout

of *Pten* reported reduced social interaction in social partition test and three chambered sociability test (Lugo *et al.*, 2014). In line with this previous study, a recent study reported short term and long term social recognition in conditional knockout of *Pten* in dorsal CA1 neurons (Chai *et al.*, 2021). Hence across different mice models of ASD, the social deficits were very variable and most of them were not reproducible. The discrepancies observed could be due to variations of background strains, protocols used, parameters assessed etc., Crabbe and colleagues had demonstrated inherent variability with respect to behavior profile in different strains of mice, in spite of controlling for apparatus, test protocols, and many environmental variables (Crabbe, Wahlsten and Dudek, 1999). In this study I had carried out assessment of social interaction in the four novel transgenic rat models using three chambered social interaction task. Results indicate comparable sociability and social novelty in *Nlgn3*$^{-/y}$ rats. Further, *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats showed reduced sociability and social novelty deficits compared to their control littermates. Time spent in exploration of social stimulus was significantly reduced in both the transgenic rats compared to their respective control littermates. The reduced exploration was more pronounced in *SynGAP*$^{-/+}$ rats which could be due to their overall general disinterest in exploration. Interestingly, *Pten*$^{-/+}$ rats show intact sociability and impaired social novelty in three chambered task. Social novelty deficit was the only convergent phenotype between*Pten*$^{-/+}$ and *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats. Altogether, more social domain behavioral assays apart from three chambered social interaction are need of the hour. Additional assays like social propinquity(Tuttle *et al.*, 2017), social recognition memory(Chai *et al.*, 2021), and empathy(Bartal, Decety and Mason, 2011) would be important to understand social deficits in these rats.

7.11. Interventional strategies to rescue behavioral deficits in transgenic rat models of ASD

Translational advantage of these rats can be realized only by rescuing the observed behavioral phenotypes. Previous research in mice have reported usage of multiple

pharmacological interventions such as mTOR inhibitors (Chen, 2019), mGlur antagonists (Gantois *et al.*, 2013; Pop *et al.*, 2014), phytochemicals like curcumin (Yu *et al.*, 2009), lovastatin (Asiminas *et al.*, 2019) etc., to ameliorate the behavioral, electrophysiological cellular and molecular phenotypes in the rodent models of ASD. In this study I have used IGF (1-3) as an interventional molecule to rescue behavioral deficit observed in *Nlgn3*$^{-/y}$ rat model. *Nlgn3*$^{-/y}$ rats consistently showed freezing deficit across contextual, auditory short term and long term fear conditioning paradigms. Previous study had found autocrine and paracrine secretion of growth hormone by baso lateral amygdala (BLA) neurons (Gisabella *et al.*, 2016). Further studies have shown that IGF signaling has role in protein synthesis by regulating mTOR signaling pathways (Magnuson, Ekim and Fingar, 2012). Previous study had reported IGF (1-3)'s ability to bind to NMDA receptor as weak agonist or co-agonist, hence might play a role in memory formation (Vaaga, Tovar and Westbrook, 2014). In this study, IGF (1-3) was injected intra-peritonially before the recall of fear memory. IGF (1-3) has short half-life in brain and can act as memory booster(Baker *et al.*, 2005). Acute IGF (1-3) injection before fear recall rescued freezing behavior deficits observed in *Nlgn3*$^{-/y}$ rats. This is an example of behavior specific interventional approach which helped to rescue the behavioral deficit. Broad symptoms based pharmacological interventional studies are required to address convergent behavioral deficits observed in different ASD models.

7.12. Convergence and divergence in behavioral phenotypes among novel transgenic rat models of ASD

Autism being highly heritable has a substantial genetic heterogeneity. This begs the question of whether there are numerous separate pathologies that manifest as autism spectrum disorder (ASD) or whether the myriad hereditary reasons coalesce around a few impaired biological pathways in the majority of people that may be therapeutically targeted. The latter, converging hypothesis has been supported by many studies led by Huda Zhogbi, Andrey Rzhetsky and Daniel Geshwind's research groups which utilized protein-protein interaction analysis, transcriptome and gene co-expression network

analysis of the existing autism-associated genes (Iossifov *et al*., 2008; Sakai *et al*., 2011; Voineagu *et al.*, 2011; Basilico, Morandell and Novarino, 2020).In this piece of work, I have behaviorally characterized four novel rat models of synaptopathies causing ASD. This is the first comprehensive study to use transgenic rats as model to evaluate behavioral manifestation of mutations in genes coding for four different post synaptic molecules implicated in ASD. All of these genes, code for the specific protein which has distinct functional role in synaptic function. This study identifies similar behavioral phenotypes in *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats. Both these rat models display hyperactivity, repetitive or stereotypic behavior in marble interaction task. Further, they both display impaired motor coordination in rotarod task. Similarly, deficits in social interaction were observed in three chambered task. Emotional learning was reduced in both of these rat lines with normal fear recall, extinction and extinction recall. Both of these rat lines did not show any difference in pain sensitivity assays. Interestingly, diverging phenotypes were observed in active place avoidance task, where *Cdkl5*$^{-/y}$ rats showed delayed acquisition of avoidance and comparable avoidance memory. However, *SynGAP*$^{-/+}$ rats showed comparable avoidance acquisition and robust avoidance memory in the same task. Further, *SynGAP*$^{-/+}$ rats showed delayed extinction of the shock zone memory during reversal training which was not observed in the *Cdkl5*$^{-/y}$ rats. Additionally, *Cdkl5*$^{-/y}$ rats showed diverging phenotype in short term object location recognition memory and novel object place task whereas other transgenic rats did not show any deficits in these tasks. Behavioral assessment of *Nlgn3*$^{-/y}$ rat revealed interesting phenotypes some of them were found to be converging and most of them diverging with respect to other three transgenic rat lines. *Nlgn3*$^{-/y}$ rat did not show any hyperactivity and repetitive behavior in marble interaction assay which was similar to phenotypes observed in *Pten*$^{-/+}$ rats in these behavioral assays. Interestingly, *Nlgn3*$^{-/y}$ rats showed reduced freezing behavior and increased flight behavior which was not seen in rest of the transgenic rat lines. Further, *Nlgn3*$^{-/y}$ rats displayed enhanced motor learning in rotarod task which was in contrast to other transgenic rat lines. These altered behaviors could be attributed to increase in acquired repetitive motor routine with underlying circuit specific

dysfunction which was previously observed in *Nlgn3*$^{-/y}$ mice (Rothwell et al., 2014). Surprisingly, *Nlgn3*$^{-/y}$ rats showed divergence with respect to social behavior in three chambered social interaction task. *Nlgn3*$^{-/y}$ rat did not show sociability or social novelty deficits whereas other three transgenic rat lines showed some form of social deficits in this task. Another point of divergence was with respect to acute pain sensitivity in *Nlgn3*$^{-/y}$ rats. *Nlgn3*$^{-/y}$ rats displayed reduced pain sensitivity whereas other transgenic rat models showed no change in pain sensitivity. Next, *Pten*$^{-/+}$rats showed divergent behavioral phenotypes like deficits in long term object location recognition task which was not observed in any of the other transgenic rat lines. Further, convergent phenotypes in the form of social novelty deficits were observed between *Pten*$^{-/+}$, *Cdkl5*$^{-/y}$ and *SynGAP*$^{-/+}$ rats. *Pten*$^{-/+}$ rats did not display any alteration in behaviors like loco-motor activity, repetitive or stereotypic behavior, motor coordination, pain sensitivity.

Recent studies have established link between these synaptic proteins which could shed light on shared pathophysiology of ASD. A recent study by Xu and colleagues showed the link between NLGN3 and PTEN proteins mediated by a scaffold protein MAGI-2(Xu *et al.*, 2019). Knocking down *Nlgn3*in cultured rat neurons resulted in up regulation of Akt/mTOR signaling which in turn led to increase in protein synthesis and dendritic branching. Thus, indicating NLGN3's role in regulating dendritic outgrowth by modulation of PTEN/Akt/mTOR signaling pathway, probably via MAGI-2(Xu *et al.*, 2019). Another study showed that introducing *Pten* heterozygous mutation resulted in normalizing structural deficits in *Fmr1* KO neurons by balancing each other's expression (Sathyanarayana *et al.*, 2022).Similarly, synaptic functions of CDKL5 also converge on to similar molecular pathways which are implicated in ASD. CDKL5 plays a role in stabilization of mature spines, long-term potentiation, regulates AKT/mTOR pathway, and regulates surface insertion of AMPA receptors on post synaptic membrane, regulation of rac1 signaling mediated actin remodeling for axon outgrowth and dendritic branching(Zhu and Xiong, 2019). Previous study had used IGF-1, as an activator of AKT/mTOR pathway, to rescue dendritic spine instability in a mouse model of CDKL5 deficiency(Della Sala *et al.*, 2016). Interestingly, CDKL5 is also expressed in adulthood

and is required for various neuronal functions like experience dependent plasticity (Terzic *et al.*, 2021). Novel gene and enzyme replacement therapy have emerged in treatment of CDKL5 deficiency disorder(Medici *et al.*, 2022). Surprisingly, like CDKL5, SYNGAP also has similar synaptic role in facilitating AMPA insertion, spine maturation and long term potentiation (Clement *et al.*, 2012; Clement, 2016). Interestingly, recent study showed adult re-expression of SYNGAP resulted in improved behavioral and electrophysiological measures of memory and seizure(Creson *et al.*, 2019). Identifying convergent molecular pathophysiology in different synaptopathies will be important to delineate defective biological processes which results in ASD. This approach would help researchers to test novel candidate drugs to ameliorate the behavioral, cellular and molecular alterations in these disorders.

7.13. Conclusion

This thesis is one amongst the very few studies (Hamilton *et al.*, 2014; Till *et al.*, 2015; Veeraragavan *et al.*, 2016; Asiminas *et al.*, 2019; Kight *et al.*, 2021; Dey and Chattarji, 2022) which have used novel transgenic rat models related to synaptic dysfunction implicated in neurodevelopmental disorders like ASD/ID. Current study provides evidence of altered behaviors across a variety of social, cognitive, sensory-motor, and emotional domains in the novel transgenic rat models of synaptopathies. Reproducibility and rigor in conducting the behavioral experiments over and above the innate biological variability is a prerequisite to get robust, replicable, behavioral phenotypes that can be used as a translational tool to evaluate potential clinical strategies (Gulinello *et al.*, 2019; Puścian and Knapska, 2022; Silverman *et al.*, 2022). To this end, this study has utilized well established behavioral protocols to test spontaneous behaviors which consistently engage well-defined, evolutionarily conserved neural circuits. This work highlights the existence of convergence and divergence among the observed behavioral traits in four genetically different models of synaptopathies. This implies that despite the genetic heterogeneity, there may be basic

pathophysiologies which could be shared and this knowledge could be utilized to create treatments that are effective for a wide range of ASD population.

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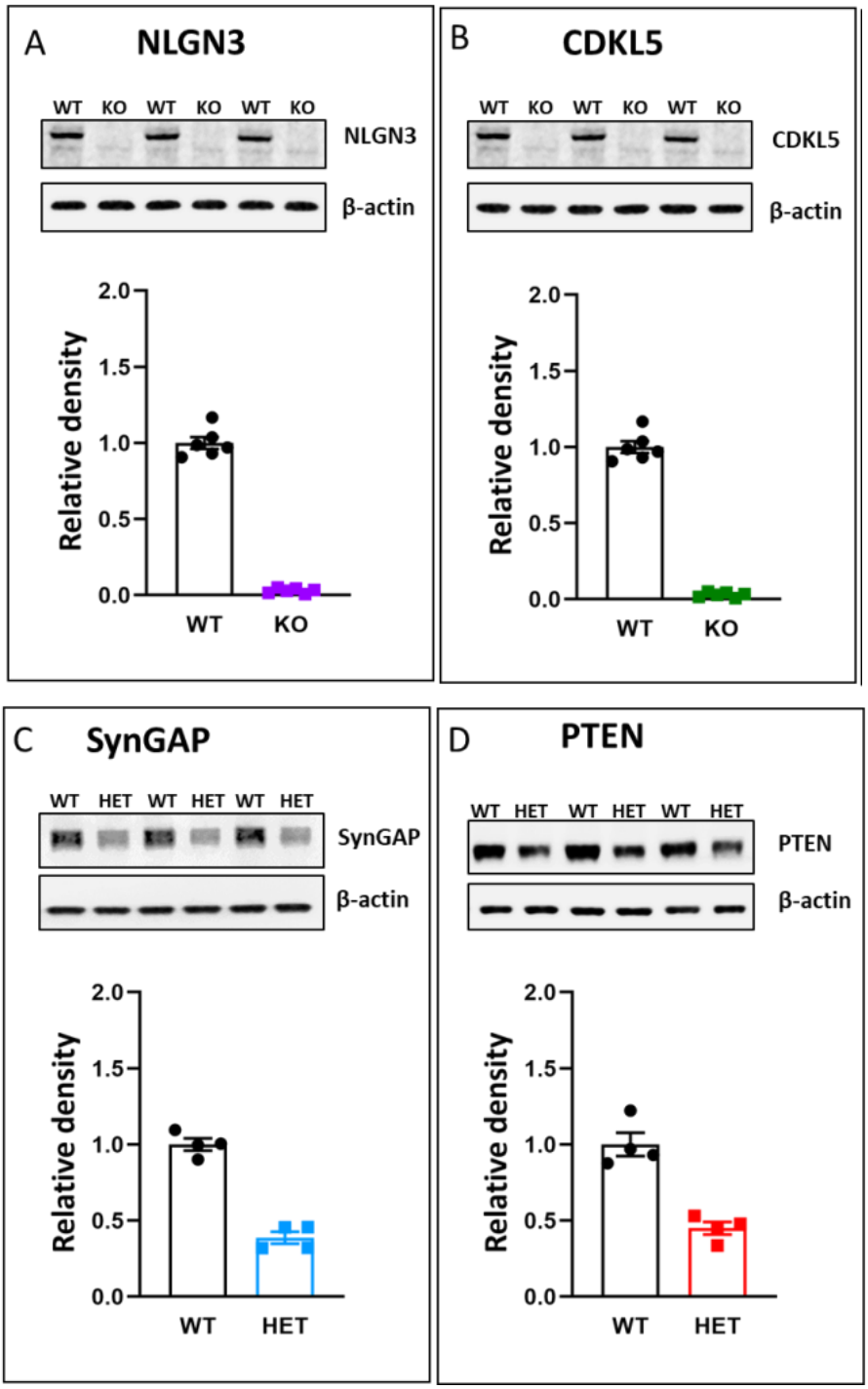
Appendix 1: Validation of the transgenic rat models



Appendix 1: Validation of transgenic rat models. Western blots with quantification. A *Nlgn3*$^{+/y}$ and *Nlgn3*$^{-/y}$ .B. *Cdkl5*$^{+/y}$ and *Cdkl5*$^{-/y}$. C. *SynGAP*$^{+/+}$ and *SynGAP*$^{-/+}$. D. *Pten*$^{+/+}$ and *Pten*$^{-/+}$.

Note: These experiments were done by Dr.Mohammed Sarfaraz Nawaz

Appendix.2. Dietary curcumin alleviates delayed onset anxiety like behavior and spinogenesis in basolateral amygdala (BLA) of rats after acute immobilization stress

A2.1. Introduction

Post-traumatic stress disorder is a psychiatric condition which occurs in few people who are subjected to physical or emotional trauma. However, not everyone who experience trauma would go on to develop PTSD but certain individuals seem to be vulnerable to this condition (*Yahuda & LeDoux., 2007*). People who get affected would display range of symptoms like anxiety, avoidance, hyper arousal, mood instability etc. Previous research in humans and rodent models has highlighted the role of amygdala in mediating these emotional symptoms (*Rauch et al., 2000, Protopopescu et al., 2005 & Adamec et al., 2012*). Past findings from our lab have shown that the single episode of stress causes a delayed increase in spinogenesis in principal neurons of basolateral nucleus of amygdala (BLA) and subsequent increase in anxiety like behavior in rats (*Mitra et al., 2005*). Further, studies have shown that this delayed increase in spine number and anxiety can be alleviated if there is an intervention prior to the stress or post-stress exposure (*Rao RP et al., 2012* & *Chakraborty et al., 2020*). However, to anticipate stress before it occurs and treat this condition by a circulating hormone like corticosterone would have many systemic effects which are not well studied. Alternative treatments are the need of the hour which have overall positive impact on the body and mind. Plant based medicines are gaining traction in treating psychiatric conditions. One such neutraceutical is Curcumin, an active compound found in turmeric extracted from a rhizome *Curcuma longa*. Curcumin is known for its anti-inflammatory, anti-cancer and anti-depressant effects (*Aggarwal & Harikumar., 2009; Kulkarni et al., 2009*). In the current study we have explored whether curcumin would rescue the delayed effects of acute immobilization stress.

A2.2. Methods

The two month old Sprague Dawley rats were used for the experiment. Two rats were housed per cage and their body weight was monitored throughout the experiment. They were kept in a 14h light/ 10 dark cycle and the experiment was done during the light-cycle. Rats were handled for 3 days prior to start of the behavioral testing. Two days prior to the handling, the rats were either habituated to the curcumin feed (1.5% curcumin in 18% protein chow) / control feed (18% protein chow) (18 g / rat / day) (*Monsey et al., 2015*). The rats continued to be on either curcumin or control feed throughout the experimental duration.

A2.2.1. Experimental groups

Rats were randomly divided into four groups.

- Group 1: Unstressed + control feed (n=16)
- Group 2: Unstressed + curcumin feed (n=14)
- Group 3: Stressed + control feed (n=18)
- Group 4: Stressed + curcumin feed (n=16)

A2.2.2. Immobilization stress protocol

Post handling rats were subjected to immobilization stress for two hours in conical plastic bags (*Vyas et al., 2002*). The rats were then released into their cages and left in the same room undisturbed for next 10 days, except for the bedding change on day 5. The corresponding control rats were housed in a separate holding room.

A2.2.3. Elevated plus maze behavior

Post ten days after acute stress the rats were assessed for anxiety-like behavior in elevated plus maze. The rats were held for 20 minutes in a holding room before the start of the experiment. The apparatus was positioned in a dark room with black curtains. The light intensity was measured to be ≈0.1 lux, 4 lux & 75 lux in closed arm,

center and open arm respectively. Rats were released in the center positioning them towards the edge of open and closed arm. The activity of the rat was recorded by overhead camera.

A2.2.4.Behavioral analysis

The off-line analysis of videos was done in blinded fashion using Boris software. The parameters included time spent & frequency of visits to open and closed arm, head dips, stretch attend postures were measured over the duration of 5 minutes.

A2.3. Morphology

Post elevated plus maze behavior, rats were anesthetized with halothane and were sacrificed was and their brain was extracted and transferred to 5% Golgi-Cox solution which comprised of mercuric chloride (5%), potassium dichromate (5%) and potassium chromate (4%), mixed in the ratio 5:5:4. The tissue was transferred to fresh Golgi-Cox solution after 24 hours. Rat brains remained in Golgi solution for the duration of 15 days. Further, post 15 days tissue was transferred to 6% sucrose solution prepared in 0.1 M PB until they were sectioned.

A2.3.1. Tissue Processing

120 µm thick coronal sections of BLA were obtained on gelatin (1%) - chrome alum (3%) coated glass slides using Leica vibratome (VT-1200S). Further, sections were developed in 5% sodium carbonate in dark for 30 minutes. Finally, sections were dehydrated in 70% alcohol for an hour and in 100 % alcohol for 3 minutes, treated with xylene (2 minutes) and mounted with DPX.

A2.3.2. Dendritic spine density analysis

The slides were coded before the analysis. Spines were counted on apical dendrites of medium spiny pyramidal neurons of basolateral amygdala (BLA). The primary dendrites arising from the main shaft were specifically chosen for the analysis. Numbers of spines

present on primary dendrite of the length 80 µm from the origin were counted. All types of spines were included in the analysis. Spine counting was done using Neurolucida image analysis software from Micro-BrightField, Williston, VT, USA and neurons were visualized by Olympus BX61 microscope (100x, 0.95 numerical aperture, Olympus BX61 from Olympus, Shinjuku-ku, Tokyo, Japan.

A2.4. Results & Discussion

Previous studies have reported that the interventions before and after stress exposure have beneficial effects with respect to delayed anxiety and spinogenesis in basolateral amygdala. Our study was set to explore if the neutraceutical like curcumin would have similar beneficial effects.

A2.4.1. Dietary curcumin alleviates anxiety like behavior post 10 days of acute stress

We re-establish in our study that the single episode of stress results in delayed increase of anxiety-like behavior. Stressed rats spent increased time in closed arm and decreased time in open arm compared to their controls (Fig 1. Unstressed-control feed Vs stressed-control feed: B. Open arm time-150.8±13.53 s Vs 103.4±13.53 s; C. Closed arm time-83.67±12.42 s Vs 125.7±12.42 s; Further, they also display increase in anxiety index (Figure.1 Unstressed-control feed Vs stressed-control feed: D. Anxiety index- 0.46±0.04 Vs 0.61±0.04) which is in accordance with previous studies. Furthermore, there was no difference in percentage open arm entries. This could be due to the fact that stressed rats would do risk assessment of open arm hence increasing total number of entries but overall spend less time in open arm. Goal of this study was to assess the effect of dietary curcumin on combating the acute stress induced effect on anxiety-like behavior. Interestingly, dietary curcumin does indeed rescue the delayed increase in anxiety-like behavior in stressed rats. Rats fed with curcumin feed spent more time in open arm, less time in closed arm, showed reduction in anxiety index and had increased percentage open arm entries (Figure 1. Stressed-Control feed Vs stressed-Curcumin feed: B. Open

arm time-103.4±13.57 s Vs 141.2±13.57 s; C. Closed arm time-125.7±12.62 s Vs 87.79±12.62 s; D. Anxiety index- 0.61±0.04 Vs 0.48±0.04). Unstressed rats fed with curcumin feed showed comparable behavioral profile as that of unstressed rats which were fed with normal control feed.

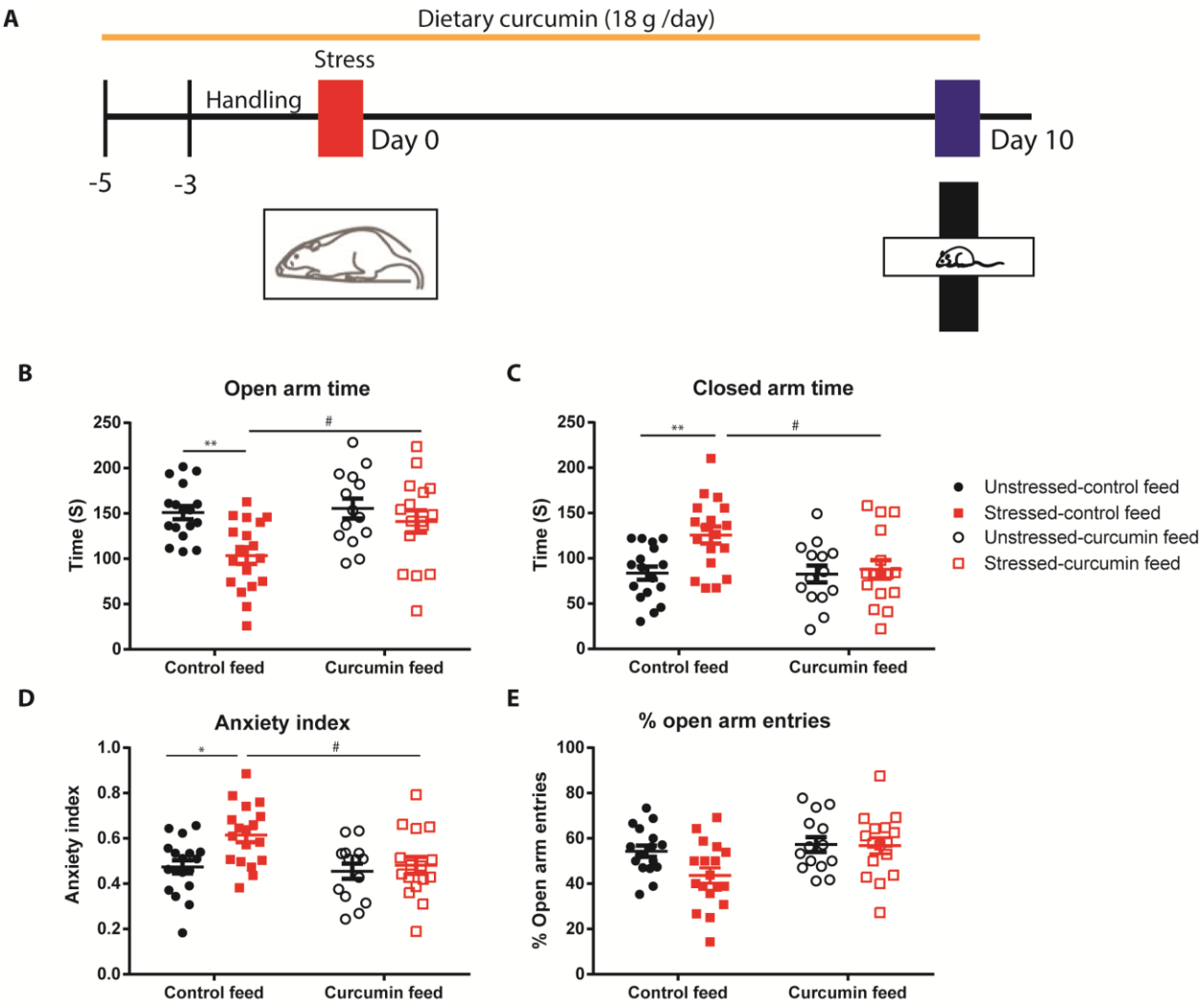


Figure 1: Dietary curcumin alleviates anxiety like behavior post 10 days after acute stress. A. Schematic of the experimental design. B. Stressed rats spent less time in open arm & dietary curcumin significantly increased open arm time in stressed rats [Unstressed - control feed (n=16); Unstressed - curcumin feed (n=14); Stressed - control feed (n=18) & Stressed - curcumin feed (n=16)]; Two way ANOVA: Interaction-$F_{(1, 61)}$

=2.76, p=0.1; Feed-$F_{(1, 61)}$ =4.51, p=0.03; Group-$F_{(1,61)}$=9.56, p=0.003. Post hoc test: Tukey's multiple comparisons test: Control-control feed Vs Stress-control feed, p=0.005**; Stress-control feed Vs Stress-curcumin feed, p=0.04#. C. Stressed rats spent more time in closed arm compared to the control rats and dietary curcumin reduces the time spent in closed arm in the stressed rats. Two way ANOVA: Interaction-$F_{(1, 61)}$ =4.07, p=0.04; Feed-$F_{(1, 61)}$ =4.53, p=0.03; Group-$F_{(1,61)}$=6.65, p=0.01. Post hoc test: Tukey's multiple comparisons test: Control-control feed Vs Stress-control feed, p=0.006**; Stress-control feed Vs Stress-curcumin feed, p=0.01#. D. Stressed rats show increase in anxiety index which is rescued by dietary curcumin. Two way ANOVA: Interaction-$F_{(1, 61)}$ =3.59, p=0.06; Feed-$F_{(1, 61)}$ =4.66, p=0.03; Group-$F_{(1, 61)}$ =7.26, p=0.001. Post hoc test: Tukey's multiple comparisons test: Control-control feed Vs Stress-control feed, p=0.006**; Stress-control feed Vs Stress-curcumin feed, p=0.02#. E. Stressed rats showed comparable percentage open arm entries and dietary curcumin increased the percentage open arm entries in stressed rats.

A.2.4.2. Dietary curcumin reduces stress induced spinogenesis in basolateral nucleus of amygdala

A single episode of stress was enough to increase spine formation in basolateral nucleus of amygdala (Figure.2. Spine number: Unstressed-Control feed (56.46±3.83); Stressed-Control feed (71.18±3.83). The observed changes were post ten days after acute stress and were in line with many previous studies in rodents (*Mitra et al., 2005, Rao et al., 2012 & Chakraborty et al., 2020*). Dietary curcumin might mediate these effects by reducing the serum corticosterone concentration & IL-6 (inflammatory cytokine) levels (*Aubry et al*., *2019*). Moreover, studies in rats have shown that the dietary curcumin reduces expression of immediate early genes like Arc/ Egr-1 which are required for amygdala dependent learning and memory (*Monsey et al., 2015*). Curcumin is also known to reduce depressive-like behaviors and prevents morphological changes in hippocampus when administered during chronic stress (*Xu et al., 2006 & 2009*). Curcumin might exert these effects by inhibiting NF-Kb pathway (Nuclear factor kappa-

light-chain-enhancer of activated B cells) *(Jobin et al., 1999).* Though, many previous studies have looked into beneficial effects of curcumin in PTSD models. Our model is novel with respect to the stressor and its delayed effects. Our study re-affirms the benefits of dietary curcumin on attenuating the delayed increase in spine density which occurs post ten days of a single stress episode (Figure.2. Spine number: Stressed-Curcumin feed (71.18±3.83); Unstressed-Curcumin feed (61.16±3.83). Furthermore, the number of spines across the length (80 µm) of primary dendrite in stressed group showed increase in spines in initial segments (10-30 µm) and dietary curcumin reduced this increase (Figure 2.D).

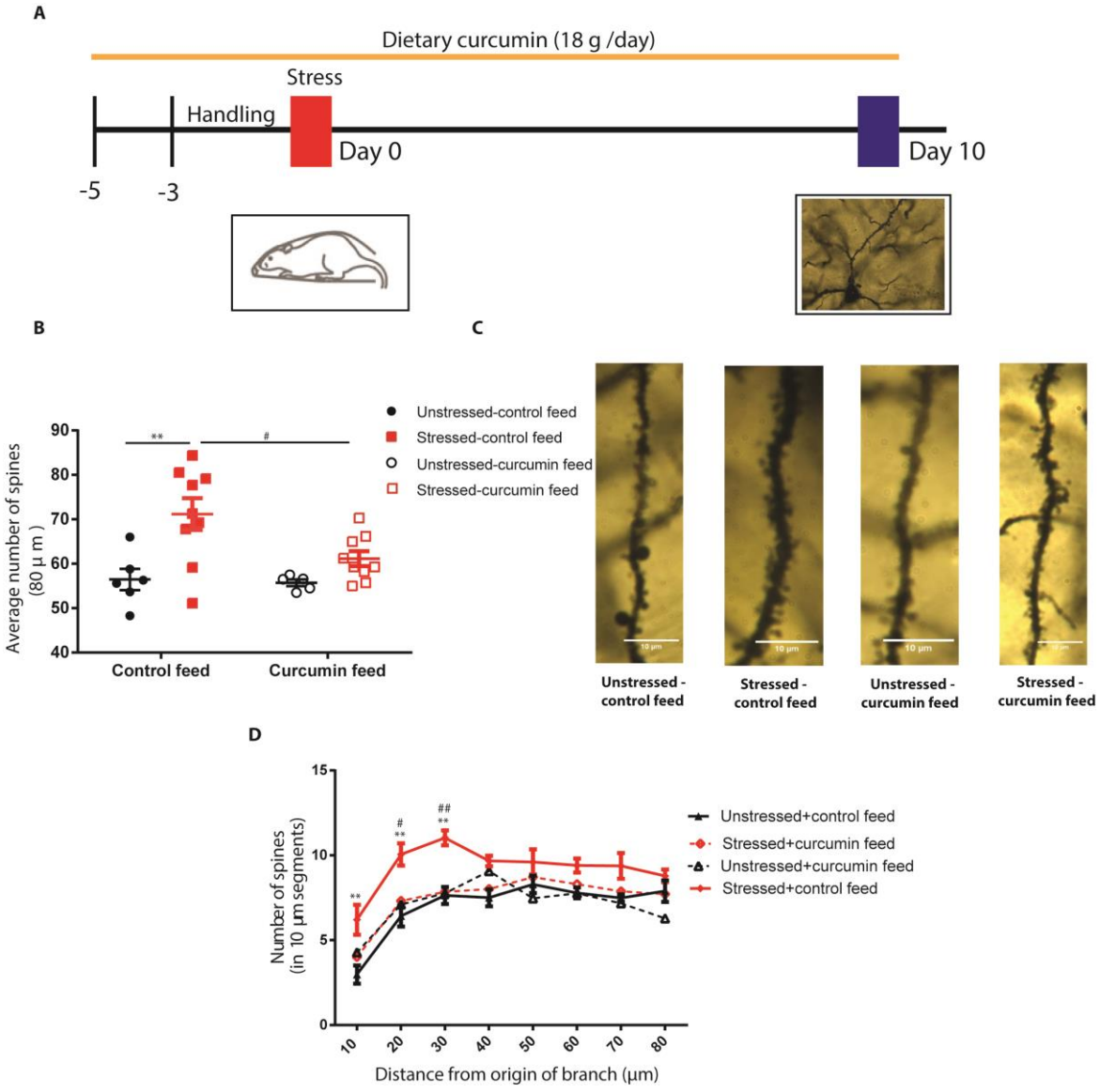


Figure 2: Dietary curcumin reduces stress induced spinogenesis in basolateral nucleus of amygdala. A. Schematic of the experimental design. B. Stress increases spine number in BLA neurons and dietary curcumin reduces the spine numbers in stressed rats. [Unstressed - control feed (n=6); Unstressed - curcumin feed (n=5); Stressed - control feed (n=8) & Stressed - curcumin feed (n=8)]. Two way ANOVA: Interaction $F_{(1, 25)}$ =2.77, p=0.10; Feed $F_{(1, 25)}$ =0.06 & Group $F_{(1, 25)}$ =13.07, p=0.001. Post hoc test: Sidak's multiple comparisons test, Unstressed - control feed Vs Stressed – control feed, p=0.004**; Stressed – control feed Vs Stressed-curcumin feed, p=0.04$^{\#}$. C. Increase in spine number was in initial segments of the primary dendrite in the stress group. Stressed rats fed with dietary curcumin showed reduction in spine numbers in the iitial segments of primary dendrite. Repeated measures Two way ANOVA: Interaction $F_{(21, 147)}$ = 1.29, p=0.18; Dentritic length $F_{(7, 147)}$ = 40.59, p<0.0001; Groups $F_{(3,21)}$ = 6.68, p=0.002. Post hoc test: Sidak's multiple comparisons test – Segment 10 µm, Unstressed - control feed Vs Stressed – control feed, p=0.002**; Segment 20 µm, Unstressed - control feed Vs Stressed – control feed, p=0.001**, Stressed - control feed Vs Stressed – curcumin feed, p=0.01$^{\#}$; Segment 30 µm, Unstressed - control feed Vs Stressed – control feed, p=0.001**, Stressed - control feed Vs Stressed – curcumin feed, p=0.005$^{\#\#}$.

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Appendix.3. Chronic administration of tianeptine reinstates avoidance response to playback of naturally aversive ultrasonic vocalizations in chronically stressed rats

A3.1. Introduction

Severe stress over time leads to psychopathology with range of debilitating symptoms like anxiety, depression, cognitive impairment etc., (*Ressler, 2010*). Rodent models of stress have provided key insights into pathobiology of stress across multiple levels of neural organization (*Chattarji et al., 2015*). At the behavioral level, most of these studies have been strictly limited to paradigms based on principles of Pavlovian conditioning. Apart from failing to provide insights into genetically-hardwired innate behaviors, these paradigms rely largely on stimuli that are not ethologically natural, e.g. exposure to electric foot-shocks. However, recently, many new ethologically relevant behavioral paradigms have emerged that use naturally occurring, ethologically relevant stimuli, e.g., predator odor induced avoidance (*Fendt et al., 2018*), visual looming stimulus induced escape response (*Daviu et al., 2020*), robogator avoidance paradigm (*Choi & Kim, 2010*) and ultrasonic aversive call playback induced avoidance task (*Shukla & Chattarji, 2022*. Former being used in the context of chronic stress recently in our lab which showed impaired avoidance behavior for 22 kHz aversive ultrasonic playbacks in rats. In our present study we have used atypical antidepressant tianeptine, a glutamatergic modulator which is shown to be efficacious both in humans and rodent models of chronic stress and depression (*McEwen & Olie, 2005*). We explored if tianeptine would rescue the avoidance response and its cellular correlates in response to aversive ultrasonic vocalizations playback in chronically stressed rats.

A3.2. Methods

Two month old male Sprague Dawley rats were used for the experiment. Rats were handled for four days prior to start of the experiment. Rats were randomly assigned to any of the following four groups.

A3.2.1. Experimental groups

- Control + vehicle (N = 14)
- Control + Tianeptine (N =16)
- Stress + vehicle (N = 18)
- Stress + Tianeptine (N = 18)

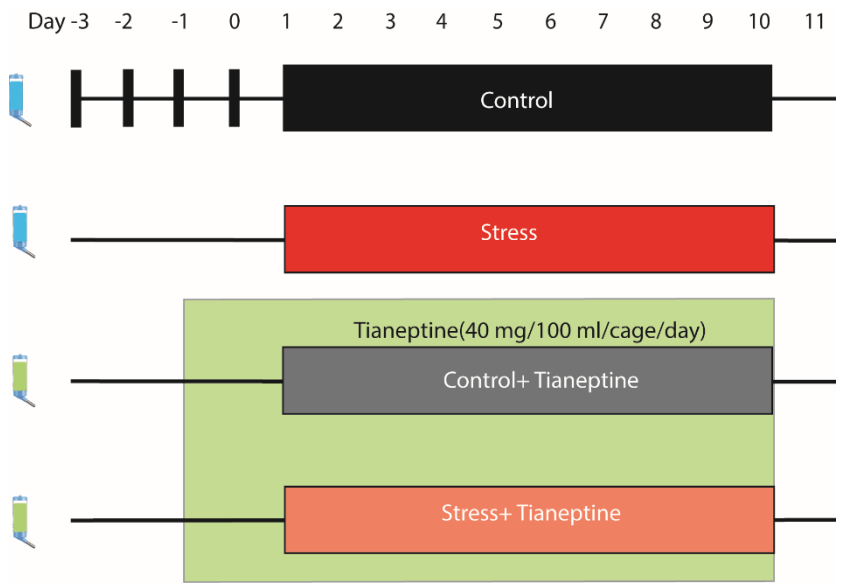
A3.2.2. Stress protocol

Rats were subjected to chronic stress for the duration of 2 hours/day for 10 consecutive days. Rats were immobilized in the conical plastic bags (*Vyas et al., 2002*). Post stress, the rats were released into their respective cages. The corresponding control rats were housed in a different room. Body weight was measured daily throughout handling and stress duration.

A3.2.3. Drug preparation

40 mg tianeptine (Stablon; Servier, Courbevoie, France) was dissolved in 100 ml of autoclaved drinking water. Rats consumed ≈50 ml/day resulting in intake of ≈20 mg tianeptine/rat/day which was in line with the previous study (*Czeh et al., 2001*). Rats either consumed tianeptine / vehicle (water) from two days prior to stress till the last day of stress (Figure.1).

Figure.1



A3.2.4. Playback of 22 kHz aversive ultrasonic vocalizations

Rats were habituated to a black plexiglass linear track (1.97m X 0.30 X 0.30 m) for 5 minutes (*habituation*). Following habituation, rats were subjected to two 3- minute episodes of playback of 22 kHz USV calls (*playback*) through an ultrasonic speaker mounted at one end of the track. Playback episodes were interleaved by a 5 minute long silent period. The behavior was recorded using overhead camera and playbacks were triggered using the Arduino Uno microcontroller board.

A3.2.5. Behavioral analysis

The linear track was divided into equal halves: the half of the track with the speaker was labeled as the proximal half, while the other half was labeled as the distal half. Location of rats was determined by tracking the recorded videos using iD tracker software (*Pérez-Escudero et al., 2014*). Time spent in each half during different phases of the behavior was estimated using custom-written MATLAB scripts. Average time spent during both playback episodes in either half of the track was calculated. Data were analyzed in GraphPad Prism statistical software package.

A3.2.6. Tissue processing & immunohistochemistry for quantifying cFos expression

Post playback or track exposure the rats were deeply anesthetized with ketamine and xylazine and perfused transcardially with 0.1 M phosphate buffered saline (PBS) and by 4% buffered paraformaldehyde (PFA). After perfusion, brains were fixed overnight in 4% PFA, then put in a sucrose gradient (10% - 20% - 30% each overnight). 50 µm thick coronal sections containing basolateral amygdala (BLA) were collected using cryotome.

- Sections were washed twice in PBS at room temperature for about 10 minutes.
- Sections were blocked in PBS with 10% NGS, 0.5% Triton, 0.05% Na-azide for 2.5-3 hours at room temperature.
- Sections were incubated in primary antibody solution ((PBS with 5% NGS, 0.3% Triton, 0.05% Na-azide, 1: 1000 mouse NeuN (Millipore) and 1: 1000 cFos (Millipore)), at 4^0 C under gentle shaking for 72 hours.
- Sections were washed 3-4 times (10-15 min/wash) in PBS at room temperature and incubated in secondary antibody solution (3% NGS, 0.1%Triton, 1: 500 dilution for all the antibodies) for 3-4 hours and were washed in PBS before mounting.

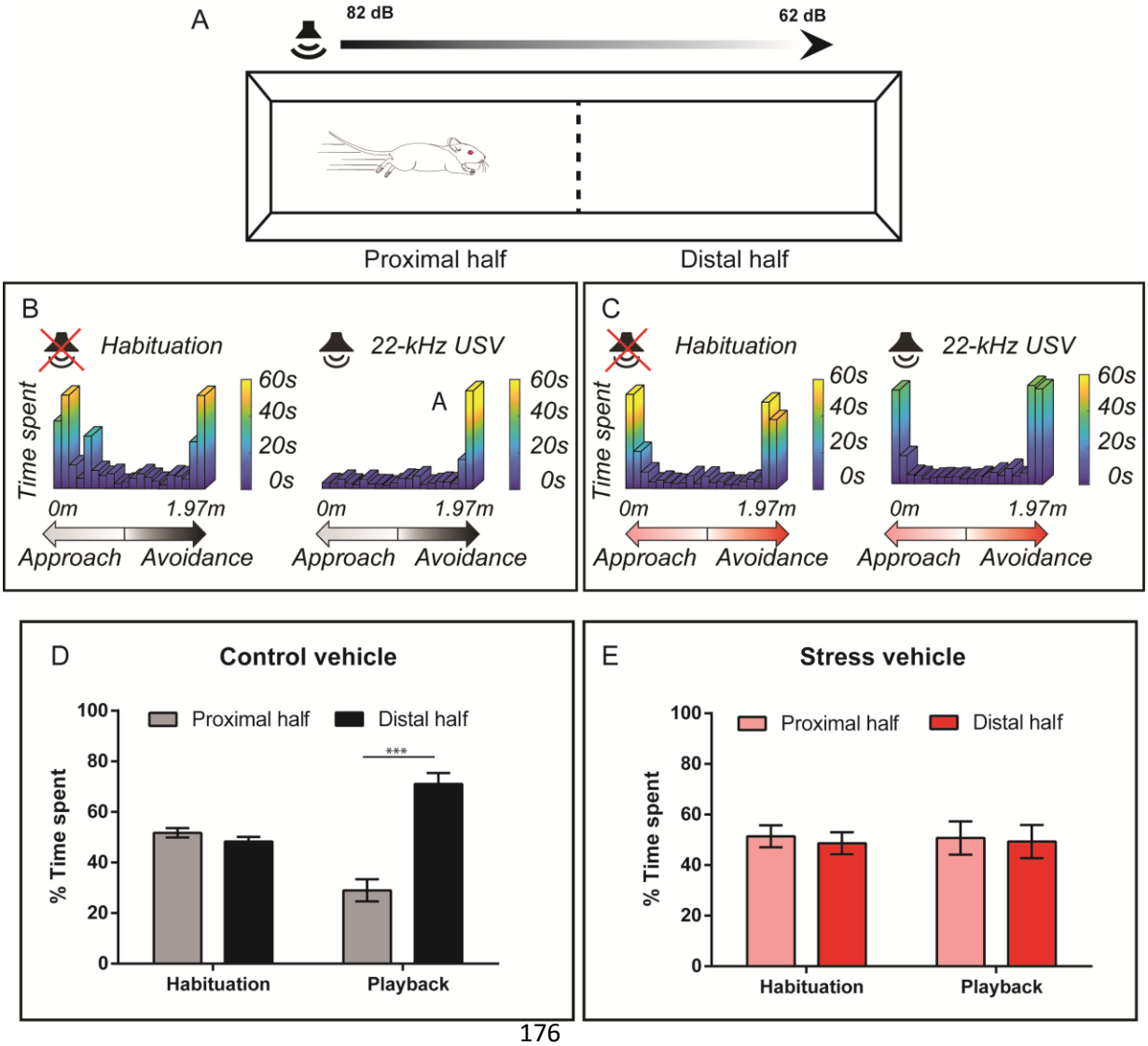
A3.3. Results & discussion

A3.3.1. Effects of tianeptine on impaired avoidance to aversive USV playbacks in chronically stressed rats

During habituation, rats from all the four groups spent equal time in the proximal and distal halves of the linear track. This shows that in the absence of any playback stimulus, rats did not have any inherent bias to either the proximal or the distal half the track. Following habituation, when vehicle-treated control rats were subjected to 22 kHz USV playback episodes, they showed robust avoidance behavior as they spent significantly more time in the distal half of the linear track (% Time spent in proximal half: 40.35±11.38 vs (% Time spent in distal half: 59.65±11.38). On the contrary, as reported previously (*Shukla & Chattarji, 2022*), vehicle-treated stressed rats failed to show avoidance to the playback of 22 kHz USVs. They spent comparable time in the

proximal and distal halves of the track during the playback episodes (% Time spent in proximal half: 51.04±6.58 vs % Time spent in distal half: 48.96±6.58). Tianeptine, an atypical antidepressant, has been to shown to be beneficial in alleviating chronic stress induced changes in dendritic growth and anxiety-like behavior (*Pillai et al., 2012*). In this context, we explored the efficacy of tianeptine to improve ill-effects of stress in ethologically relevant behavior task. While there was no effect of chronic tianeptine treatment on 22 kHz USV playback-induced avoidance behavior in control rats (% Time spent in proximal half: 42.96±3.89 vs % Time spent in distal half: 57.04±3.89), it reinstated the same in rats previously exposed to chronic stress track (% Time spent in proximal half: 35.75±10.27 vs % Time spent in distal half: 64.25±10.27).

Figure.2



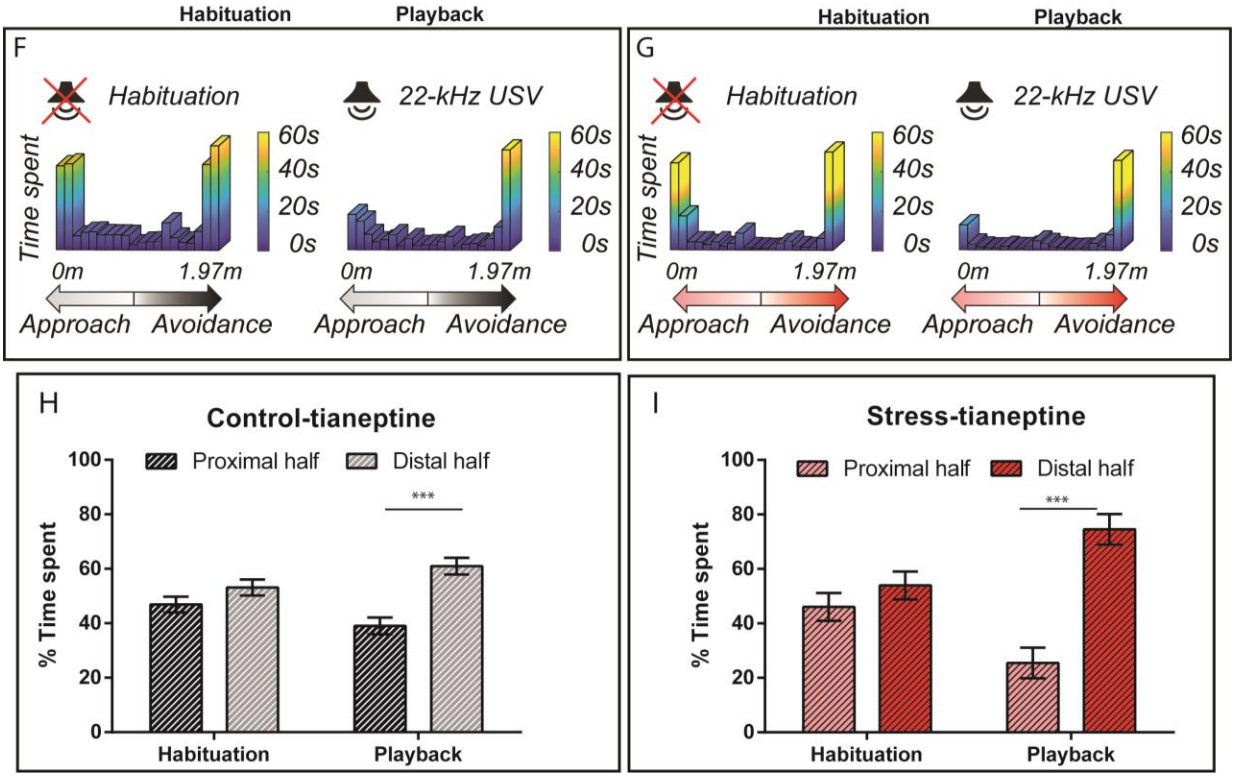


Figure. 2: A. Schematic of the linear track. (B, C) Time spent by a representative vehicle-treated control rat (B) and a representative vehicle-treated stressed rat (C) along the track during habituation (left) and playback episodes (right). D. Time spent by vehicle-treated control rats in the proximal and distal halves of the track. Two-way RM ANOVA followed by Sidak's multiple comparisons test, location: $F_{(1, 13)} = 18.87$, *** $p < 0.001$; playback: $F_{(1, 13)} = 0.09177$, $p = 0.77$; location x playback: $F_{(1, 13)} = 20.27$, $p <$ ***0.001. E. Time spent by vehicle-treated stressed rats in the proximal and distal halves of the track: Two-way RM ANOVA followed by Sidak's multiple comparisons test, location: $F_{(1, 14)} = 0.04$, $p = 0.84$; playback: $F_{(1, 14)} = 2.318$, $p = 0.15$; location x playback: $F_{(1, 14)} = 0.02$, $p = 0.89$. (F, G) Time spent by a representative tianeptine-treated control rat (F) and a representative tianeptine -treated stressed rat (G) along the track during habituation (left) and playback episodes (right). H. Time spent by tianeptine-treated control rats in the proximal and distal halves of the track. Two-way RM ANOVA followed by Sidak's multiple comparisons test, location: $F_{(1, 15)} = 7.754$, *$p < 0.05$; playback: $F_{(1, 15)} = 0.0$, $p >$ 0.9999; location x playback: $F_{(1, 15)} = 5.856$, *$p < 0.05$. I. Time spent by tianeptine-

stressed control rats in the proximal and distal halves of the track. Two-way RM ANOVA followed by Sidak's multiple comparisons test, location: $F_{(1, 12)} = 12.11$, $^{**}p < 0.01$; playback: $F_{(1, 12)} = 0.4059$, $p = 0.5360$; location x playback: $F_{(1, 12)} = 8.823$, $^{*}p < 0.05$.

A3.3.2. Effects of tianeptine on cFos expression in BLA elicited by aversive USV playbacks in stressed rats

Previous study in rats had shown that USV playback activates immediate early genes like cFos in BLA cells (*Sadananda et al*., 2009). Recently, study from our lab had shown that chronic stress leads to reduction in cFos activation upon 22 kHz USV playbacks (*Shukla & Chattarji., 2022*). In the current study, we are assessing the effect of tianeptine on aversive USV induced cFos activation in BLA in stressed rats. To this end, we have finished the tissue processing for immunohistochemistry for cFos. Confocal imaging is ongoing which will be followed by cell counting and data analysis. Representative confocal images of cFos expression in BLA (Figure 3).

Figure. 3

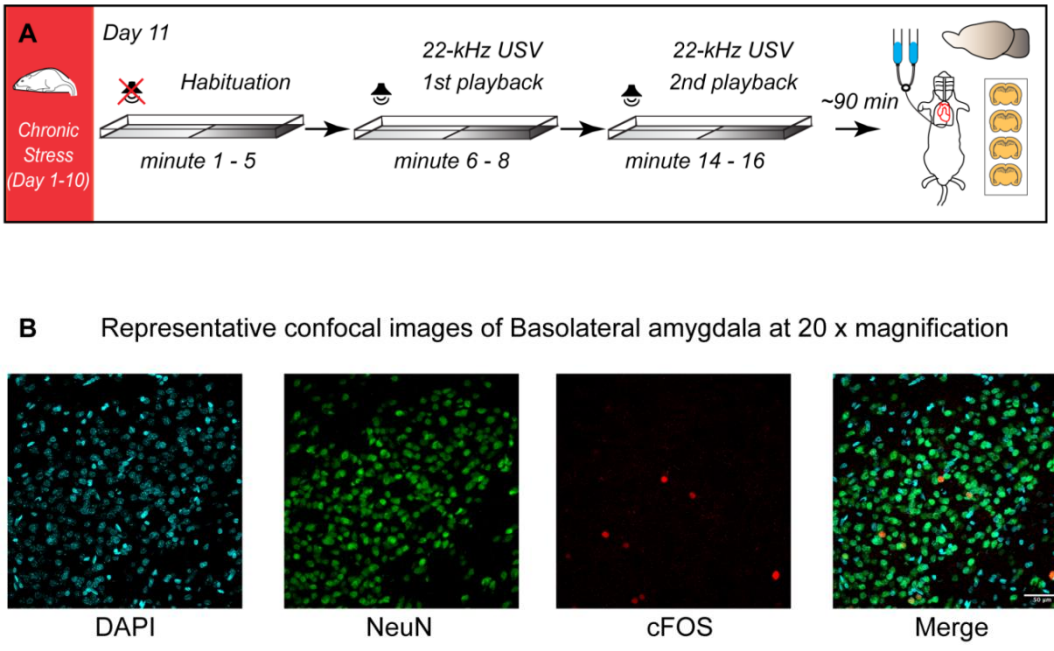


Figure.3: A. Schematic of experimental design for cFos immunohistochemistry. B. Representative confocal images showing expression of DAPI, NeuN, cFos.

A3.4. References

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