Altered plasticity and hunting behavior in Shank3 mutant mice

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Abstract

Altered plasticity and hunting behavior in Shank3 mutant mice

A dissertation presented to the Faculty of the
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By Chelsea Groves Kuhnle

Autism associated disorders (AADs) affect tens of millions of people worldwide and encompass Autism Spectrum Disorder (ASD) and conditions such as Rett, Fragile X and Phelan McDermid Syndromes. This class of conditions are developmental neurological disorders that share common symptoms such as OCD-like behavior, difficulty learning language, sensory hypersensitivity, and social divergence, though the severity of these symptoms varies widely both within ASD and between differing AADs. As AAD symptoms manifest during development, one overarching theme of Autism research focuses on understanding the ways in which genetic factors associated with AADs affect plasticity mechanisms associated with the maturation of neural circuits. It is hoped that by understanding the effect of mutations to AAD-associated genes on neural plasticity and the behavior of circuits, it will be possible to relate these neural underpinnings to the behavioral symptoms associated with Autism. In this thesis I present work contributing to such an understanding of the loss of Shank3, a gene associated with Phelan McDermid syndrome and linked to severe cases of ASD. This work demonstrates that Shank3 is necessary for normal cortical homeostatic plasticity and circuit maturation in the visual cortex, and presents a profile of Shank3 loss in learning and performing the complex ethological behavior of cricket hunting.

In the first section of this work, I investigated the effects of Shank3 loss in the visual cortex. While it was known prior to our study that Shank3 loss blocks Hebbian long term potentiation in the hippocampus, it was not known whether Shank3 is also necessary for homeostatic plasticity in central circuits. We showed through these experiments that Shank3 is indeed required for normal homeostatic plasticity during circuit maturation in the visual cortex, demonstrating that its loss impairs synaptic scaling, intrinsic homeostatic plasticity, firing rate homeostasis, and ocular dominance plasticity. By simultaneously investigating how fundamental homeostatic mechanisms as well as how circuit properties such as ocular dominance plasticity respond to Shank3 loss, we contribute evidence that the stability of circuits in the visual system is affected by loss of Shank3 due to disabled homeostatic mechanisms. We additionally show that both synaptic scaling up and the overgrooming phenotype observed in Shank3 knockout (KO) mice can be therapeutically rescued, contributing evidence that Shank3 abolishes homeostatic mechanisms *via* the GSK3 pathway.

Next, I investigated the effect of Shank3 loss on cricket hunting. While AADs are associated with a wide range of symptoms including learning difficulties and increased time in planning goal-directed actions expected to affect complex ethological tasks, it is only recently that hunting has been established as a paradigm for investigating such behaviors in mice. By investigating the behavior of both wild type and Shank3 KO mice during five days of cricket hunting, we find that both naive and experienced Shank3 KO mice hunt crickets less efficiently than their wild type littermates. Through detailed investigations of the behavior of both strains, we find that such differences are not a result of locomotive motor deficits or neophobia. Rather, they are caused by the ability of wild type mice to more rapidly and dynamically respond to their

prey. We also find evidence that experienced wild type mice perform distinctive actions during cricket attack which Shank3 KO mice did not acquire as a result of training.

Through a series of investigations examining the effect of Shank3 loss on homeostatic plasticity, the behavior of visual cortical circuits and tactics employed by mice during cricket hunting, the work presented in this thesis thus establishes a profile of Shank3 deficiency in mice. These findings are consistent with multiple hypothesized mechanisms by which Shank3 loss affects hunting behavior, including perturbation of the superior colliculus necessary for mediating several aspects of hunting performance and loss of plasticity in the basal ganglia necessary for acquisition of goal directed actions. It is hoped that future work will build on these results to show which factors are responsible for effects of Shank3 loss on complex ethological behavior, and that this will contribute to our broader understanding of neurodivergence.

Preface

In this thesis, I present work which I performed with colleagues while a graduate student in the Neuroscience program in the Brandeis Department of Biology. The goal of these investigations is to better understand the mechanisms by which Shank3 contributes to autistic-like behaviors in mice. To do this, we study Shank3 loss in visual cortical circuits to understand its effects on homeostatic plasticity mechanisms, and study the same mouse model in a cricket hunting test to investigate the contribution of this gene to complex ethological behavior. In **Chapter 1**, I introduce features of Autism Associated Disorders (AADs) and Shank3 as a gene which is implicated in AADs. I also discuss the visual system and measurement of ocular dominance plasticity as a model system for investigating perturbations to plasticity mechanisms, and cricket hunting as an experimental paradigm for studying ethological behavior in a controlled setting. **Chapter 2** presents findings that *Shank3* is required for a set of homeostatic plasticity mechanisms in the visual system and that loss of this gene renders cortical circuits more vulnerable to perturbations than normally developing circuits. **Chapter 3** demonstrates that mice without Shank3 hunt less efficiently both before and after training, and that these differences appear to be related to flexibility in cricket pursuit as well as ability to learn novel behaviors. In **Chapter 4** I discuss these experimental findings and ways in which they contribute to our understanding of the mechanism by which Shank3 loss gives rise to homeostatic plasticity deficits as well as behavioral differences. Finally, I discuss further experiments that can be done to explore these mechanisms in **Chapter 5**.

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Chapter 1: Introduction

Autism Associated Disorders (AADs) and circuit development

Aberrant homeostatic plasticity as a unifying feature of autism

Autism Spectrum Disorders (ASDs) are a class of neurodevelopmental conditions associated with a divergent set of behavioral symptoms such as repetitive behaviors, difficulty in interpreting and responding to social cues, and limited or narrow interests. About 1.68% of the population of the United States is diagnosed with an ASD (Hodges et al. 2020), and together with Intellectual Disability (ID) and other Autism Associated Disorders (AADs) this category of developmental conditions affects tens of millions of people worldwide.

A characteristic shared by many ASD models is an atypical balance of excitation and inhibition (E/I) in central circuits. The hypothesis that an increased E/I ratio is a unifying feature of ASDs (Rubenstein and Merzenich 2003) would seem to explain common features of these disorders such as the relative prevalence of epilepsy in autistic individuals (Bolton et al. 2011) and hyperexcitability of sensory cortices in some autism animal models (Takare and Sweeney, 2017; Zhang et al. 2014). However, reduced excitation (Dani et al. 2005) and regionally dependent variation of E/I ratio (Goncalves et al. 2017) have also been observed in ASD mouse models, calling this hypothesis into question. Rather than conceiving of ASDs as the result of global E/I imbalances, an alternative is to conceptualize them as a set of local E/I perturbations interacting with homeostatic responses which may also be perturbed as a result of the disorder (Nelson and Valakh 2015). These interacting perturbations are thought to lead to atypical function as neural circuits develop. Such a view would indeed predict differential perturbations to E/I ratio across brain regions, as observed in AADs.

This perspective centers plasticity mechanisms regulating excitation and inhibition, the contribution of these mechanisms to circuit development, and perturbations to these mechanisms in AADs. Plasticity mechanisms fall into two categories known as Hebbian and Homeostatic. Hebbian plasticity refers to a set of associative, input-specific processes that tend to be self-reinforcing and occur over relatively short timescales. Homeostatic plasticity refers to a set of mechanisms that act to return neurons to an activity set point when their activity is perturbed. One of these mechanisms, synaptic scaling, utilizes changes in synaptic strength to alter input onto cells bidirectionally in the opposing direction of the activity perturbation, thus adjusting firing rate back to a predetermined set point (Turrigiano et al. 1998).

Evidence from the etiology of several AADs suggests that aberrant homeostatic plasticity during developmental critical periods may cause deficits in circuit function (Verma et al. 2019). In particular, mutations of genes linked to AADs have been shown to cause altered homeostatic plasticity. Rett syndrome, for example, is an AAD caused by a mutation of the gene MeCP2. Mice with MeCP2 mutations were shown to have altered E/I balance favoring inhibition in networks of cortical neurons (Dani et al. 2005). Furthermore, downregulation of MeCP2 blocks activity-dependent synaptic scaling in rat cortical (Blackman et al. 2012) and hippocampal (Qiu et al. 2012) neurons, impairing homeostatic plasticity. Additionally, loss of MeCP2 was shown to cause precocious onset and close of the visual critical period (Krishnan et al. 2015). Another autism-associated gene, *Fmr1*, is linked to Fragile X syndrome (FXS). This syndrome is associated with hyperactivity and seizures, consistent with a view that E/I balance is shifted toward excitation in some circuits. Mouse Fmr1 KO models show that cortical intrinsic homeostatic plasticity is disrupted by loss of this gene (Bulow et al. 2019), though this appears to differentially affect cells in other brain regions such as the hippocampus (Booker et al. 2020).

This altered plasticity may be functionally relevant, as Fmr1 KO mice also show aberrant potentiation in response to monocular deprivation during visual critical periods (Dolen et al. 2007). Taken together this evidence demonstrates support for the view that aberrant homeostatic plasticity alters maturation of circuits during developmental critical periods and that this causes local shifts in the balance of inhibition and excitation, leading to symptoms of AADs.

Sensory hypersensitivity and impaired multisensory integration in AADs

Altered sensory processing is a hallmark of AADs. One example of this is hypersensitivity, which is observed across a range of sensory modalities in those with AADs. For example, children with ASD show increased pain sensitivity and touch sensitivity (Riquelme et al. 2016). Auditory hypersensitivity is also common in those with ASD (Zachary et al. 2021). Hypersensitivity is also found in AADs beyond ASD. For example, individuals with FXS demonstrate auditory hypersensitivity, altered acoustic startle response, and increased tactile defensiveness (Rais et al. 2018).

The origin of sensory hypersensitivities in AAD individuals is not well understood. For one, it is debated whether hypersensitivity results from changes in sensory processing or from higher-level responses to sensory information. Additionally, altered plasticity in sensory systems leading to hyperexcitability may affect sensory processing and result in hypersensitivity (Bülow et al. 2019). While some measures like visual acuity appear to be identical between ASD and typically developing (TD) individuals (Albrecht et al. 2014), those with ASD do appear to show a higher variation in tactile temporal resolution (Ide et al. 2019). It is suspected that auditory hypersensitivity may be the result of decreased inhibitory processing in cortical circuits (Matsuzaki et al. 2014) but other studies suggest that it may alternatively be a conditioned

response to aversive sounds (Lucker et al. 2013). Additionally, some components of sensory hypersensitivity originate in the peripheral nervous system. Specifically, tactile hypersensitivity in monogenic mouse models of autism has been linked to abnormalities in somatosensory neurons (Orefice et al. 2016; Orefice et al. 2019).

Beyond hypersensitivity of individual sensory modalities, those with AADs often demonstrate impaired multisensory integration. A metastudy found that impaired audiovisual integration is prevalent in ASD individuals, more common in children, and was correlated with the severity of other ASD symptoms (Feldman et al. 2018, Brandwein et al. 2015). There is evidence that in socially relevant tasks such as recalling the names of others, impaired multisensory integration hinders the ability of those with ASDs to perform the task (Curti et al. 2020). There is also evidence that disruption to typical visual processing contributes to differences observed in autistic individuals when processing social cues. Visually evoked potential (VEP) studies show that autistic children have an enhanced response to high spatial frequencies compared to non-autistic children, indicating a disruption in typical visual processing (Vlamings et al. 2010). Additionally, autistic individuals have more difficulty remembering facial identity and discrimination than non-autistic individuals (Weigelt et al. 2012). Children with ASD also showed lower behavioral facilitation with multisensory audiovisual input in non-social tasks (Brandwein et al. 2013; Stevenson et al. 2014).

The detailed cell-level origin of atypical sensory processing in developed circuits of ASD individuals is not well understood. Due to the evidence above that plasticity is impaired by mutations in genes related to AADs and because AADs are developmental disorders, it is likely that the impairment of varying plasticity mechanisms affects relevant circuits during development to cause these effects. This is consistent with findings that, for example, ASD

individuals exhibited diminished visual cortical plasticity (Ellis et al. 2021). Furthermore, reduced multisensory integration in individuals with AADs is consistent with findings that plasticity in the superior colliculus (SC) during development determines reactivity to cross-modal stimuli (Xu et al. 2012). There is also evidence that the development of multisensory integration according to normal milestones is delayed in ASD but that developmental deficits are eventually ameliorated later in life (Becker 2018 et al.), underscoring the relationship between impaired development and ASD.

In Chapter 2 we add to the growing evidence for this framework for understanding impaired circuit function in AADs, showing that Shank3 impairs two forms of homeostatic plasticity and that this affects the maturation of visual circuits.

Impaired goal-directed action in AADs

Impaired circuit development in AADs is also revealed in studies examining the learning and performance of goal-directed actions. These studies generally found that such movements were more imprecise and exaggerated in AAD than in TD individuals. Autistic young adults took longer to prepare and execute movements, had more temporal and spatial variability during initial movement, and moved more slowly than TD controls (Glazebrook et al. 2006). Such individuals situationally require more time to plan and execute goal-directed movements (Longuet al. 2011). In a test analyzing subjects' sinusoidal arm movements, ASD individuals' movements exhibited greater jerk than TD controls, and higher acceleration (Cook et al. 2013). Autistic young adults furthermore took longer to prepare movement and showed more variability during initial movement on an aiming task. When the difficulty of the task was modulated by changing the size of the two targets and distance between them, these differences were found to

be more pronounced (Glazebrook et al. 2006). Six-year-old children with ASD showed higher movement initiation latency than TD controls in a sequential goal-directed peg insertion task (Backstrom et al. 2019). Autistic individuals were also able to learn new motor skills with training, but after learning showed a longer latency to begin the motion and more temporal variability at the start of motion (Foster et al. 2019). They also demonstrate inflexible responses in performing goal-directed actions in response to changing reward outcomes (Alvarez et al. 2016).

Studies attempting to disambiguate results of motor action tests have shown nuance in the type of action planning that is impaired in autism, the origins of these effects, and the ways in which they evolve during late development. For example, a study aiming to disambiguate movement type within the context of autism movement planning found that ASD individuals show higher movement latency only in three-dimensional tasks, suggesting that the additional time is related to the need to plan unconstrained high-dimensional movement specifically (Zheng et al. 2019). Additionally, while it has been suggested that the lack of flexibility in movement planning results from an overreliance on habitual behavior, other work has found that ASD does not affect the balance between goal-directed and habitual behavior in some tasks (Geurts et al. 2013). Furthermore, while ASD individuals in late adolescence show an improvement in movement smoothness, they still exhibit hesitation in chaining motor actions (Fukui et al. 2018). This suggests that some amount of the deficit occurring during early development is ameliorated in later life. This highlights the importance of understanding impairment of plasticity mechanisms operating on different timescales.

Rather than being the result simply of motor deficits, it is believed that impairment in learning and performance of goal-directed actions is in part related to dysfunction of striatal

plasticity in individuals with AADs. Cortical afferents to the basal ganglia and the striatum in particular are crucial for the performance of goal-directed action (Balleine et al. 2015; Insel et al. 2017; Hart et al. 2018). There is evidence that acquisition of new goal-directed actions requires plasticity in the posterior dorsomedial striatum (Shan et al. 2014). Thus, striatal disruption and aberrant striatal plasticity in particular might be expected to affect learning new goal-directed actions. In Chapter 3, we examine learning of a complex, ethological goal-directed behavior in an autism model known to affect the function of striatal and other circuits.

Shank3 is implicated in autism associated disorders (AADs)

Involvement of Shank3 in AADs

Shank3 mutation is among the most common genetic causes of AAD, being present in 0.69% of individuals with ASD, and 2.12% of those with ID (Leblond et al. 2014). Haploinsufficiency of Shank3 is furthermore a highly penetrant risk factor for the AAD Phelan McDermid Syndrome (PMS, Costales et al. 2015). PMS is a debilitating neurodevelopmental disorder that most commonly results from a 22q13.3 deletion, a deletion at locus q13.3 on chromosome 22 which eliminates Shank3 as well as other genes. Its symptoms overlap significantly with other AADs, and include motor deficits, intellectual impairment, difficulty with language, auditory hypersensitivity, sleep difficulties, and a higher risk of developing seizure disorders or mood disorders, such as bipolar disorder (Harony-Nicolas et al. 2015; Kolevzon et al. 2014; Mieses et al. 2016; Droogmans et al. 2019). Interestingly, Shank3 mutations in non-autistic individuals have additionally been associated with speech impairments (Manning et al. 2021), demonstrating the diversity of effects linked to this gene.

Role of Shank3 at the synapse

Shank3 is a scaffolding protein localized at the postsynaptic density of glutamatergic synapses (Montiero and Feng 2017). Homer and Shank are among the most abundant scaffolding proteins in the PSD of these neurons, and together form a complex which provides a superstructure interacting with other proteins (Hayashi et al. 2009). Shank3 is rich in domains facilitating protein-protein interactions (Sheng et al. 2000), and proteomic analysis has shown that numerous proteins exhibited altered levels in the striatal post synaptic density (PSD) as a result of Shank3 loss. Shank3 loss significantly decreases Homer1 in the PSD (Reim et al. 2017). Furthermore, Shank3 is involved in recruiting components of mGlu, AMPA and NMDA receptors to the postsynaptic density (Bozdagi et al. 2010) which was consistent with early hypotheses that the gene played a role in controlling synaptic transmission. There is an increasing amount of evidence demonstrating the validity of this view. Shank3 KO in mice has been shown to lower synaptic strength in striatal neurons (Peça et al. 2011) and a different mouse model has been used to show that impaired cortico-striatal signaling in Shank3 KO is the result of mGluR5 deficiency in the PSD (Wang et al. 2016). Furthermore, behavioral and circuit deficits were reversed in Shank3 KO mice through the application of an mGluR5 agonist (Wang et al. 2016; Vicidomini et al. 2017). It has also been shown that Shank3 KO impairs Hebbian long term potentiation (LTP) in mouse (Bozdagi et al. 2010) and rat (Song et al. 2019) hippocampal neurons.

Numerous Shank3 mutant mouse models have been developed, and display a range of phenotypes (Peça et al. 2011; Wang et al. 2011; Gogolla et al. 2014; Mei et al. 2016; Jaramillo et al. 2015; Lee et al. 2015; Luo et al. 2017; Balaan et al. 2019; Guo et al. 2019; Vyas et al. 2020). Behaviorally, *Shank3* loss in mice tends to result in overgrooming, slower ambulation, impaired

motor coordination, anxiogenic behavior, inhibited social interaction, and altered vocalization (Peca et al. 2011). While wild type littermates generally prefer to interact with novel cage mates over investigating novel objects, the *Shank3* KO mice show essentially no difference in their interaction levels between the object and novel mouse (Wang 2011). It should be noted that such social deficits are not found in all Shank3 KO models, likely because these models express varying levels of different Shank3 isoforms which induce subtly different behavioral effects (Mei et al. 2016; Balaan et al. 2019). Additionally, Shank3 KO mice have been observed to groom themselves to the point of injury in a compulsive manner that mimics the obsessive compulsive-like symptoms observed in many forms of autism (Peca et al. 2011).

Before the work described in Chapter 2, it was known that Shank3 is necessary for normal functioning of Hebbian plasticity but not of homeostatic plasticity mechanisms such as synaptic scaling and intrinsic homeostatic plasticity. We show therein that in mice, Shank3 is necessary for normal homeostatic plasticity in visual cortical cells and that additionally, mouse knockout lines undergo aberrant ocular dominance plasticity. This work contributes to the state of knowledge surrounding Shank3 specifically and the role of homeostatic plasticity in AADs more generally.

The visual system as a model for studying developmental plasticity

The visual critical period

Vision is one of the best characterized sensory modalities and is central to many crucial tasks performed by animals in the wild, from identifying and pursuing prey to determining when they themselves might become prey. The visual system has been studied across a wide variety of species and developmental ages. In mammals, vision matures during a well-defined set of critical

periods early in development. Because critical periods have been extensively characterized across many species and can be manipulated using a number of established techniques, the visual system has been widely used as a model for studying plasticity in the brain. In this work, we sought to better understand the role of Shank3 in visual system development at the molecular, functional and behavioral levels.

Critical periods are epochs of heightened plasticity wherein networks in the brain mature and stabilize. Proper maturation relies on both correct gene expression as well as experience during the critical period. Manipulation of both of these factors can be used to perturb the critical period in order to study its normal progression as well as developmental disorders. Critical periods have been identified in circuits mediating a wide range of functions, from those processing sensory information to those governing higher-level functions such as phonology and language syntax rules (Harlow et al. 2010; Barkat et al. 2011; Levelt et al. 2012; Thompson et al. 2020; Werker et al. 2015; Woodard et al. 2020).

Historically, critical periods have frequently been studied by manipulating sensory experience. In the 1960s-70s, Hubel and Wiesel manipulated visual drive in kittens by depriving one eye of visual input, a procedure known as monocular deprivation (MD), over periods as short as 3-4 days (Wiesel et al. 1963; Hubel et al. 1963). Through the use of single unit recordings in the visual cortex, they were able to determine the distribution of cellular responses driven by visual input from each eye and use this distribution to quantify ocular dominance (OD). When Hubel and Wiesel compared cellular responses to contralateral and ipsilateral eyes in visual cortex of kittens following an MD procedure, they found that MD reduced response to the deprived eye and induced a shift in OD away from that eye. This effect was measured after the deprivation period had ended, demonstrating persistent experience-dependent shifts in circuit

behavior. This OD shift occurred when MD was performed on juvenile kittens but not mature cats, demonstrating a visual critical period that closes before maturity. Studies have since catalogued similar critical periods in a wide range of animals such as primates, ferrets and mice, and in brain regions governing different sensory modalities such as the auditory and barrel cortices (Issa et al. 1999; Horton and Hocking, 1997; Gordon and Stryker, 1996; Chang and Merzenich, 2003; Fox, 1992).

Mice have been frequently used as a model for studying visual critical periods for several reasons. Firstly, the kinetics of the mouse visual critical period are well characterized (Espinosa and Stryker; 2012). Secondly, these kinetics resemble those of other mammals, allowing knowledge gained from studying this system to inform our understanding of cortical plasticity in other species (Gordon and Stryker; 1996). Thirdly, many tools have been optimized in mice allowing questions to be asked through genetic manipulation and a wide range of characterization techniques. The visual critical period in mice differs somewhat from other model organisms such as cats in the degree to which plasticity is active in different brain regions. In the mouse visual system, signals generated by retinal ganglion cells in the eyes are relayed to the thalamus, and then along thalamocortical projections innervating L4 of primary visual cortex. There is evidence that the murine visual critical period involves plasticity in both the visual cortex and thalamocortical afferents (Hooks and Chen; 2020). This contrasts with cats, in which experience-dependent plasticity during the corresponding critical period occurs primarily in the visual cortex alone as previously mentioned (Wiesel and Hubel; 1963).

The opening and closing windows for critical periods are controlled by gene expression. The visual critical period in mice is known to require expression of the glutamic acid decarboxylase GAD65 as shown in work using transgenic lines, preventing the canonical shift in

OD that results from MD in the wild type (Hensch et al. 1998; Iwai et al. 2003). GAD65 produces Gamma-Aminobutyric acid (GABA), the primary inhibitory neurotransmitter throughout the central nervous system. In *GAD65* knockout mouse lines, administering diazepam to restore inhibition induces the critical period. This demonstrates that triggering of the critical period is highly dependent on levels of cortical inhibition (Fagiolini and Hensch, 2000), which is consistent with the fact that critical periods occur concomitantly with the maturation of inhibitory networks (Zhang et al. 2011).

Both Hebbian and homeostatic plasticity mechanisms are involved in the visual cortical response to MD. During the first three to four days after deprivation, response to visual drive from the deprived eye decreases as a result of long-term depression (LTD), weakening synapses conjoining decorrelated pre- and postsynaptic neurons (Espinosa and Stryker 2012). This decorrelation is the result of activity in the visual cortex being driven primarily by the open eye during deprivation, so that weak signals resulting from spontaneous firing in visual circuits associated with the deprived eye do not drive cortical activity and are thus not temporally correlated with cortical firing (Hooks and Chen 2020). Over a longer time scale, beyond about 3-4 days, homeostatic plasticity dominates the subsequent response to MD (Mrsic-Flogel et al. 2007; Kaneko et al. 2008). Reduced visual drive resulting from eye closure causes decreased activity in the downstream visual system and homeostatic scaling compensates for this reduction (Lambo and Turrigiano 2013). Evidence suggests that the mechanism for synaptic scaling up is that low calcium levels caused by infrequent depolarization induce a signaling cascade resulting in AMPA excitatory receptor insertion at synapses (Turrigiano 2008), though a comprehensive pathway has not yet been described. Scaling up increases the firing rate of affected neurons, and

in MD induces a partial rebound in cortical response to the deprived eye and an overall increase to drive from the non-deprived eye (Hooks and Chen 2020).

In Chapter 2 we investigate the role of Shank3 in regulating homeostatic plasticity in the visual system, examining both synaptic scaling and ocular dominance plasticity. This work shows that Shank3 is necessary for both of these forms of plasticity, demonstrating that the gene plays a vital role in the maturation of visual cortical circuits.

Hunting as a goal-directed ethological behavior

Characterization of cricket hunting behavior in mice

Cricket hunting by mice is a goal-directed ethological behavior that is mediated by sensory input. It was shown that mice hunt crickets under normal circumstances primarily using vision but that audition can be used for hunting as well, in particular to make up for impaired vision in dark conditions (Hoy et al. 2016). A more detailed understanding of the use of visual input to guide hunting has emerged from several recent studies. Firstly, these showed that during approach, eye movements are used primarily to stabilize head motion. However, unlike the visual strategies of creatures with a fovea, change of visual focus is mainly performed through head motion with saccades acting to amplify these directional shifts and then stabilize the field of view (Michaiel et al. 2020). Secondly, it was shown that proficient hunting relies on binocular integration of signals from both eyes, with ablation of a small minority of retinal ganglion cells necessary for binocular vision sufficient to substantially decrease hunting effectiveness (Johnson et al. 2021). It was also shown that the decision by a mouse to freeze or approach a visual stimulus is driven by combinations of features of that stimulus, and the features triggering hunting change as the mouse gains experience hunting live crickets (Procacci et al. 2020).

Recent work has also revealed that the superior colliculus (SC) plays a substantial role in hunting behavior. A circuit between this region and the zona incerta is involved in triggering the onset of predatory hunting based on sensory information (Shang et al. 2019). It was separately found that GABAergic neurons in the zona incerta play a role in mediating appetitive behavior (Zhao et al. 2019). Additionally, the details of how the SC modulates approach behavior have been explored, showing that widefield vertical neurons mediate prey detection and narrow field neurons are used for accurate orienting to the prey (Hoy et al. 2019).

While the SC mediates approach, several other circuits have been shown to mediate attack behavior. These include GABAergic circuits in the basal forebrain (Cai et al. 2020), and neurons in the medial preoptic area projecting to the ventral periaqueductal gray (Park et al. 2018). Additionally, the amygdala stimulates biting behavior through projections to the reticular formation in the brainstem, and approach behavior through projections to the midbrain periaqueductal gray matter (Han et al. 2017). Furthermore, optogenetically stimulating projections from the mouse lateral hypothalamus to the periaqueductal gray also drove predatory behavior (Li et al. 2018)

This work establishes that ethological, goal-directed predatory hunting behavior is a complex process triggered by sensory information and which involves coordination between many brain regions. As the detailed behavior of identifiable neuronal populations can have a direct effect on macroscopically observable hunting behaviors (Hoy et al. 2019), this task provides an avenue for assessing the results of circuit-level perturbations on high-level actions. We therefore characterized the hunting behavior of Shank3 KO mice in Chapter 3, in order to assess the contribution of Shank3 to the learning and efficient performance of this behavior.

Avenues by which Shank3 KO may affect hunting

In order to hunt, a mouse must integrate information across sensory modalities, rapidly interpret and respond to the behavior of its prey, and engage its own motor system for successful pursuit and capture. As described in the previous section, this requires multisensory integration and coordination between several brain regions. There are thus several reasons to suspect that Shank3 loss may impair hunting ability in mice.

A priori, impairment of hunting ability as a result of Shank3 loss would be unsurprising because of the performance of goal-directed tasks by individuals with AADs. As described above, AADs are associated with more variability in starting and stopping goal directed actions, lack of flexibility in response to changing circumstances, increased hesitation as complex actions are planned, and an inability to smoothly chain successive actions together. As planning and flexible response are required for efficiency in hunting, impairment of hunting ability might be expected in Shank3 KO mice on this basis alone.

At the circuit level, Shank3 loss leads to long-range effects on circuits and signaling which may inhibit the circuit-level integration required for hunting. Shank3 KO mice display impaired multisensory integration, with Shank3 KO models exhibiting hyperreactivity to tactile sensory input, altered audio-tactile responses, and weakened auditory responses (Chen et al. 2020; Gogolla et al. 2014; Engineer et al. 2018). Furthermore, Shank3 KO rats had a lower relative volume of SC, suggesting that Shank3 loss impairs development of this region required for high-level behavioral control mediated by sensory information (Golden et al. 2020). Loss of Shank3 in mammals also induces long-range disruptions such as hypoconnectivity in the prefrontal cortex, global circuit abnormalities associated with learning disabilities (Pagani et al.

2019; Zhou et al. 2019), and diminished signal transmission in the prefrontal cortex (Frost, 2021).

Additionally, Shank3 KO mice exhibit circuit-level and phenotypic differences which may be expected to inhibit their ability to learn goal-directed tasks such as hunting. Goal-directed behavior is mediated by corticostriatal activity (Insel et al. 2017; Corbit et al. 2018, Hart 2018-2) as described above, and acquisition of new goal-directed actions induces and is thought to rely on striatal plasticity (Shan et al. 2014, Perrin et al. 2019, Fisher et al. 2020, Matamales 2020). Shank3 KO mice exhibit altered cortico-striatal synapse size, lowered cortico-striatal synaptic strength, and lower striatal spine density (Peca et al. 2011; Mei et al. 2016; Jaramillo 2016), which may therefore impair the efficient learning and performance of goal-directed tasks. Furthermore, mGluR5 is involved with acquisition of goal directed actions (Chiamulera et al. 2001) and Shank3 KO might therefore impair the acquisition of actions required for hunting.

While plasticity is associated with the acquisition of new behaviors and with AADs as described above, a detailed understanding of how autism-associated mutations contribute to difficulty in learning high-level tasks is lacking. In Chapter 3 we therefore sought to assess the performance of Shank3 KO mice in learning to hunt crickets, a complex ethological task, in order to elucidate the role of Shank3 in learning to perform goal-directed behaviors. We found that both naive and experienced Shank3 KO animals hunt less efficiently than their wild type littermates, showing that Shank3 plays a role in both the learning and performance of goal-directed actions.

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Chapter 2: Autism-Associated Shank3 Is Essential for Homeostatic Compensation in Rodent V1

Preface

This chapter is a version of Tatavarty et al. 2020, a paper entitled *Autism-Associated Shank3 Is Essential for Homeostatic Compensation in Rodent V1* published in Neuron (DOI: https://doi.org/10.1016/j.neuron.2020.02.033). My contribution to this work included the experimental design, execution, data analysis and written description of results for the ocular dominance plasticity experiments presented in Figure 4 (C-F).

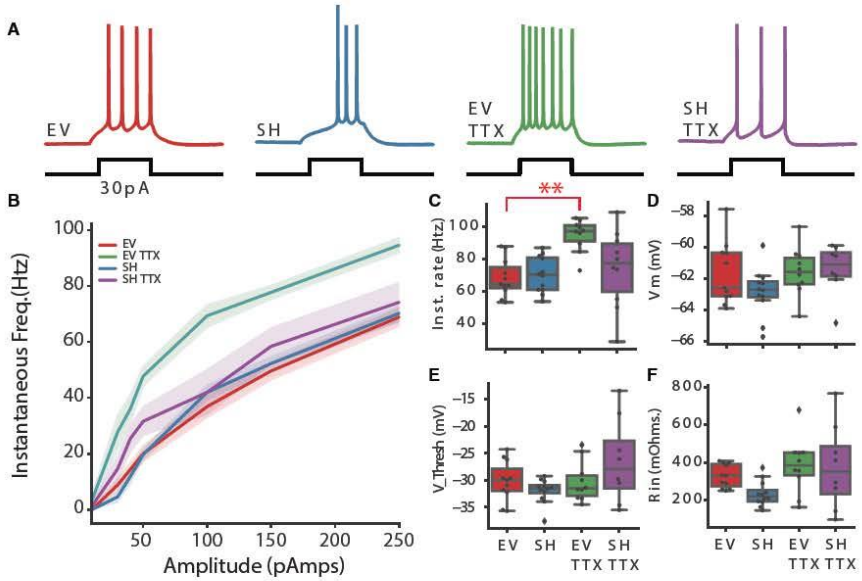

Figure 2. Shank3 Loss Prevents Intrinsic Homeostatic Plasticity

(A) Example firing for neurons under the indicated conditions.

(B) Average *f I* curves for the indicated conditions. Shaded areas around curves denote standard error.

(C) Average instantaneous firing rates for the indicated conditions during the highest current injection (250 pA) (n: EV = 11, EV TTX = 10, SH = 12, SH TTX = 8).

(D F) Passive neuronal properties across conditions, showing Vm (D), VThresh (voltage threshold for action potential firing) (E), and Rin (F).

See also Figures S2 and S4.

Ibata et al., 2008). Synaptic scaling could be rescued by co-expression of an RNAi-resistant form of Shank3 (Figure 1E, resistant [RES]), ruling out off-target effects of the short hairpin RNA (shRNA). Thus, a 50% loss of Shank3 (analogous to haploinsufficiency in human shankopathies) is sufficient to completely abolish synaptic scaling, and this requirement is cell autonomous. As a second means of testing the necessity of Shank3 in synaptic scaling, we made cultures from visual cortices of *Shank3b*$^{-/-}$ (Figures 4F and 4G), *Shank3b*$^{+/-}$, or WT littermate mice (Figure S4E). Although neurons from *Shank3b*$^{-/-}$ and *Shank3b*$^{+/-}$ mice had similar basal mEPSC amplitudes as WT littermate neurons (as reported previously; Chiesa et al., 2019; Peça et al., 2011), in KO neurons, TTX induced only a small and non-significant increase in mEPSC amplitude (Figures 1F and 1G; Figure S4E).

Shank3 Is Required for Homeostatic Regulation of Intrinsic Excitability

Shank3 interacts with ion channels as well as synaptic scaffold proteins (Yi et al., 2016), but the possibility that intrinsic homeostatic plasticity is impaired in shankopathies has not been explored. To determine whether Shank3 is essential for intrinsic homeostatic plasticity, we knocked down Shank3 sparsely in individual cultured neurons as before (Figures S1A and S1B), blocked spiking with TTX for 24 h, and then probed intrinsic excitability by generating firing rate vs current *(f-I)* curves in the presence of synaptic blockers (Figure 2). After TTX treatment, the same current injection evoked more action potentials in EV neurons (Figure 2A), and the entire FI curve shifted significantly upward and leftward (Figure 2B), as expected (Desai et al., 1999). In contrast, TTX had no effect on excitability in Shank3 KD neurons (Figures 2B and 2C), indicating that intrinsic homeostatic plasticity is absent. Interestingly, although in hippocampal pyramidal neurons Shank3 KO reduces a hyperpolarization-activated cation current (I_h) and, thus, increases input resistance (Yi et al., 2016), in these V1 pyramidal neurons Shank3 KD had no significant effect on passive neuronal properties (Figures 2D–2F) or on basal intrinsic excitability (Figures 2B and 2C); similarly, input resistance was not altered in L2/3 pyramidal neurons from Shank3 KO mice relative to WT littermates (Figure S2F).

Synaptic and Intrinsic Homeostatic Plasticity Defects Caused by Shank3 KD Can Be Restored by Li Treatment

Li has wide efficacy to treat many neurological disorders, but the mechanism of action is debated (Pisanu et al., 2016). Recent

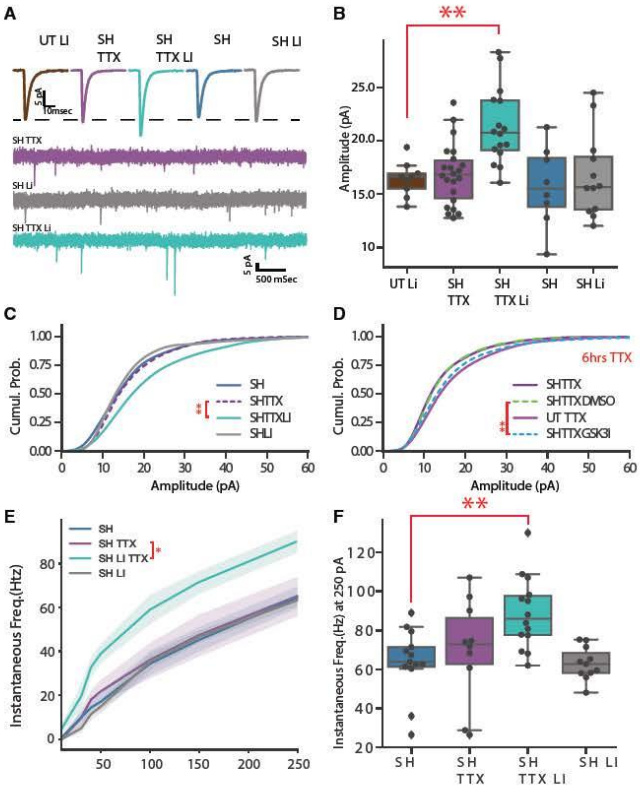

Figure 3. Li Treatment Rescues Intrinsic and Synaptic Homeostatic Plasticity

(A) Average mEPSC traces (top) and example raw traces (bottom) from control or KD neurons treated with Li or Li and TTX for 24 h.

(B) Mean mEPSC amplitudes (n: Li = 9, SH TTX = 22, SH TTX Li = 16, SH = 8, SH Li = 12).

(C) Cumulative histograms of mEPSCs for the conditions in (B).

(D) Cumulative histograms from neurons treated for 6 h with TTX and DMSO (vehicle) or TTX and 5 μM GSK3 inhibitor (GSK3i) (n: UT [untransfected] TTX = 30, SH TTX = 25, SH TTX DMSO = 31, SH TTX GSK3i = 30. SH TTX GSK3i versus SH TTX DMSO (**).

(E) Li rescue: *f I* curves for the indicated conditions.

(F) Average firing rates from the conditions in (E) at the highest current injection (250 pA) (n: SH = 13, SH TTX = 10, SH TTX Li = 14, SH Li = 11).

See also Figure S3.

case studies have raised the possibility that Li might alleviate some neuropsychiatric symptoms in human shankopathies (Darville et al., 2016; Egger et al., 2017; Serret et al., 2015), leading us to wonder whether Li might rescue the defects in homeostatic plasticity induced by Shank3 loss. After 24-h incubation with a therapeutic dose (1 mM) of LiCl, Shank3 KD neurons were again able to fully express synaptic scaling (Figures 3A–3C). Li did not exert these effects through simple global enhancement of synaptic scaling because Li treatment had no effect on the magnitude of scaling in control neurons with a normal Shank3 complement (Figures S3A–S3D). Further, Li had no significant effect on basal postsynaptic (Figures S3B and S3C) or neuronal (Figures S2A and S2B) properties. Strikingly, Li treatment was also able to rescue intrinsic homeostatic plasticity (Figures 3E and 3F); the TTX-induced shift in the FI curve for Shank3 KD neurons treated with Li was similar to that of control neurons (curves not significantly different; Figure S3G).

In addition to rescuing synaptic scaling after Shank3 KD, Li treatment was also able to rescue synaptic scaling in KO mouse cultures (Figure S3F), suggesting that Li is functioning downstream of Shank3 in the signaling pathways that regulate synaptic scaling. Li acts as a direct and indirect inhibitor of GSK3 through inhibitory phosphorylation of Ser9/21 (Freland and Beaulieu, 2012); we therefore tested whether a highly specific GSK3 inhibitor (GSK3i), BRD0320, which blocks GSK3 alpha and beta but not other closely related kinases (Wagner et al., 2016), could rescue synaptic scaling. Like Li, BRD0320 was able to rescue synaptic scaling in Shank3 KD neurons (Figure 3D), suggesting that the GSK3 pathway is dysregulated by Shank3 loss in neocortical pyramidal neurons and that this dysregulation contributes to the observed defect in synaptic scaling.

Shank3 KO Mice Have Deficits in FRH and OD Plasticity

A well-described function of synaptic scaling and intrinsic homeostatic plasticity *in vivo* is to slowly restore firing rates (FRH) and rebalance sensory drive following sensory perturbations (Hengen et al., 2013; Kaneko et al., 2008; Keck et al., 2013; Mrsic-Flogel et al., 2007; Turrigiano, 2017). Given that loss of Shank3 disrupts both of these major forms of homeostatic plasticity, we wondered whether compensatory plasticity during MD *in vivo* would also be disrupted. Initially we examined FRH by implanting critical-period Shank3 KO or littermate WT mice with multielectrode arrays in the V1m, performed MD as described (Hengen et al., 2013, 2016), and followed changes in firing rates over time in freely behaving animals. In both WT and KO animals, the distributions of baseline firing rates were wide, as expected (Darville et al., 2016; Peixoto et al., 2016), and were only subtly different between WT and KO littermates (Figure 4A).

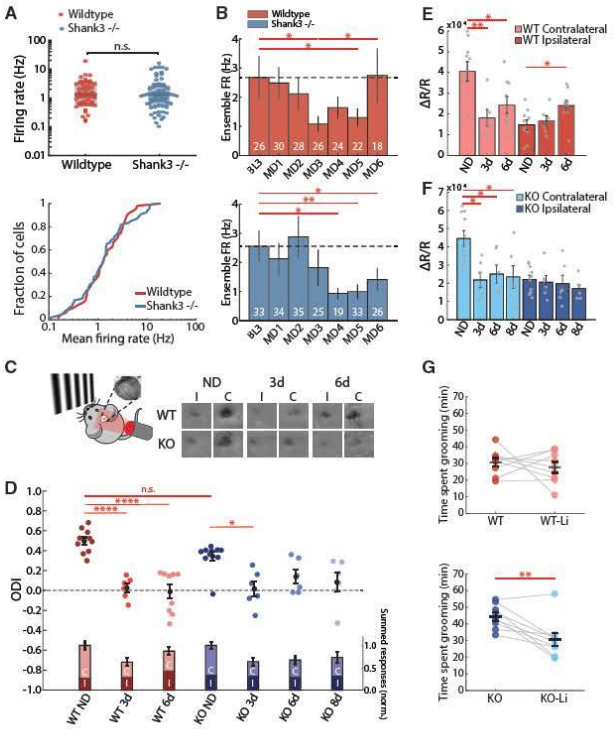

Figure 4. Shank3 Is Required for FRH and OD Plasticity *In Vivo*

(A) Baseline mean ensemble firing rates (top) and cumulative distribution of firing rates (bottom) for V1 neurons in freely behaving Shank3b WT mice and KO littermates.

(B) Ensemble firing rate (hertz) across baseline and 6 day MD. Bars show mean ± SEM; numbers indicate the number of cells recorded on that day.

(C) Schematic of intrinsic signal optical imaging (left) and representative changes in reflectance in response to drive through either the ipsilateral (I) or contralateral (C) eye for both genotypes after no (ND), 3 days, or 6 days of MD.

(D) ODI values for each genotype (WT and KO) for ND, 3 day, or 6 day MD. Inset: summed responses from both eyes, normalized to ND conditions by genotype.

(E and F) Responses to C or I visual drive in WT (E) or KO (F) animals.

(G) Cumulative time spent grooming during a 3 h period, before and after 1 week Li treatment for WT mice (top, n = 9) and Shank3 KO littermates (bottom, n = 9). Grey lines connect data from same mouse; black lines and error bars indicate mean ± SEM.

See also Figure S4.

In freely behaving WT mice, MD resulted in biphasic changes in firing of V1m neurons, as expected (Hengen et al., 2013, 2016): first a drop in mean firing rates that reached a minimum after 3 days of MD (because of suppressive Hebbian mechanisms induced by blurred vision through the closed eye; Smith et al., 2009), followed by homeostatic restoration of firing rates back to baseline values (Figure 4B). This drop was a little slower than described previously in the rat V1m, where the minimum firing rate is on MD 2 (Hengen et al., 2013, 2016). The behavior of neurons in Shank3B KO mice was strikingly different from WT neurons: mean firing rates dropped as for WT animals (although more slowly, reaching a nadir after 4 days of MD), and there was no recovery in firing rates over the ensuing 2 days (Figure 4B). In WT animals, the entire distribution of firing rates initially shifted to the left and then recovered, so that, by MD6, the distribution was statistically indistinguishable from baseline (Figure S4B). In contrast, in Shank3 KO mice, the distribution shifted to the left, and there was little recovery by MD6 (Figure S4A).

In the V1b, MD induces shifts in visual drive from the two eyes that is also biphasic; 3-day MD induces a Hebbian depression of closed (contralateral [C]) eye responses, followed by a homeostatic increase in responsiveness to the open (ipsilateral [I]) eye (Espinosa and Stryker, 2012; Frenkel and Bear, 2004; Gordon and Stryker, 1996; Kaneko et al., 2008; Smith et al., 2009) so that total drive from the two eyes after 6-day MD is restored close to the non-deprived (ND) condition (Figure 4D, WT inset, Mrsic-Flogel et al., 2007). Together, these changes result in shifts in the OD index (ODI; defined as (C−I)/(C+I); Figures 4C and 4D). As a second means of measuring homeostatic recovery in Shank3 KO mice, we therefore performed MD on KO and WT littermates and measured OD shifts in the V1b using intrinsic signal imaging (Figures 4C and 4D; Cang et al., 2005; Moore et al., 2018). In both WT and KO mice, MD shifted ODI toward the open eye (Figure 4D), but the contribution of the two eyes to this shift was distinct in the two genotypes (Figures 4D, inset, 4E, and 4F). Both WT and KO mice exhibited the normal loss of responsiveness to the C eye after 3-day MD, but although WT mice showed the expected increase in I eye responses after 6-day MD (Figure 4E), Shank3 KO mice failed to show this homeostatic increase (Figure 4F). An important question is whether homeostatic compensation is missing in Shank3 KO mice or whether the compensation is simply slower. To address this, we performed MD for 8 days in KO mice and again measured responsiveness to visual stimulation of the two eyes; even after longer deprivation, there was no homeostatic increase in I responsiveness (Figure 4F). Thus, loss of Shank3 severely compromises the ability of neocortical neurons to undergo homeostatic recovery after perturbations to sensory drive.

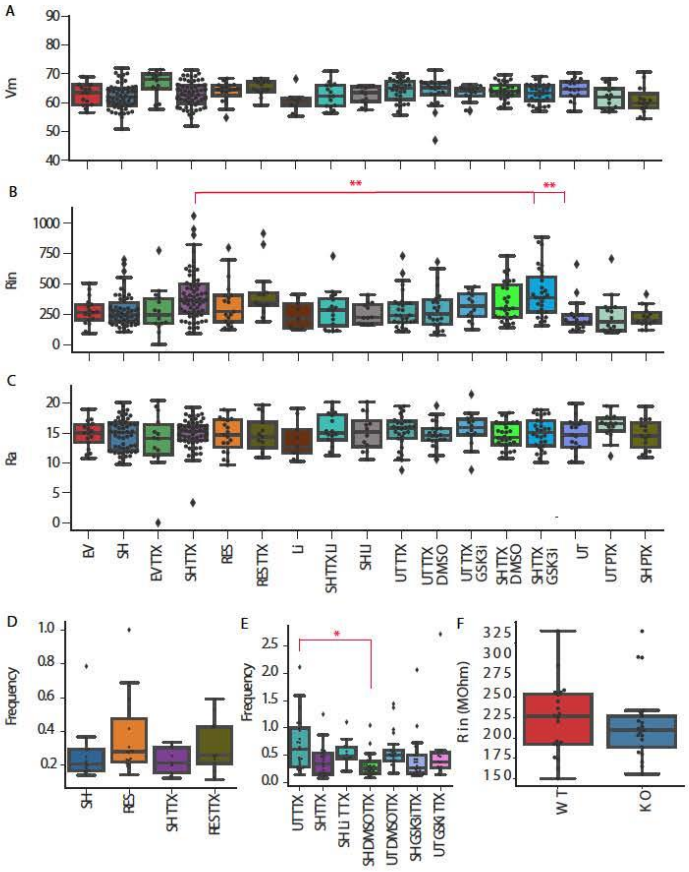

Fig. S2 (related to Fig 1,2): Pooled Passive properties for all conditions. (**A**) Resting membrane potential (Vm), (**B**) Input resistance (Rin) (**C**) Access Resistance (Ra). One-way ANOVA followed by Bonferroni's post hoc test. * = indicated conditions different from each other, $p<0.05$. In general there were no systematic differences in passive properties due to Li, TTX, or Shank3 KD. (**D, E**) Frequency analysis for matched conditions recorded from sister cultures. One-way ANOVA followed by BC. * Different from control $p<0.05$. (**F**) Input resistance (Rin) measured during whole cell recording from KO or WT mouse L2/3 pyramidal neurons in V1. (*n*, WT=21, KO=22; WT vs KO (N.S.) two tailed students t-test).

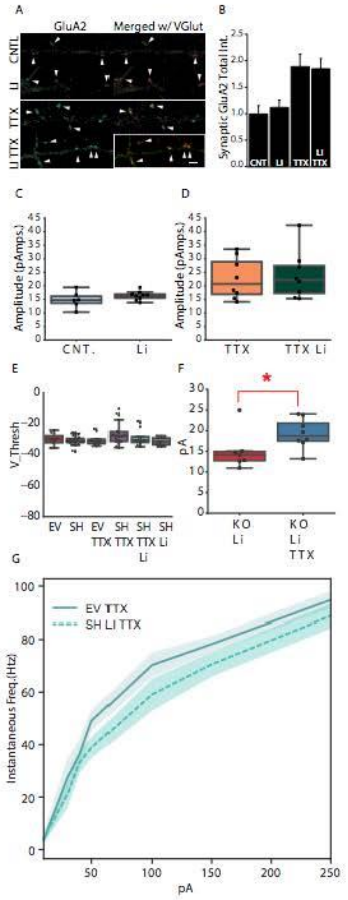

Fig. S3 (related to Fig. 3,4): Lithium does not impact magnitude of synaptic scaling, and rescues scaling in Shank3 knockout neurons. (**A**) Untransfected neurons treated with Li, TTX or both for 24 hrs and immunolabled for GluA2 and VGluT1. (**B**) Quantification of synaptic GluA2 intensity (*n* Control=28, TTX=29, Lithium=27, Lithium TTX=22; Control vs Lithium (N.S.), TTX vs Li TTX (N.S.) two tailed t-test. (**C**) Mean amplitude of mEPSCs recorded from control or Li treated neurons (24 hrs). Li data has been re-plotted from Fig 3B for ease of comparison (*n*, CNT = 6, Li = 9). (**D**) Mean amplitude of mEPSCs recorded from untransfected cells treated with TTX alone or TTX and Li (*n*, TTX = 8, TTX Li = 8). (**E**) Pooled data from intrinsic excitability current clamp experiments was analyzed for changes in threshold for firing (V_Thresh). (N.S.). (**F**) Mean amplitude of mEPSCs recorded from Shank3$^{-/-}$ KO neurons treated with Li or Li and TTX (3 days Li, 24 hrs TTX) (n, KO Li = 7, KO Li TTX = 8) one tailed t-test. (**G**) FI curves for EV TTX and SH Li TTX conditions; the two curves are not significantly different.

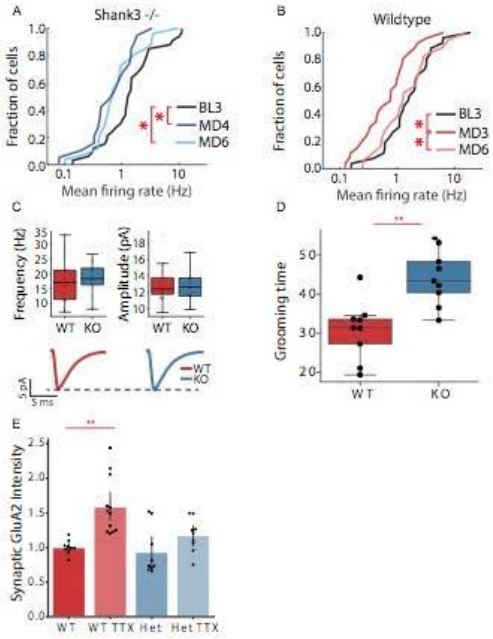

Fig. S4 (related to Fig. 2,4): Characterization of the Shank3 knockout: Cumulative distributions of mean firing rates comparing baseline, early, and late MD for Shank3 knockout **(A)** and wildtype neurons **(B)**; Anderson-Darling test followed by BH-FDR. **(C)** Frequency (left) and amplitude (right) of V1 L2/3 pyramidal neuron mEPSCs (*n*, WT and KO = 24; two-sample t-test (N.S). Average mEPSC waveforms from WT or KO mice. **(D)** Mean grooming time replotted from Fig 4G for comparison. WT vs KO Two-sample t-test, p = 0.0012. **(E)** Synaptic scaling measured using changes in synaptic GluA2 intensity, for WT and Shank3$^{+/-}$ mouse V1cultures treated with TTX for 24 hrs. (n, WT=10, WT TTX=12, Het=9 Het TTX=9, Kruskal-Wallis test followed by Dunn's test, WT vs WT TTX , p= 0.0018)

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Table S1. Details of statistical comparisons. Related to Fig.1-4, S1-4.

Fig	n	Statistical test	P value
1D	EV=17, EV TTX=15, SH=32, SH TTX=22	Kruskal Wallis with Bonferroni's	KW p= 0.0001; EV vs EV TTX p=0.0001; vs SH TTX p=0.092; SH TTX vs EV TTX p=.024; EV vs SH p=0.792
1E	EV=17, EV TTX=15, SH=32, SH TTX=22, RES= 19, RES TTX=13	one way ANOVA with Bonnferroni's	ANOVAp= 0.0001, EV vs EV TTX, p= 0.006; vs SH TTX p=.933; vs Res TTX p= 0.018
1F	Control=19, PTX=19, SH PTX=18	Kruskal Wallis with Bonferroni's	KW p=0.001, Control vs PTX, p=0.0001; vs SH PTX p=0.208
1G	Control=19, PTX=19, SH=17, SH PTX=18	two-tailed Student's t-test	Control vs PTX, p=0.041; SH vs SH PTX, p=0.684
1I	WT=11, WT TTX=20, KO=15, KO TTX= 18	two-tailed Student's t-test	WT vs WT TTX p=0.0150 KO vs KO TTX p=0.127
2C	EV=11, EV TTX=10, SH=12, SH TTX=8	one way ANOVA with Bonnferroni's	ANOVA p=0.001; EV vs EV TTX, p=0.001, vs SH, SH TTX, p=1.0
2D	EV=11, EV TTX=10, SH=12, SH TTX=8	one way ANOVA	ANOVA p=0.18
2E	EV=11, EV TTX=10, SH=12, SH TTX=8	one way ANOVA	ANOVA p=0.08
2F	EV=11, EV TTX=10, SH=12, SH TTX=8	one way ANOVA with Bonnferroni's	ANOVA p=0.0411; EV vs EV TTX = 0.9, EV vs SH= 0.56, EV vs SH TTX = 0.9, SH vs SH TTX = 0.132
3B	Li = 9 SH TTX = 22, SH TTX Li = 16, SH = 8, SH Li = 12,	one way ANOVA with Bonnferroni's	ANOVA p=0.0001; LiCl2 vs SH Li TTX, p=0.006; vs SH TTX, p=1, vs SH, p=1; vs SH Li, p=1
3C	SH TTX = 22, SH Li = 12, SH TTX Li = 16, SH = 8	Kruskal Wallis with Bonferroni's	KW p=0.0001; SH TTX Li vs SH TTX, SH, and SH Li p=0.0001; vs SH vs SH TTX, p=0.854
3D	UT TTX= 30, SH TTX= 25, SH TTX DMSO = 31, SH TTX GSK3i = 30	Kruskal Wallis with Bonferroni's	KW p=0.0001; SH TTX vs SH TTX DMSO, p=1; SH TTX vs UT TTX, p = 0.0001; SH TTX vs SH TTX GSK3i, p=0.004
3E	SH TTX=10, SH TTX Li =14	One tailed Student's t-test	SH TTX vs SH TTX Li p=0.033
3F	SH=13, SH TTX=10, SH TTX Li =14, SH Li=11	one way ANOVA with Bonnferroni's	ANOVA p= 0.003, SH vs SH TTX Li, p=0.006; vs SH TTX, p=1.0, vs SH Li, p=1.0.
4A Top	WT = 51, KO = 68	Wilcoxon rank sum test	p = 0.750

4A Bottom	WT = 51, KO = 68	Anderson Darling test	p = 0.631
4B	WT=25 (BL3), 26 (MD3), 18 (MD6). KO=33 (BL3), 19 (MD4), 26 (MD6)	Kruskal-Wallis test. One tailed pairwise Wilcoxon rank sum tests, p values adjusted with Benjamini Hochberg FDR procedure	All timepoints BL3 through MD6 WT= 0.0379, KO=0.008 ***WT:*** BL3 vs MD1=0.3123, BL3 vs MD2=0.2055, **BL3 vs MD3=0.0111**, BL3 vs MD4=0.1008, **BL3 vs MD5=0.0430**, BL3 vs MD6=0.3123 **MD3 vs MD6=0.0425**, MD4 vs MD6=0.2630 ***KO:*** BL3 vs MD1=0.1475, BL3 vs MD2=0.5379, BL3 vs MD3=0.1962, **BL3 vs MD4=0.0070**, **BL3 vs MD5=0.0425**, **BL3 vs MD6=0.2630**, MD3 vs MD6=0.7829, MD4 vs MD6=0.4034
4D	WT ND = 10, WT 3d = 6, WT 6d = 9, KO ND = 10, KO 3d = 6, KO 6d = 6, KO 8d = 6	two-way ANOVA with Tukey's multiple comparison test	ANOVA p= 0.0001 ND:WT vs. 3d:WT p <0.0001, ND:WT vs. 6d:WT p <0.0001, ND:WT vs. ND:KO p = 0.5069, ND:KO vs. 3d:KO p = 0.0143
4E	WT ND = 10, WT 3d = 6, WT 6d = 9,	one-way ANOVA with Tukey's multiple comparisons test	ANOVA p= 0.0053 Contralateral: WT nd vs. wt 3d p = 0.0076, WT nd vs. wt 6d p = 0.0313; ANOVA p= 0.03 Ipsilateral: WT nd vs. wt 3d p = 0.8870, WT nd vs. wt 6d p = 0.0321
4F	KO ND = 10, KO 3d = 6, KO 6d = 6, KO 8d = 6	one-way ANOVA with Tukey's multiple comparisons test	ANOVA p= 0.0045 contralateral: KO nd vs. KO 3d p = 0.0128, KO nd vs. KO 6d p =0.0379, KO nd vs. KO 8d p = 0.0227; Ipsilateral: ANOVA p=0.7278 KO nd vs. KO 3d p = 0.981, KO nd vs. KO 6d p = 0.954, KO nd vs. KO 8d p = 0.6739
4G	WT= 9 KO=9	Wilcoxon sign-rank test.	WT vs WTLi p = 0.8203; KO vs KOLi p = 0.0117.
S1B	EV=34, SH=27	Two-tailed Student's t-test	p=0.0001
S1D	EV=23, SH=26, EV TTX= 23, SH TTX= 24	one way ANOVA with Bonnferroni's	ANOVA p= 0.002 EV vs SH, SH TTX p=1, vs EV TTX p=0.043
S1F	EV=17, SH=18, EV TTX= 17, SH TTX=15	one way ANOVA	ANOVA p=0.353,
S1G	EV=17, SH=15, EV TTX= 15, SH TTX= 12	one way ANOVA	ANOVA p= 0.547
S2A,B,C	pooled across all mEPSC recordings EV =17, SH=58, EV TTX= 14, SH TTX=67, RES=19, RES	one way ANOVA with Bonnferroni's	ANOVA p= 0.0001, UT vs SH TTX p=.01, vs SH TTX GSK3i p=0.009 UT vs EV,EV TTX, RES, RES TTX, Li, SH TTX Li, SH Li, UT TTX, UT TTX DMSO, UT

	TTX=13, Li=9, SH TTX Li=16,SH Li=12, UT TTX=29, UT TTX DMSO=22, UT TTX GSK3i=14, SH TTX DMSO=30, SH TTX GSK3i=30, UT=19, UT PTX=16, SH PTX=15		TTX GSK3i, SH TTX DMSO, UT PTX, SH PTX, p=1
S2D	SH=17, RES= 19, SH TTX = 9, RES TTX = 13	one way ANOVA	Anova p=.0729
S2E	UT TTX=25, SH TTX = 25, SH TTX Li=12 SH TTX CompA = 30, UT TTX CompA = 22, SH TTX CompB = 30, UT TTX CompB = 14	one way ANOVA with Bonnferroni's	UT TTX vs SH TTX =0.094, vs SH TTX Li= 1, SH TTX CompA=0.004, vs , UT TTX CompA = 1, vs SH TTX CompB = 0.1 vs UT TTX CompB = 1
S2F	WT=24, KO=24	Two-tailed Student's t-test	Rin: p=0.640
S3C	Control=28, TTX=29, Lithium=27, Lithium TTX=22	Two-tailed Student's t-test	Control vs Lithium: p=0.93; Lithium TTX vs TTX: p=0.50
S3D	TTX=8 TTX Li=8	Two-tailed Student's t-test	TTX vs TTX Li=0.77
S3E	pooled across all Intrinsic excitability experiments EV =11, SH=25, SH TTX=19, SH TTX LI = 13, EV TTX = 11	one way ANOVA with Bonnferroni's	ANOVA p= 0.006 EV vs SH TTX, p=0.170; vs EV TTX, vs SH TTX Li,vs SH Li, vs SH p=1; UT vs UT Lip=0.29; Li vs Li TTX p=0.77
S3F	KO Li = 7, KO Li TTX = 8	One tailed Student's T test	p=0.034
S3G	Replotted WT TTX from Fig 2 and SH TTX Li from fig 3	One tailed Student's T test	p=.079
S4 A,B	WT=25 (BL3), 26 (MD3), 18 (MD6). KO=33 (BL3), 19 (MD4), 26 (MD6)	Pairwise Anderson-Darling tests followed by Benjamini-Hochberg procedure (FDR = 0.05)	WT: BL3 vs MD3, p=0.003*; MD3 vs MD6, p=0.025*; BL3 vs MD6, 0.704. KO: BL3 vs MD4, p=0.028*; MD4 vs MD6, p=0.603; BL3 vs MD6, p=0.029*. *: test significant after FDR procedure All p values non adjusted.
S4C	WT=24, KO=24	Two-tailed Student's t-test	Freq: p = 0.788; Ampl: p = 0.564
S4D	Replotted from Fig 4 for comparison. WT= 9, KO = 9	Two-tailed Student's t-test	p=0.0012
S4E	WT=10, WT TTX= 12, Het=9, Het TTX=9	Kruskal-Wallis test with post hoc Dunn's multiple comparison test.	Kruskal-Wallis test. p=0.0001 WT vs WT TTX = 0.001, WT vs Het= 0.99, WT vs Het TTX = 0.824, Het vs Het TTX = 0.36

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Chapter 3: Shank3 knockout mice exhibit altered behaviors in cricket hunting

Preface

This chapter is a draft of a manuscript entitled *Shank3 knockout mice exhibit altered behaviors in cricket hunting* intended to be submitted shortly. My contribution to this work included design, execution, and data analysis of all experiments as well as authoring the initial draft of the manuscript.

Manuscript Abstract

Individuals with autism associated disorders such as Phelan-McDermid syndrome often exhibit different strategies or characteristics when solving complex tasks. Phelan-McDermid syndrome arises from haploinsufficiency of the glutamatergic synaptic protein Shank3. To gain insight into the influence of Shank3 on performance of ethologically relevant complex tasks, we examined characteristics of Shank3 knockout (KO) mice and their wild type littermates as they learned to hunt crickets. Shank3 KO mice initially showed interest in examining live and dead crickets, but required more exposure before they began consuming them as food compared to wild type animals. After five days of cricket hunting, both genotypes learned the task and Shank3 KO animals were only slightly slower compared to wild type littermates. Compared to wild type mice, Shank3 KO mice exhibited different characteristics when pursuing crickets that defied explanation as a simple motor deficit. Although both genotypes moved at the same average speed when approaching a cricket, Shank3 KO mice paused more often during their approaches, did not begin their final accelerations toward crickets as early, and made movements

that did not close the distance gap to the cricket as quickly as wild type mice. Therefore, Shank3 KO mice recapitulate some behavioral characteristics of individuals with autism associated disorders performing complex tasks such as slower action initiation and completion, which may allow some of the underlying neural circuit mechanisms to be uncovered in future studies.

Manuscript Introduction

Individuals within a population develop different strategies for solving ethological tasks. Humans with autism spectrum disorder (ASD) require more time to plan and execute goal-directed movements, exhibit more temporal and spatial variability during initial movement, and move at slower speeds (Longuet et al. 2011; Glazebrook et al. 2006). In addition, ASD individuals demonstrate less flexible responses in performing goal-directed actions (Alvarez et al. 2016), and diminished audio-visual multisensory integration (Stevenson et al. 2014, Feldman et al. 2018, Brandwein et al. 2015). Enhanced responsiveness to auditory signals has also been reported (Bonnel et al. 2003; Stanutz et al. 2012). The neural underpinnings of these differences remain unclear.

Some conditions related to ASD have a monogenic origin, which facilitates studies of relationships between phenotype and genotype in animal models. Many ASD cases as well as the Autism-Associated Disorder (AAD) Phelan-McDermid syndrome are caused by haploinsufficiency of Shank3 (LeBlond et al. 2014; Costales et al. 2015), a scaffolding protein found at glutamatergic synapses (Montiero and Feng, 2017) that is required for normal homeostatic and Hebbian plasticity in rodent models (Tatavarty et al. 2020, Bozdagi et al. 2010, Song et al. 2019). Shank3 KO mice display impaired multisensory integration, with Shank3 KO models exhibiting hyperreactivity to tactile sensory input, altered audio-tactile responses, and

weakened auditory responses (Chen et al. 2020, Gogolla et al. 2014, Engineer et al. 2018). While plasticity is associated with the acquisition of new behaviors and many monogenic autism models exhibit altered plasticity (Blackman et al. 2012, Nelson et al. 2015, Soden et al. 2010), a detailed understanding of how autism-associated mutations contribute to difficulty in learning high-level tasks is lacking. We therefore sought to assess the performance of Shank3 KO mice in learning a complex task in order to elucidate the role of Shank3 in learning to perform goal-directed behaviors.

Cricket hunting is an ethologically relevant behavior that likely requires integration of activity across many brain regions in order for animals to achieve proficiency. The primary visual cortex (V1) is among the relevant brain regions which may be impaired in Shank3 KO mice as vision is known to be crucial for efficient prey capture (Hoy et al. 2016; Johnson et al. 2021), and Shank3 loss disrupts homeostatic compensation in the visual cortex as shown in Chapter 2 (Tatavarty et al. 2020). Visual acuity (Noutel et al. 2011; Durand et al. 2012) and depth perception (Felgerolle et al. 2019) are additionally known to be impaired in MeCP2 and Fmr1 mutant mice, respectively, which exhibit altered cortical homeostatic plasticity as do Shank3 KO mice. V1 in turn innervates the superior colliculus (SC), a multisensory hub crucial for regulating predatory hunting behavior as well as other goal-directed visual activities (Furgio et al. 2010; Zhen et al. 2017; Hoy et al. 2019; Wang et al. 2020). Connections from superior colliculus to the basal ganglia are critical for switching between exploratory and hunting behaviors (Shang et al. 2019, Huang et al. 2020), and Shank3 KO mice exhibit altered cortico-striatal synapse size, lowered cortico-striatal synaptic strength, and lower striatal spine density (Peca et al. 2011; Jaramillo et al. 2016; Mei et al. 2016).

We allowed Shank3 knockout mice and their wild type littermates to hunt crickets over a period of five days. Shank3 KO mice were initially less likely to recognize crickets as prey and initiate hunting, but they learned to perform this behavior over several sessions. By day five, Shank3 KO mice captured and consumed as many crickets as wild type animals and were only a little slower in overall time-to-capture. However, there were two major differences in the approaches of Shank3 KO and wild type animals. First, Shank3 KO mice paused much more frequently during their approaches. Second, while Shank3 KO and wild type mice exhibited the same average and peak speeds during approaches, wild type mice showed much greater modulation of their speed, increasing their speed progressively as they neared a cricket in a way that Shank3 KO mice did not. Therefore, just as neurotypical individuals and autism spectrum disorder individuals exhibit different behavioral strategies when performing goal-directed tasks (Alvarez et al. 2016, Longuet et al. 2011; Glazebrook et al. 2006), wild type and Shank3 KO mice developed cricket hunting behaviors with distinct features. The circuit mechanisms underlying these differences can be explored in future experiments.

Methods

Experimental Model and Subject Details

All procedures were approved by the Brandeis University Institutional Animal Care and Use Committee, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all experiments, mice of both sexes were used. No differences were noted between males and females and data were combined. We used the homozygous Shank3B knockout mouse introduced by Peça et al. (2011), obtaining founder mice from Jackson labs

(Stock No: 017688). Genotyping was done based on primers as previously described (Peça et al. 2011). All animals began habituation between the ages of p26-p28 and data were combined.

Live Cricket hunting

Littermate pairs of animals were moved to the behavioral testing room two days before testing and housed as previously described (Hengen et al. 2013). Cedar chip bedding, standard chow, water, huts and several toys were provided in the enclosure. A twelve-hour circadian cycle was maintained, with dark hours between 7:30 pm and 7:30 am. Animals were weighed each day and monitored for health indicators. During the second night of habituation, mice were deprived of standard chow for no more than 16 hours total and five decapitated crickets were left in the enclosure. Ad libitum access to water continued during food deprivation. On the morning of the third day, testing began. The enclosure in which animals were housed was used additionally as the arena for hunting. Animals were moved to a carrier after weighing, while the arena was prepared. Bedding was disposed of and Fisherbrand™ Absorbent Underpads were taped down both on the underside and at the edges of the arena. On each corner of the arena, a cricket dispenser was placed and loaded so that an Arduino motor system could be used to robotically dispense crickets. Dispensers on opposite corners of the arena were programmed to rotate simultaneously so that animals would not be able to predict which dispenser the cricket was dropping from. Animals were first habituated to the arena for several minutes before a cricket was released. Behavior was recorded using a Logitech C920x HD Pro Webcam and Synapse (TDT) software. The duration of each hunting session was either three hours, or up to 6 crickets per session, whichever was shorter. One hunting session per day, per animal, was performed. Once both animals completed the hunting task, the absorbent padding was removed from the

arena and fresh bedding was laid down along with food, huts, and toys. Water was available ad libitum throughout the experiment. After the first day of hunting, animals were food deprived without access to standard chow or dead crickets each night for a maximum of 16 hours. Animals were weighed each day and removed from the experiment if weight loss exceeded 20% of the starting weight, and continually monitored during experiments for signs of pain or distress. Five days of testing were performed per mouse. Animals were sacrificed following the fifth day of testing in accordance with the IACUC approved protocol.

Approach and consumption during dead cricket and cereal trials

The procedure for dead cricket consumption experiments was similar to that described for live cricket hunting. Littermate pairs of mice were habituated in an enclosure, and food deprivation was performed overnight following the second day of habituation. For these tests, mice did not have access to dead crickets during food deprivation. Dead crickets used for consumption trials were decapitated the preceding night. The arena was prepared and behavior was recorded as described in the live cricket hunting tests. A mouse was placed in the arena and crickets were dispensed one at a time by hand. Crickets were left in the arena for mice to consume at will. The duration of a session was three hours or after a maximum of 5-6 crickets were consumed. Mice were tested in sequence, with two dead cricket consumption trials performed per day. A single day of testing was performed for each mouse. After animals completed the dead cricket test, pieces of brightly colored sugary cereal (Froot Loops, Kellogs) were weighed and dispensed into the arena by hand one at a time. This test lasted for a maximum of one hour or until three total Froot Loops were given to the animal. Remaining Froot Loops

were again weighed following consumption. Animals were sacrificed following Froot Loop tests in accordance with the IACUC approved protocol.

Data Analysis

Videos recorded during live cricket hunting and dead cricket consumption trials were manually scored in order to determine the times of a) cricket release, b) mouse initially orienting to the cricket's position, c) cricket capture, and d) cricket consumption. Additionally, for days one and five of the live hunting experiments, approach periods were manually scored. Approaches were taken as periods during which the animal actively pursued the cricket, which may or may not include interceptions or end in capture. Based on manual scoring, time to capture per trial was calculated as the time between cricket release and capture. The number of approaches per trial was evaluated as the number of manually scored approaches between cricket release and capture.

DeepLabCut (DLC, Mathis et al. 2018; Nath et al. 2019) was used to assess cricket position as well as the mouse position and orientation during trials. Still frames from hunting videos were chosen and manually labeled with cricket position and the position of the mouse body, head, nose, left ear and right ear to produce training and test data. A ResNet-50 neural network was trained on these manually labeled datasets. Following training, the performance of the network was evaluated using test data. Outlier frames were chosen and manually labeled for subsequent training iterations. Nine training iterations were performed, and the resulting network exhibited a test error of 2.0 pixels (equivalent to 0.18 cm). This network was then used to analyze the positions of mouse and cricket markers during live cricket hunting and dead cricket consumption experiments.

Outputs from DLC were analyzed using a Matlab script. Periods of under 50 frames (2.5 seconds) in which the DLC network confidence was below 98% were interpolated. Additionally, cricket positioning was improved by designating 'jump' frames as frames in which the decoded cricket position moved more than 10 cm between one frame and the next. 'Jump sequences' were considered to be time intervals consisting of multiple 'jump' frames with 10 frames or fewer between any individual jump frame and following jump frame. Cricket position was interpolated between the first and last position in each 'jump sequence.' Speed was calculated based on the change in mouse body position between one frame and the next, and smoothed by applying a sliding window average over two frames. Azimuth was calculated using cricket position, and the mouse head and nose position. Mouse-cricket-distance (MCD) was calculated as the distance between the mouse nose and cricket position. Interceptions were taken as frames during which the MCD fell below a threshold of 5 cm. Time immobile was calculated as time during which the mouse speed was below an immobile threshold of 1 cm/s. Distant approaches were calculated as approaches for which the MCD on the first frame of the approach was greater than a threshold value of 15 cm.

Statistics

A Wilcoxen rank test was used for analysis in **Fig.1 D-F**, as these data corresponded to a small, discrete numeric variable (number of crickets captured or consumed) which was not normally distributed. For all other data presented as bar charts, including **Fig.1 G-J, Fig. 2, Fig. 3**, and **Fig. 4 A, B, F**, a two-tailed t-test was used for statistical analysis. A value of $p=0.05$ was used as a threshold for statistical significance. In producing the graphs shown in **Fig. 4 G-H**,

sequences of mouse speed data were identified in the second preceding and following cricket interception. A speed distribution was obtained for each frame in this sequence over the four conditions analyzed (namely naive wild type and knockout animals, and experienced wild type and knockout animals). For both naive and experienced mice, genotype speed distributions at each corresponding frame in the sequence were compared using a two-tailed t-test.

Results

Shank3 mutant mice require more trials to learn to capture crickets and take longer to capture crickets after learning.

Cricket hunting behavior was assessed in Shank3 mutant mice and their wild type littermates. Mouse hunting behavior was recorded with an overhead camera in a clear plexiglass arena of 14x14 inches (**Fig. 1A**). Each trial began when a cricket was dispensed in one of four corners of the arena, and the behavior of both mouse and cricket was recorded on video. Sessions ended after the mouse had performed 5-6 trials (consuming 5-6 crickets), or after three hours, whichever came first. Before hunting, animals were habituated for two days and were food-deprived for one night before testing (**Fig. 1B**). Animals were tested over five sessions, with one session occurring on each of five successive days.

Differences in the two genotypes were evident on the first day of hunting. The vast majority of wild type mice readily engaged in hunting and ate live crickets on the first day, while most Shank3 KO animals did not eat any live crickets on the first day (**Fig. 1 D, E**). To determine whether part of this initial reluctance to hunt represents reduced motivation to

consume a novel food source, in a separate set of experiments we assessed the likelihood of consuming dead crickets or treats (Froot Loops). Shank3 KO mice were more variable than wildtype in the number of dead crickets consumed, and also consumed a smaller amount of novel treat, although these differences were not statistically significant (**Fig 1F,G**). This suggests that Shank3 mice are more variable in their willingness to consume a novel food, which could contribute to their initial reluctance to hunt.

Despite this initial slowness to hunt, over the course of 5 hunting sessions Shank3 KO animals eventually learned to capture and eat live crickets (**Fig. 1D, E**). Wild type mice exhibited a rapid reduction in the distance they travelled before capturing a live cricket (**Fig. 1H**) as well as the time they took to do so over the 5 days (**Fig. 1I**), while Shank3 KO animals improved more slowly. The initial differences in time to consumption between the genotypes were also present in experiments with dead crickets (**Fig. 1J**). By day 5, Shank 3KO animals were more efficient and captured and consumed all 5-6 live crickets provided (**Fig. 1E**), but took about twice as long to do so as wild type animals (**Fig 1I**). Thus, Shank3 KO animals are able to learn a complex behavior requiring extensive sensorimotor integration, but never become as efficient as their wildtype littermates.

Naïve WT animals make more approaches during hunting than Shank3 mutants

In order to understand what accounted for the differences in time to capture between genotypes, we carefully examined the instances where mice made an active "approach" to a cricket. These approaches were manually scored for days 1 and 5 for both genotypes. "Interceptions" were defined as times when the distance between the mouse's nose and the cricket's body fell below a threshold of 5 cm. After an interception, it was possible that the

cricket would be captured and eaten, or that the cricket would escape. If the mouse immediately continued to pursue a cricket after it escaped, then this was considered part of the same approach. Therefore, an approach might contain many interceptions. Approaches might end in a capture, or might end with a successful escape if the mouse did not continue an active pursuit after an unsuccessful interception.

On day 1 there were substantial differences in the number of active approaches by the two genotypes, with wild type mice making more approaches than KO mice (**Fig. 2A**). Additionally, on Day 1 both genotypes made many cricket interceptions (**Fig. 2B**). By day 5, the overall number of approaches and interceptions required to capture a cricket decreased (**Fig. 2A-B**) concomitant with an improvement in hunting performance.

The greater number of approaches of wild type mice on day 1 might reflect a higher intrinsic interest in crickets of wild type mice compared to Shank3 KO mice, or lower initial fear of crickets in wild type mice as compared to Shank3 KO mice. Alternatively, the fewer approaches of Shank3 KO mice might reflect differences in sensory processing or motor planning in Shank3 KO mice. To examine these possibilities, we observed naive wild type and Shank3 KO mice as they interacted with dead crickets (**Fig. 2C**). The genotypes exhibited an equal propensity to investigate dead crickets, indicating that the differences in their interactions with live crickets is unlikely to result from differences in intrinsic interest or fear of novel objects.

In order to understand whether a sensory deficit or a motor planning deficit might underlie the differences between the genotypes, we examined whether experienced mice would learn to start their approaches to crickets from a farther distance. We assessed sensitivity to cricket presence by calculating the fraction of approaches starting with a Mouse-cricket-distance

(MCD) greater than 15 cm (**Fig. 2D**). Both genotypes showed a numerical increase in this metric with training, suggesting that they became more able to recognize crickets as prey from a distance with experience. However, only wild type animals showed a statistically significant increase in the fraction of distant approaches. Shank3 KO mice began their approaches when they were closer to the cricket than wild type mice, suggesting some deficit in sensory processing or motor planning speed.

Shank3 KO mice exhibit increased periods of immobility compared to wild type mice

In an effort to account for the differences in time to capture between wild type and Shank3 KO mice, we analyzed other features of the approaches. Even though the genotypes made similar numbers of approaches on day 5, the shorter time to capture and shorter path lengths traveled by wild type mice suggested that they were using time more efficiently than Shank3 KO mice.

We examined the time spent immobile during approaches (**Fig. 3A**) and found a striking difference: Shank3 KO mutants spent considerably more time immobile (movement <1cm/s) during approaches than their wild type littermates (WTD1 mean 0.44% time immobile, WTD5 0.037%, KOD1 0.77%, KOD5 0.26%). That is, while wild type mice smoothly pursued the crickets, the Shank3 KO mice exhibited a move-and-pause strategy during approach periods. Both genotypes showed an increase with training in the amount of distance travelled between one period of immobility and the next (**Fig. 3B**). This indicates that both genotypes learned more active approach strategies over the course of the experiment.

Shank3 KO mice also spent considerably more time immobile in between bouts of cricket hunting, when they were free to explore the arena. In these periods when crickets were not

present, Shank3 KO mice were immobile 39% of the time, compared to 27% of the time for wild type mice (**Fig. 3C**).

Experienced Shank3 KO exhibit less modulation of speed during hunting than wild type mice

We next sought to understand whether the speed of the mice when moving differed between wild type and Shank3 KO mice. Speed was calculated from mouse body position assessed using DLC and averaged over two frames (Methods). "Moving speed" was taken to be the average speed calculated over manually scored approach periods.

When no cricket was present, Shank3 KO mice moved in the arena at slightly lower speeds than wild type mice (**Fig. 4A**). However, during live cricket approach, the average speeds of naive mice from both genotypes were identical, and both genotypes exhibited statistically identical increases in average approach speed (**Fig. 4B**).

We were curious to understand how the genotypes could exhibit identical average approach speeds on day 5, when wild type mice exhibited faster capture times than Shank3 KO mice. We found that wild type mice, in addition to spending less time immobile, were also more efficient when they did move. To quantify this, we calculated the average rate of change of the Mouse-to-Cricket Distance (dMCD/dt) during each approach, which is a measure of how quickly the mouse is closing on the cricket. Example MCD profiles during approach on day 5 are shown for wild type (**Fig. 4C**) and KO (**Fig. 4D**) mice, along with the corresponding profiles of dMCD/dt (**Fig. 4E**). Arrows in **Fig. 4C, D** correspond to interceptions. On day 1, mice of both genotypes exhibited highly inefficient movements, with a positive dMCD/dt indicating that the mice are less effective at approaching the cricket than the cricket is at escaping (WTD1 mean -0.164 cm/s, KOD1 mean 1.00 cm/s **Fig. 4F**). However, by day 5, both genotypes exhibited an

overall negative dMCD/dt (WTD5 mean -3.26 cm/s, KOD5 mean -0.905 cm/s), indicating that they had learned to close the distance to the cricket more effectively. Also on both days 1 and 5, wild type mice exhibited a more negative dMCD/dt than Shank3 KO mice. These results indicate that wild type mice were more efficient at closing the distance gap to the cricket compared to Shank3 KO animals.

In order to better understand how wild type mice closed the distance gaps more efficiently, we examined the speed of each mouse one second before and after an interception. All frames over this time range were averaged, for both naive (**Fig. 4G**) and experienced (**Fig. 4H**) mice of both genotypes. We found that wild type mice approached crickets more rapidly immediately before interception and that this difference was statistically significant for all frames during naive trials and for many frames during experienced trials. We additionally found that while experienced mice of both genotypes rapidly increased their speed as they were intercepting the cricket, wild type mice show an additional locomotive burst about 500ms before interception. This suggests that wild type mice learned to modulate their speed more efficiently as they began to intercept a cricket.

Discussion

We observed marked differences between wild type and Shank3 KO mice as they learned to hunt crickets. Shank3 KO mice initially captured fewer crickets and required more time and longer paths to do so (**Fig 1C-E, H-I**). After five days of hunting, smaller but significant differences in time to capture and path length persisted. We observed that experienced Shank3 KO mice exhibited substantially more periods of immobility (pauses) as compared to their wild type littermates, and also that wild type mice modulated their approach speeds in a manner

that Shank3 KO mice did not. Thus, while Shank3 mice are capable of learning this complex task they differ in strategy and efficiency from their wildtype littermates.

Shank3 KO mice showed similar interest in crickets to wild types

In principle, the initial reluctance of Shank3 KO animals to hunt crickets could reflect lower interest in crickets than wild type animals or greater short-term or long-term fear of crickets. However, we did not find evidence for either of these potential explanations. During our experiments with dead crickets, both genotypes showed a statistically identical number of interactions with novel dead crickets (**Fig. 2C**), which suggests that differences in performance cannot be attributed to a simple difference in interest or some type of neophobia that inhibits their examination of novel objects. This result is consistent with literature findings that wild type and Shank3 KO mice spent similar amounts of time exploring novel objects outside their nests (Wang et al. 2011).

Nevertheless, despite their interest in interacting with novel dead crickets, Shank3 KO animals consumed fewer of these crickets as compared to wild type mice (**Fig 1F**). Further, Shank3 KO mice consumed less novel fruity cereal than wild type mice (**Fig 1G**). These results suggests that Shank3 KO animals either a) exhibit a deficit in identifying a novel object as a potential food source, b) have a fear or other reluctance to actually ingest a novel food, and/or c) would like to eat the novel food but have difficulty carrying out the planning or the movements necessary to achieve the consumption. Any of these factors or a combination could have contributed to the lower performance of Shank3 KO mice on the first day of cricket hunting (**Fig 1C-E, H-I**). Regardless of naive KO behavior, statistical differences in the number of live crickets eaten (**Fig. 1D**) and probability of capture (**Fig. 1E**) disappear after one to two days of

hunting. This suggests that any contribution from these effects may affect the initial stages of hunting but is eventually overcome by Shank3 KO animals.

Wild type mice exhibit more dynamic hunting tactics

Shank3 KO and wild type mice exhibited substantial differences in pursuit movements during hunting, but these differences defy an easy characterization as a simple motor deficit.

The two genotypes exhibited a pronounced difference in the time spent immobile – that is, paused. Both genotypes showed a decrease in the amount of time spent immobile during approaches as a result of experience over the five days, but time immobile nearly disappeared for wildtype animals, while knockout mice continued to exhibit substantial pauses. These pauses could reflect sensory and/or motor planning deficits, such that knockout animals need more time to process the sensory information about the cricket or to plan their next moves.

Wild type and knockout animals also exhibited substantial differences in their patterns of movement when they did move. Interestingly, the average speeds (**Fig 4B**) of both genotypes during approaches were not significantly different. However, over the course of an approach, wild type mice moved more efficiently, reducing their distance to the cricket more quickly than Shank3 KO mice (**Fig 4F**). The genotypes also exhibited major differences in how experienced mice modulated their speeds in the final second leading to an interception (**Fig 4H**). While Shank3 KO animals exhibited only one burst of acceleration as they reached the cricket, wild type mice moved faster 1 second before an interception and showed an additional increase in speed about 500 ms before interception. That is, experienced wild type mice employed an extra locomotive burst precisely when it was most needed: as they neared their peak speed, only centimeters away from their prey. This pattern was not present in wild type mice on the first day

of hunting (**Fig. 4G**), so it represents a distinctive hunting tactic that wild type mice learned but knockout mice did not.

Shank3 KO mice exhibited lower levels of baseline ambulation when no cricket was present (**Fig. 3C**). This is consistent with previous reports, which show that Shank3 KO mice travel more slowly than their wild type counterparts during open field tests (Mei et al. 2016, Wang et al. 2011, Guo et al. 2019).

Therefore, while it appeared that Shank3 KO mice were capable of moving as quickly as wild type mice, they paused more, exhibited less efficient approaches, and did not modulate their speed in the same manner as wild type mice.

Possible circuit mechanisms

Shank3 KO mice exhibited two major differences from wild type mice in cricket hunting. First, while Shank3 KO mice were interested in crickets, they appeared slow to recognize them as prey to be hunted and eaten (**Fig 1D-E, 2A-C**). Second, they paused more and were less efficient at modulating their speeds during approaches (**Fig 3,4**).

One explanation consistent with these data is that visual function is impaired by Shank3 loss. As homeostatic compensation in the primary visual cortex was shown in Chapter 2 to be disrupted in Shank3 KO animals (Tatavarty et al. 2020), it is feasible that visual processing deficits lead to lack of recognition of the cricket as prey as well as increased hesitation during hunting. This is consistent with the fact that vision (Hoy et al. 2016) and specifically binocular vision (Johnson et al. 2021) are known to be required for effective cricket hunting, and are impaired in other monogenic mouse models of autism exhibiting altered cortical homeostatic plasticity (Noutel et al. 2011; Durand et al. 2012; Felgerolle et al. 2019).

The superior colliculus has long been known to be involved in predatory hunting behavior as well as other goal-directed visual activities (Furgio et al. 2010; Wang et al. 2020). Global inactivation of the superior colliculus significantly degrades overall hunting performance (Zhen et al. 2017) while selective inactivation of defined cell types in the superior colliculus inhibits distinct aspects of prey approach and capture (Hoy et al. 2019), establishing that detailed circuit interactions mediated by this brain region have direct and well-defined effects on hunting behavior. Interestingly, the superior colliculus is involved in triggering the onset of predatory hunting (Shang et al. 2019, Huang et al. 2020), and Huang et al. found that inactivation of the pathway from the superior colliculus to the substantia nigra compacta disables hunting but does not disable exploratory behavior. It is possible that differences in superior colliculus or substantia nigra signaling or their connections might underlie the behavior differences reported here. While data is lacking in the literature as to the effect of Shank3 on SC signaling, Shank3 KO rats exhibit a lower relative volume of SC, suggesting that Shank3 loss may alter development of this brain region (Golden et al. 2020).

Shank3 KO hunting behavior shows parallels with symptoms of autism-associated disorders

The genotype-dependent differences in both performing and learning cricket hunting observed in our experiments are analogous to some symptoms of autism-associated disorders. Firstly, Shank3 KO mice exhibited a greater amount of time immobile during hunting (**Fig. 3A**). Experienced Shank3 KO mice also pursued crickets less rapidly and efficiently (**Fig. 4F**). This is reminiscent of the behavior of individuals with ASD, who take more time to initiate goal-directed actions and move more slowly during the initiation of those actions (Alvarez et al. 2016; Longuet et al. 2011; Glazebrook et al. 2006). Further, individuals with ASD exhibit irregularities

in audiovisual integration (Stevenson et al. 2014; Feldman et al. 2018; Brandwein et al. 2015), and the availability of multisensory information (auditory and visual) improves cricket hunting in wild type mice (Hoy et al. 2016). This suggests that Shank3 contributes to effective learning of this complex ethological task in part by allowing mice to learn to rapidly integrate information about the position of their prey and respond to cricket movements expeditiously.

Another parallel between the behaviors observed in our study and symptoms of autism-associated disorders is that wild type animals exhibit a greater degree of improvement on multiple efficiency metrics than Shank3 KO animals. The lower learning proficiency observed according to all of these metrics is consistent with learning disability classically associated with Phelan-McDermid syndrome and other autism-associated disorders (Kolevzon et al. 2014), and furthermore generalizes deficits in instrumental learning (Bey et al. 2018; Wang et al. 2016) as well as spatial learning and memory (Jaramillo et al. 2015; Jaramillo et al. 2017) found in Shank3 KO mice to complex ethological behavior.

Conclusion

Our data indicate that there are substantial differences in cricket hunting efficiency between Shank3 KO mice and wild type mice. Rather than being the effect of simple motor deficits or differences in interest in novel objects, our data suggest that Shank3 KO mice have difficulty rapidly integrating and responding to sensory information, and either do not learn or cannot execute efficient sensorimotor patterns that could improve their performance. Future work is needed to map these observations to synapse and circuit-level disruptions due to Shank3 loss.

Figures

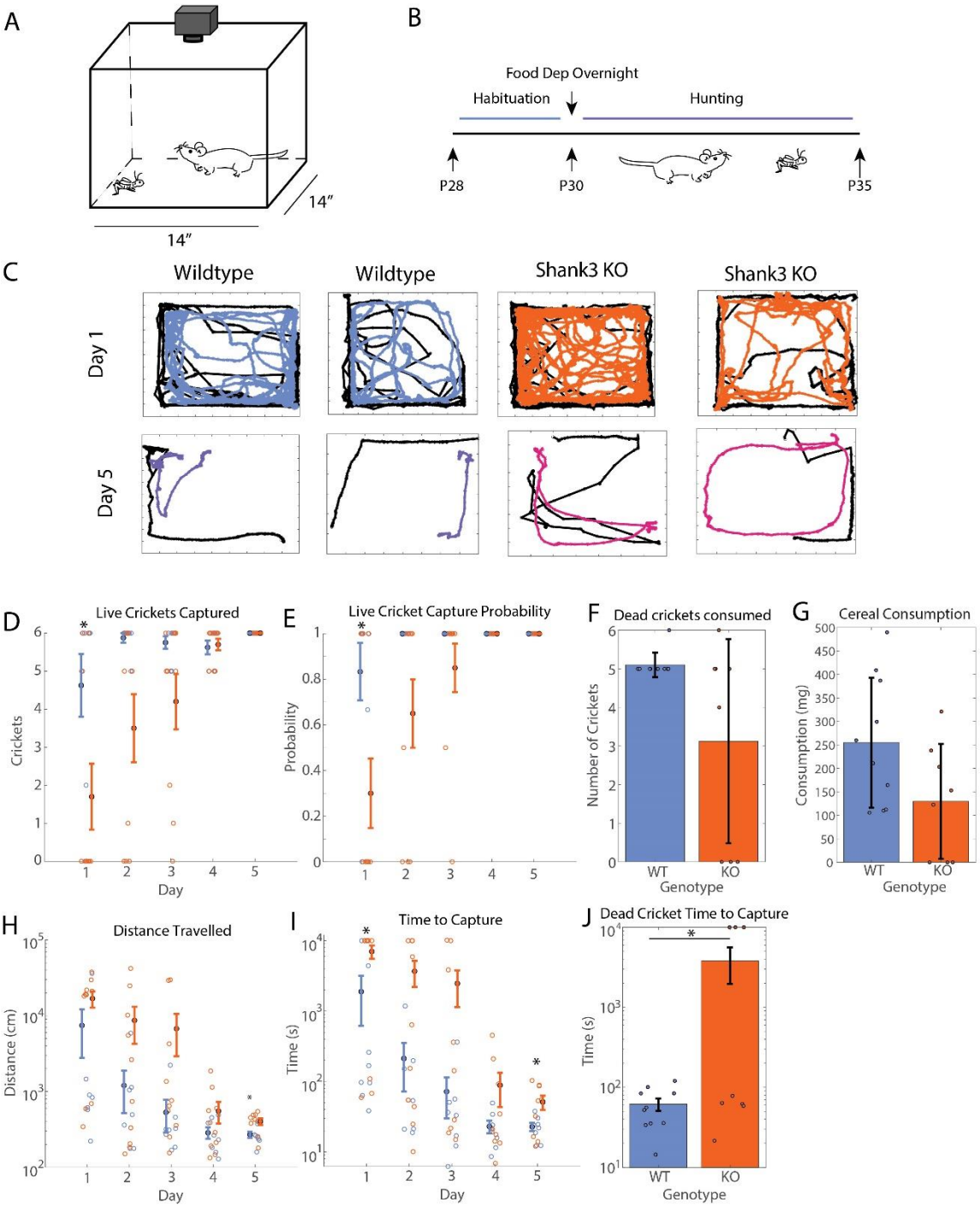
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Figure 1: Shank3-KO animals initially consume fewer crickets than wildtype littermates and improve at hunting more slowly (A) Schematic of recording setup for hunting sessions. Camera records behavior of mouse and cricket in a given session. Videos are later analyzed using Deep Lab Cut (DLC) in order to track individual features on the animals. (B) Experiment timeline whereby animals are habituated for two days in advance of hunting in littermate pairs. Food deprivation happens overnight the second night before beginning hunting. (C) Representative traces of mouse and cricket position during hunting trials by naive and experienced animals, in which the black trace corresponds to cricket position and the colored line corresponds to the mouse position. In graphs D-E and H-I, open dots are individual observations, filled dots represent averages across multiple sessions and error bars represent standard error. (D) Number of live crickets captured per session. Stats for WT vs KO, Number of crickets captured per day, Day 1 p=0. 042, Day 2 p=0. 052, Day 3 p=0. 142 (Wilcoxen). (E) Fraction of cricket trials concluding in successful capture per session. Stats for WT vs KO, Capture probability, Day 1 p=0. 046, Day 2 p=0. 137, Day 3 p=0. 588 (Wilcoxen). (F) Number of dead crickets consumed by session, p=0.079 (Wilcoxen). Without training, shank3 KO mice consume fewer dead crickets than wild type mice. (G) Mass of sugary cereal consumed by session, p=0.062 (t-test). (H) Median mouse distance travelled per hunting session, across multiple live cricket trials, Day 1 p=0.145, Day 2 p=0.156, Day 3 p=0.168, Day 4 p=0.205, Day 5 p=0.016 (t-test). After training, wild type mice traveled less distance to capture crickets compared with Shank3 KO mice. (I) Median time to capture per hunting session, across multiple live cricket trials. Maximum time was plotted as 10,000 seconds in the case that animals did not capture any crickets during their allotted 3 hours. Stats for WT vs KO, Time to capture, by session, Day 1 p=0.023, Day 2 p=0.055, Day 3 p=0.127, Day 4 p=0.216, Day 5 p=0.042. After training, wild type mice were

slightly faster than Shank3 KO mice. (J) Time to capture dead cricket, median by session p=0.035 (ttest). Shank3 KO mice took substantially longer time to eat dead crickets than wild type mice.

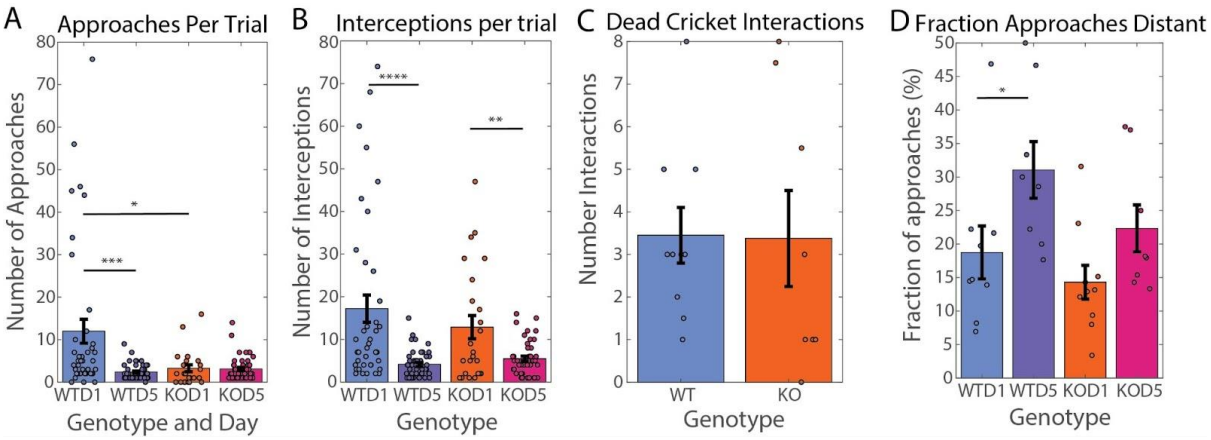

Figure 2: Both wild type and Shank3 KO animals gain some proficiency at cricket hunting and reduce overall number of interceptions per trial. (A) Number of approaches before cricket capture, per trial. All stats calculated using two-sample t-test WTD1-KOD1 p=0.022, WTD5-KOD5 p=0.127, WTD1-WTD5 p=3.57e-4, KOD1-KOD5 p=0.839. (B) Number of interceptions before cricket capture, per trial. WTD1-KOD1 p=0.352, WTD5-KOD5 p=0.080, WTD1-WTD5 p=2.89e-05, KOD1-KOD5 p=0.001. (C) Number of interceptions per dead cricket, median by session p=0.953 (ttest). (D) Fraction of approaches for which the mouse to cricket distance exceeded 15 cm at start of approach, median per day. WTD1-KOD1 p=0.346, WTD5-KOD5 p=0.133, WTD1-WTD5 p=0.050, KOD1-KOD5 p=0.074.

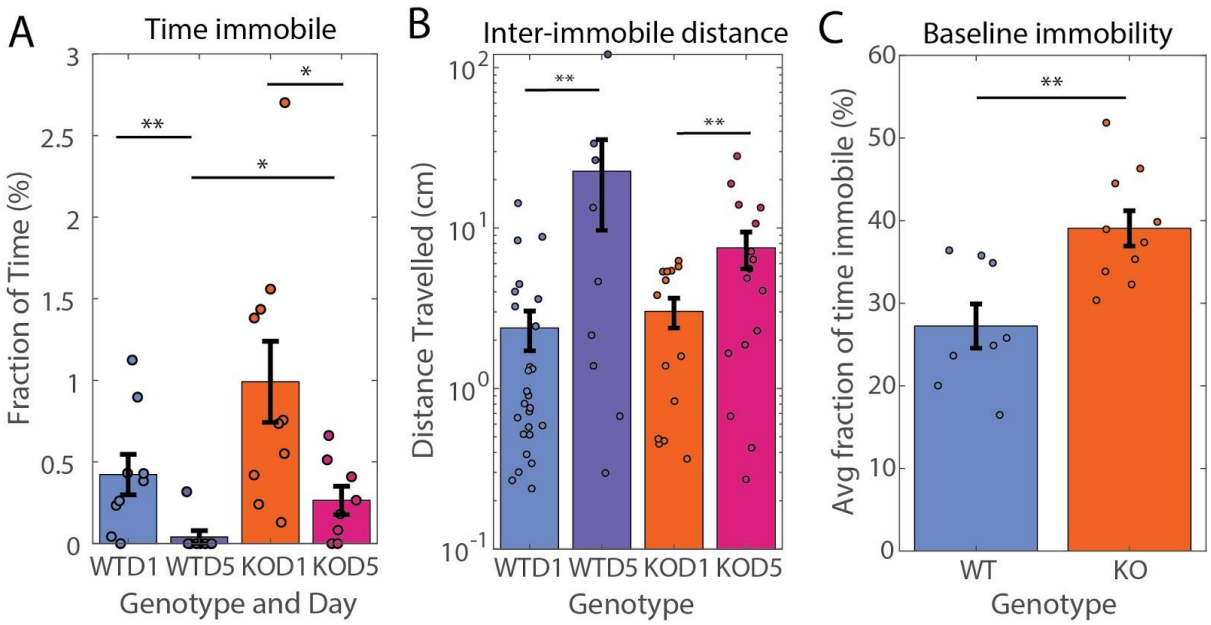

Figure 3: Shank3 KO mice exhibit more frequent periods of immobility than wild type mice. (A) Fraction of time immobile during approach per session, averaged over all approaches. Bars show average value and error bars indicate standard error. Both genotypes spent less time immobile with training, but Shank3 KO animals continued to exhibit substantial time immobile after training whereas wild type animals exhibited very little time immobile during approaches. Calculated using two-sample t-test, WTD1-KOD1 p=0.064, WTD5-KOD5 p=0.034, WTD1-WTD5 p=0.014, KOD1-KOD5 p=0.023. (B) Mean distance travelled between periods of immobility. Wild type animals traveled farther than Shank3 KO animals before pausing. Bars show average value and error bars indicate standard error. Calculated using two-sample t-test, WTD1-KOD1 p=0.531, WTD5-KOD5 p=0.140, WTD1-WTD5 p=0.011, KOD1-KOD5 p=0.047. (C) Average fraction of time immobile in the absence of appetitive stimulus, by session. Time immobile Calculated using two-sample t-test, p=0.003. Shank3 KO mice spent more time immobile when no cricket was present.

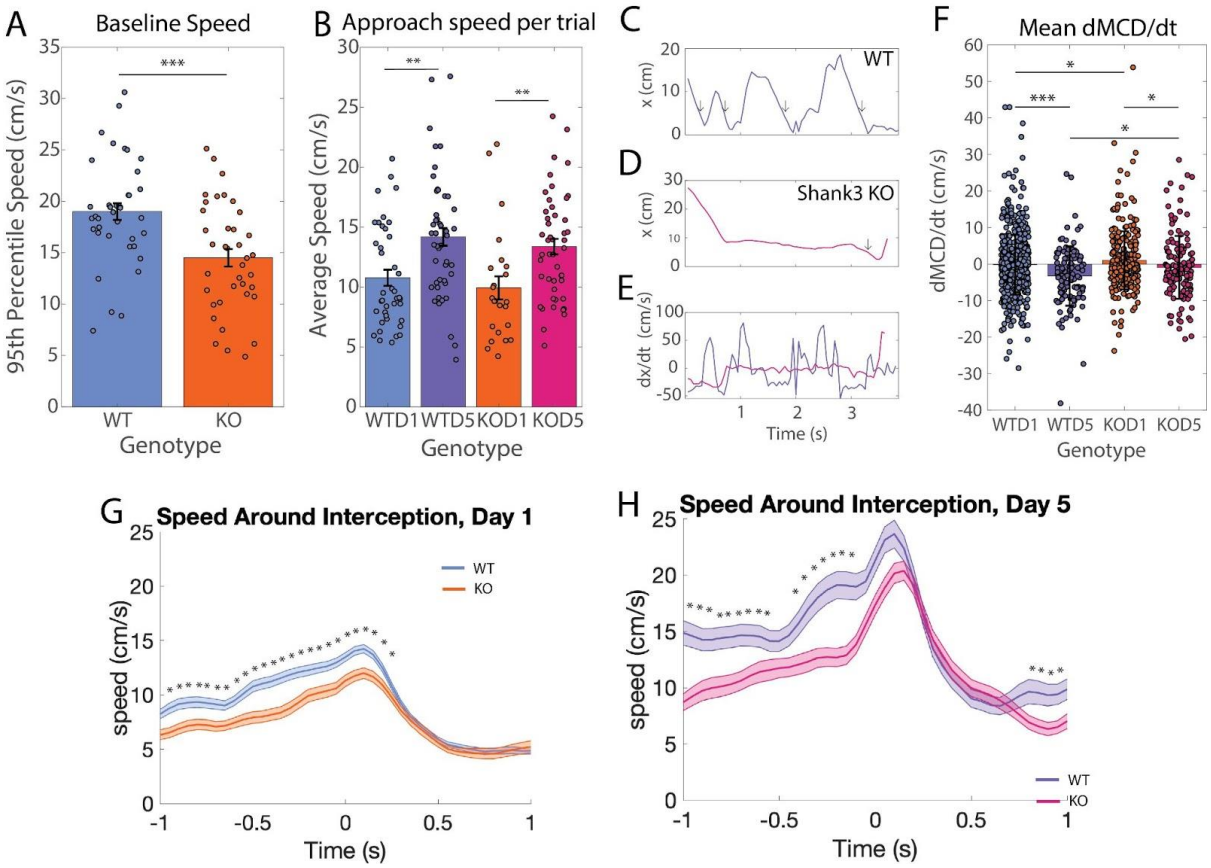

Figure 4: Shank3 KO animals exhibited similar average speeds as wild type animals during approaches, but were slower to close in on crickets and exhibited less modulation of their speed immediately before interceptions. (A) 95th percentile speed in the absence of appetitive stimulus, by session. 95th percentile speed p=2.78e-4. Baseline speed was slower in Shank 3KO animals. (B) Average speed during approach, median over approaches during a trial. Wild type and Shank3 KO mice did not differ in average speed during approaches. WTD1-KOD1 p=0.459, WTD5-KOD5 p=0.410, WTD1-WTD5 p=1.12e-3, KOD1-KOD5 p=3.29e-3. (C,D) Representative traces of the distance between mouse nose and cricket (mouse to cricket distance, x) during the course of one approach, for both wildtype (C) and Shank3-KO (D) mice after training. Vertical arrows indicate interceptions. (E) Derivative of mouse to cricket distance with respect to time, moving average over one second, for traces shown in (C) and (D). (F) Mean

derivative of MCD with respect to time averaged over each approach period. Error bars indicate standard deviation. Calculated using two-sample t-test, WTD1-KOD1 p=0.048, WTD5-KOD5 p=0.026, WTD1-WTD5 p=2.29e-4, KOD1-KOD5 p=0.023. Experienced wild type mice exhibited lower values than experienced Shank3 KO mice, indicating that wild type animals closed the distance gap to the cricket more efficiently. (G-H) Mouse body speed one second before and after interception, averaged over all interceptions for a given genotype for both naive (G) and experienced (H) mice. Shaded region indicates standard error. Asterisks indicate frames for which $p<0.05$ as calculated using a two-sample t-test. Wild type mice began to ramp up their speed about 500 ms before intercepting a cricket, while Shank3 KO mice did not greatly increase their speed until about 200 ms before intercepting a cricket.

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Chapter 4: Discussion

Summary

The work presented in the previous chapters outlines a profile of Shank3 deficiency in mice which encompasses deficits in synaptic scaling, intrinsic homeostatic plasticity, ocular dominance plasticity, and proficiency at both performing and learning a goal-directed behavior. Before the work described here, it was known that Shank3 is required for Hebbian plasticity mechanisms in the hippocampus (Bozdagi et al. 2010; Song et al. 2019), and interactions between Shank3 and other proteins in the postsynaptic density (PSD) of glutamatergic synapses pointed to a role for this protein in the maturation of central circuits. Specifically, Shank3 was known to be localized in the PSD (Montiero et al. 2017), and to be involved in the recruitment of receptor subunits necessary for synaptic transmission (Bozdagi et al. 2010; Wang et al. 2016). Furthermore, Shank3 mutations are prevalent in individuals with neurodevelopmental disorders such as Autism Spectrum Disorder (ASD), Phelan McDermid Syndrome (PMS) and Intellectual Disability (ID) which are associated with perturbed circuit maturation (Leblond et al. 2014; Costales et al. 2015), and perturbation of homeostatic plasticity appears to play a role in other monogenic models of autism (Verma et al. 2019; Blackman et al. 2012; Qiu et al. 2012; Krishnan et al. 2015; Bülow et al. 2019). However, it was not known before this work whether Shank3 is required for homeostatic plasticity or the high-level visual and goal-directed functions studied here.

This work provides compelling new evidence that mutations to Shank3 play a role across a range of deficits associated with Autism Associated Disorders (AADs). At the circuit level, these deficits include impaired homeostatic plasticity and ocular dominance plasticity,

representing an inability of visual circuits to properly recover following perturbation. This is likely in turn to contribute to local imbalances of excitation and inhibition. Observed impairments also include deficits in hunting behavior which may be the result of perturbed sensation, perturbed sensory integration, delay in triggering subtasks required for hunting, or the acquisition and regulation of goal-directed behaviors. In establishing this profile, the work suggests a set of paths forward for better understanding the etiology of ASD and AADs.

In Chapter 2, we probe the effects of Shank3 loss on homeostatic plasticity in the visual cortex and the maturation of visual circuits during the critical period. In a global Shank3 KO model and also a short hairpin RNAi Shank3 KD model, we show that Shank3 is required for both synaptic scaling up, and for intrinsic homeostatic plasticity in V1 pyramidal neurons. However, manipulation of Shank3 levels had no measured effect on the basal miniature excitatory postsynaptic current amplitude or on basal intrinsic excitability, showing that Shank3 loss affects homeostatic changes in synaptic and firing properties rather than perturbing basal properties. We then showed that Shank3 loss blocks homeostatic recovery of both firing rate and response to visual drive during monocular deprivation. As a result, the canonical shift in ocular dominance resulting from monocular deprivation was altered in these mice. This established a dependency between the functional properties of circuits in the visual system and Shank3 dependent homeostatic plasticity.

Furthermore, we investigated the mechanism by which Shank3 affects homeostatic plasticity through a series of rescue experiments using lithium chloride and the GSK3 inhibitor BRD0320. The fact that these treatments were able to rescue both synaptic scaling and the overgrooming observed in Shank3 KO mice suggests that Shank3 loss causes both of these effects through the GSK3 pathway. This finding both indicates routes for development of new

therapeutic interventions, and sheds light on the mechanism through which Shank3 regulates synaptic plasticity. This work therefore helps to elucidate Shank3 function at the levels of pathways required for typical development, effects of these pathways on the synaptic and intrinsic properties of V1 pyramidal neurons, and the results of these effects on the development of visual circuits.

In Chapter 3, we extended this line of inquiry to investigate the effects of Shank3 loss on the learning and performance of cricket hunting. We showed that mice without Shank3 hunt less effectively and less efficiently: they initially consume fewer of the crickets available to them, require more time to capture crickets even when experienced, and travel longer paths before successful capture. The detailed behavior revealed by these experiments further underscores differences between the two strains. Wild type mice modulate their speed more productively during cricket approach, spend less time immobile, and initially make more approach attempts per cricket hunting trial. Even though both wild type and Shank3 KO mice are able to learn to improve their hunting skills through repeated trials, wild type mice show a greater degree of improvement across a range of metrics, and differences between the strains in metrics such as the amount of time taken to capture crickets and the mean change in mouse-to-cricket distance integrated over time. Furthermore, a detailed analysis of mouse movement immediately before a cricket interception reveals that wild type animals learn a distinct hunting pattern allowing them an extra locomotive burst as they approach the cricket. Together, these data provide compelling evidence that Shank3 enables learning of skills required for efficient and effective cricket hunting.

Implications for AADs

By presenting novel data characterizing Shank3 KO mice, the experiments described in this thesis are relevant to several aspects of the etiology of AADs. Firstly, they provide evidence for a mechanism by which Shank3 mutations may cause local imbalances of excitation to inhibition in central circuits: specifically, deficient homeostatic plasticity. As homeostatic mechanisms such as synaptic scaling and intrinsic homeostatic plasticity act to return neuronal activity to a set firing rate following perturbation, deficiencies in these mechanisms leave circuits vulnerable. The experiments presented in Chapter 2 specifically show the inability of visual cortical neurons to homeostatically increase their firing rate following TTX treatment. This implies that during development, such cells may thus have lower firing rates than would be expected in normally developing mice in the face of persistent perturbation. The lack of ocular dominance plasticity showed the analogous effect on a circuit level, in which response to visual drive failed to compensate for monocular deprivation in Shank3 KO mice.

These results additionally underscore the fact that AADs likely affect the balance of excitation and inhibition differentially, in subtle and contingent ways resulting from a complex interplay of Hebbian and homeostatic plasticity mechanisms. Given that it was already known that Shank3 loss impairs Hebbian plasticity in the hippocampus, the discovery that it also impairs homeostatic plasticity in V1 demonstrates that Shank3 loss likely affects circuit development through multiple distinct routes. As both Hebbian and homeostatic plasticity simultaneously respond to and affect firing, the net effect of a Shank3 mutation may be circuit-dependent. Further complicating the situation, the effect of Shank3 loss on plasticity is thought to be substantially dependent on cell type and brain region. Analogously, loss of the gene *Fmr1* associated with Fragile X syndrome (FXS) appears to impair intrinsic homeostatic plasticity in

cortical (Bulow et al. 2019) but not in hippocampal (Booker et al. 2020) cells. Shank3 loss in different brain regions does indeed incur different electrophysiological and proteomic effects which give rise to varied behavioral phenotypes (Bey et al. 2018), but data on plasticity specifically in these regions is lacking. If investigations of the effect of Shank3 loss on plasticity mechanisms in different brain regions reveal similar dependency, this will contribute to widespread variation in the net effects of Shank3 loss on excitation and inhibition.

A further source of variation is due to differences in Shank3 mutations and isoforms. In humans, haploinsufficiency of Shank3 due to a 22q13.3 deletion is sufficient to cause the debilitating condition PMS and a variety of mutations to Shank3 are linked to ASD, ID, and speech impairment in non-autistic individuals (Leblond et al. 2014; Costales et al. 2015; Manning et al. 2021). In mouse models of Shank3 perturbation, different mutations are known to result in varying phenotypes, which is believed to be primarily caused by changes to the relative and absolute levels of different Shank3 isoforms depending on the mutation studied (Wang et al. 2016; Mei et al. 2016; Peça et al. 2011; Vyas et al. 2020). It is possible that loss of different Shank3 isoforms affects plasticity mechanisms differentially, contributing to the complexity of studying the effects of Shank3 loss. Furthermore, homozygous Shank3B$^{-/-}$ animals were used in these experiments rather than heterozygous Shank3B$^{-/+}$ animals because the homozygous Shank3 KO is known to demonstrate a more robust behavioral phenotype. Whereas in humans, haploinsufficiency of Shank3 is sufficient to cause PMS, heterozygous Shank3$^{+/-}$ mice are known to perform similarly to wild type animals at a range of behavioral measures, including rotarod, ultrasonic vocalization, open field and instrumental conditioning tests (Wang et al. 2016). However, as we found in Chapter 2, acute knockdown of Shank3 in mouse visual cortical cells to 50% of its baseline value is sufficient to completely abolish synaptic scaling. This suggests that

unknown processes in mice partially compensate for Shank3 loss resulting in the observed Shank3B$^{-/+}$ behavioral phenotype.

It is notable that we did not find differences in basal neuronal properties of visual cortical neurons as a result of Shank3 loss, in contrast with other work that did find deficits in cortico-striatal transmission in the same mouse strain (Peça et al. 2011). It is possible that these differences are due to the fact that different circuits were examined in the two studies. An alternative explanation is the ages of the mice involved. Primary cortical neurons in our experiments were harvested on postnatal day 1-3 and tested after 7-11 days *in vitro* while samples taken from 6-7 week old adult mice were used by Peça et al. If deficits in transmission due to Shank3 loss are largely the result of perturbed plasticity, it is to be expected that said deficits would emerge during development when circuits are rapidly changing and plasticity mechanisms are most active. Thus, it is not surprising that young neuronal cultures would be unperturbed, while perturbations would evolve during the course of development and be present in adult circuits. This corresponds with the fact that symptoms in ASD and other neurodevelopmental diseases appear during childhood development.

Furthermore, the results presented in Chapter 2 provide a possible explanation for the hunting deficits examined in Chapter 3. As it has been demonstrated that hunting is a visually driven task (Hoy et al. 2016; Johnson et al. 2021), it would be expected that degraded visual function should impair hunting performance. Thus, deleterious impacts of Shank3 loss in visual cortical circuits may directly account for the hunting deficits observed. Normal visual function relies on numerous developmental processes which occur during the visual critical period. These include refinement of receptive fields necessary for visual acuity (Fagiolini et al. 1994; Prusky and Douglas 2003), as well as binocular matching of orientation preference, and direction

selectivity (Li et al. 2008; Rochefort et al. 2011; Wang et al. 2010; Gu et al. 2016; Jenks et al. 2020). It is known that such experience-dependent development relies on Hebbian plasticity mechanisms (Rucci et al. 2004; Smith et al. 2007; Huberman et al. 2008). Strong theoretical arguments as well as a growing body of experimental evidence suggest that Hebbian plasticity, in the visual cortex as well as in other brain regions, must be balanced by homeostatic plasticity in order for circuits to remain functional during and after development (Turrigiano et al. 2000; Kaneko and Stryker 2017; Turrigiano 2017). Therefore, demonstrating that Shank3 loss impairs homeostatic plasticity mechanisms in the visual cortex as shown in Chapter 2 implies that experience-dependent developmental processes necessary for normal visual function will be perturbed in the absence of some Shank3-independent compensatory homeostatic process. Such developmental impairment is consistent with visual functional deficits observed in other monogenic mouse models of autism known to exhibit perturbed homeostatic plasticity (Noutel et al. 2011; Durand et al. 2012; Felgerolle et al. 2019). Visual impairment induced by deficient visual cortical homeostatic plasticity could in turn lead to the hunting deficits observed in Chapter 3, as the mice are having difficulty visually tracking the cricket.

The experiments described in this thesis are also relevant to understanding the origins of sensory hypersensitivity and impaired multimodal sensory integration. These symptoms are both strongly associated with AADs and their etiology is incompletely understood. Our results contribute to this in three ways. Firstly, these experiments demonstrate a route by which Shank3 loss affects the development of visual circuits and their ability to stabilize following perturbations: specifically, impairment of homeostatic plasticity and the homeostatic portion of ocular dominance plasticity in the visual cortex. Similar developmental deficits may directly contribute to sensory processing differences in neurodivergent individuals. Secondly, showing

that Shank3 loss impairs homeostatic plasticity in the visual cortex provides a basis for studying its effects in other circuits which process sensory data such as the auditory cortex as well as perturbations to somatosensory neurons outside the central nervous system. If Shank3 loss impairs homeostatic plasticity in other brain regions, this provides a mechanism by which it may broadly give rise to sensory processing deficits. This may further our understanding of the etiology of these symptoms and also contribute novel biomarkers of AADs. Thirdly, we show that Shank3 KO mice are less able than wild type mice to rapidly and dynamically respond to cricket motion during hunting and that differences in hunting performance persist after training. Abnormal sensory processing may be a factor in these deficits. If so, then further study of this mouse model may allow a better understanding of the underlying contributing circuits and necessary cellular roles in abnormal sensory processing.

It has been argued that autism affects the balance between repetitive and flexible behaviors (Corbit et al. 2018), although this is contested as a general feature of AADs (Geurts et al. 2014). Regardless, it is clear that OCD-like symptoms are common in AADs and that individuals with ASD are situationally less flexible in planning and carrying out goal directed actions (Bodfish et al. 2000; Alvares et al. 2016). Consistent with this, overgrooming is a common symptom of many Shank3 KO mouse models (Peça et al. 2011; Mei et al. 2016; Balaan et al. 2019). Our detailed analysis of the hunting behavior of our Shank3 KO mice line suggests that these animals are less able to dynamically respond to cricket motion, reminiscent of ASD tests in which participants took longer to plan actions. We also show that Lithium acts to restore homeostatic plasticity and simultaneously to ameliorate overgrooming in Shank3 KO mice. By showing that Shank3 loss leads to overgrooming *via* a route that can be rescued by Lithium, this increases the specificity of our understanding of the pathways by which Shank3 loss leads to

behavioral deficits as compared to previous Shank3 rescue studies (Mei et al. 2016), specifically indicating that it may involve the GSK3 pathway targeted by Lithium.

The data presented additionally shed light on the role of Shank3 in enabling the acquisition of goal-directed actions. Deficits in acquiring and performing such actions are observed in ASD and ID (Glazebrook 2006; Longuet al. 2011; Cook et al. 2013; Backstrom et al. 2019; Foster et al. 2019; Zheng et al. 2019), and in some subjects Shank3 loss may be related to such deficits. Acquisition of goal directed actions is associated with plasticity in the basal ganglia, and in particular the posterior dorsomedial striatum (Shan et al. 2014). Additionally, performance of such actions is mediated by corticostriatal pathways (Corbit et al. 2018; Insel et al. 2017). Shank3 loss is known to affect corticostriatal signaling (Wang et al. 2016) and to impair synaptic strength and medium spiny neuron morphology in the striatum (Peça et al. 2011). The knowledge that Shank3 is required for normal Hebbian and homeostatic plasticity in other brain regions therefore suggests that impairment of plasticity in the striatum may inhibit acquisition of goal directed actions in Shank3 mutants. The hunting data presented demonstrates that goal-directed action is indeed impaired in these mice, giving reason to further probe the neural underpinnings of complex behavioral phenomena such as hunting in the presence of perturbations to Shank3 expression.

Chapter 4 References

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Chapter 5: Future Directions

Additional Hunting Experiments

The data presented in Chapter 2 show that Shank3 is required for normal synaptic scaling, intrinsic homeostatic plasticity, firing rate homeostasis, and ocular dominance plasticity. The hunting experiments presented in Chapter 3 established that Shank3 KO mice initially make fewer approach attempts, spend more time immobile both before and after training, and respond less rapidly and dynamically to the actions of their prey. Future work remains to be done to understand the relationships between the phenomena observed, connecting the effects of Shank3 loss to the pathways, circuits, and behaviors affected by absence of this protein. It is hoped that working towards a more complete mechanistic model of the behavioral phenotype of Shank3 KO mice will yield both an improved theoretical understanding of the neurological underpinnings of AADs, and novel therapies.

Firstly, in order to understand the link between sensory circuit maturation and the observed hunting behaviors, it would be useful to perform hunting experiments using juvenile wild type and Shank3 KO mice under sensory deprivation conditions. These conditions may include hunting in the dark and with plugged ears as described by Hoy and colleagues (Hoy et al. 2016) or under a monocular deprivation condition as also previously studied (Johnson et al. 2021). It is known from these sources that mice rely on binocular vision to hunt prey effectively, and that their hunting effectiveness is significantly diminished under deprivation conditions. By comparing the amount of time taken to capture crickets in each condition, it should be possible to assess the relative contribution of each sensory modality, as well as binocular integration of visual information, to hunting for each strain. If such experiments were to find that Shank3 KO

deficiencies in hunting performance persist in sensory deprivation conditions, this would establish that other processes, such as attention deficits or motor deficits, are involved in the strain dependent performance differences observed.

In order to further investigate the circuit-level underpinnings of the observed hunting behavior, and to relate this behavior to perturbed cortical homeostatic plasticity in Shank3 KO mice, a Shank3 conditional knock-in model could be used. Two studies examining such conditional knock-in animals demonstrate that restoration of Shank3 during both development and in adult mice is able to rescue the majority of observed behavioral deficits, including repetitive grooming and social interaction deficits (Mei et al. 2016; Jaramillo et al. 2020). Restoration in adult mice was also shown to rescue striatal signaling deficits, but did not rescue motor coordination deficits or anxiety as measured by rearing time and frequency (Mei et al. 2016). This technique thus enables experimental separation between developmental requirements for and acute effects of Shank3. This thus provides an avenue to explore whether perturbed homeostatic plasticity in the visual system as discussed in Chapter 2 gives rise to the hunting deficits outlined in Chapter 3. While presence of Shank3 during the visual critical period may be necessary for the receptive field (RF) refinement, it is expected that restoration of Shank3 after the visual critical period would not improve RF properties as they require additional plasticity mechanisms active during the critical period which are not regulated by Shank3. Thus, if the hunting deficits observed are largely the result of visual deficits due to perturbed cortical homeostatic plasticity, we would not expect a conditional knock-in experiment to rescue hunting deficits. However, if the observed hunting deficiencies are due to disrupted goal-dependent action, we would expect these behaviors to be rescued along with striatal signaling (Mei et al. 2016). Thus, a conditional knock-in experiment comparing the hunting behavior of wild type,

KO animals, and KO animals with restored Shank3 would aid in interpreting the origins of observed hunting deficits in light of observed deficits in cortical homeostatic plasticity.

Hunting experiments exploring rescue of hunting behavior in Shank3 mutant mice would provide additional clues as to the origins of differences in hunting performance and possible therapeutic strategies. A starting point would examine the ability of either Lithium Chloride or BRD0320 to rescue the hunting performance of mice using the same genetic model as used in the tests described in Chapter 3. As shown by our data in Chapter 2, both of these drugs are able to rescue synaptic scaling and Li treatment is also able to rescue overgrooming behavior. This is consistent with reports of Li proving an effective therapy in some humans with AADs resulting from Shank3 mutation (Serret et al. 2015; Egger et al. 2017). An alternative rescue strategy would employ a conditional knock-in model such as that described by Mei and colleagues (Mei et al. 2016). In this model, a Cre-dependent switch is used allowing Shank3 expression levels to be restored through administration of tamoxifen. This model would avoid confounding results through off-target effects of Li, though it may introduce confounds associated with tamoxifen toxicity. Additionally, the Shank3 knockout model used by Mei et al. exhibited motor deficits during the rotarod test which were not detected in the model used for the work presented in this thesis (Peça et al. 2011), suggesting that the hunting phenotype may also be different for this alternate strain and would thus need to be evaluated.

Another experiment which would allow assessment of the effect of Shank3 loss on complex behavior is examining the social transmission of hunting behavior. Social transmission of food preference has been widely studied in mice (Wrenn et al. 2004), rats (Posadas-Andrews et al. 1983), monkeys (Hikami et al. 1990) and birds (Suboski et al. 1984). Additionally, behavioral strategies for acquiring food including foraging techniques (Page et al. 2006) and

social transmission of tool use (Kopps et al. 2014) have been documented in animals. Social transmission of behavior such as food preference is known to rely on activity in the prefrontal cortex (Loureiro et al. 2019), and altered activity in this region (Sacai et al. 2020) including that induced by Shank3 loss (Frost et al. 2021) is known to disrupt social behavior. This set of experiments would first examine whether naive wild type mice are able to learn hunting behavior by observing an experienced mouse hunt. If this is shown to be possible, the experiment would be repeated with naive Shank3 KO mice. As Shank3 KO mice show reduced interest in social behavior (Luo et al. 2017; Baalan et al. 2018; Guo et al. 2019), it is expected that they may be less able to learn based on social demonstration due to Shank3 loss. If, by contrast, Shank3 KO mice are able to learn hunting from a littermate this would have implications for our understanding of biomarkers related to AADs. Namely, such a result would show that simple social interactions, as measured by a three-chamber test, cannot be extrapolated to more complex interactions such as the transmission of goal-directed behaviors.

It may additionally be interesting to examine paw and jaw motion using multiple cameras, tracking attack and grasping behavior in three-dimensional space using Deep Lab Cut (Bova et al. 2019). Using this technique, it may also be possible to track jaw motion as the mouse intercepts the cricket and assess whether Shank3 loss induces a hesitation in the triggering of jaw motion during attack (Han et al. 2017). These experiments would add to our analysis of approach speed immediately before interception in Chapter 3, which shows that experienced wild type mice attack crickets with a distinctive pattern of speed affording them an extra locomotive burst. While it is possible to assess such a strategy using an overhead camera, other details of attack may be better understood using the multiple camera setup described. If Shank3 KO mice are seen to hesitate in their grasping or jaw behavior, this would suggest that triggering

of attack is impaired by Shank3 loss. It is also possible that somatosensory differences or motor differences in these mice, which are known hallmarks of ASD (Orefice et al. 2016; Orefice et al. 2019), cause hesitation or altered attack strategy as a result of Shank3 loss which would be observable in an enhanced setup allowing three-dimensional tracking. These findings would further expand our understanding of the effects of Shank3 loss in detailed strategy employed in ethological hunting behavior.

Multimodal Sensory Integration

A major focus of future investigations determining the circuit-level effects of Shank3 loss on hunting should be circuits involving the Superior Colliculus (SC) as a possible route by which the observed deficits arise. Beyond a finding that the developed rat SC exhibits a lower relative volume as a result of Shank3 loss (Golden et al. 2020), little is known about the effect of Shank3 mutation or loss on the SC. However, the region is known to be involved in several functions necessary for hunting. Notably, the SC performs visual, auditory and somatosensory multisensory integration (Wang et al. 2017; Cuppini et al. 2018; Stein et al. 2020) and these functions depend on experience during early development (Xu et al. 2012; Wang et al. 2020-2; Xu et al. 2017). Multisensory integration in the SC is then used to drive behaviors necessary for hunting. An example of this is SC-mediated control of eye movement (Wang et al. 2017), perturbations of which are a possible route according to which hunting performance in mice may be diminished given the importance of detailed eye movement in targeting during hunting (Michaiel et al. 2020). Additional evidence that distinct cell populations in the SC mediate prey detection and orienting (Hoy et al. 2019) supports the hypothesis that processes in the SC are necessary for the rapid and dynamic response to prey motion which our experiments show is

lacking in Shank3 KO mice. If Shank3 loss interferes with plasticity in the SC, it may prevent mice from making multisensory associations which drive these behaviors. Understanding the contribution of these processes to efficient hunting could be done by sensory perturbations to wild type and Shank3 KO mice during development such as noise rearing, which is known to perturb the development of multisensory integration in the SC (Xu et al. 2017). An alternate method which would assess the contribution of Shank3 loss to eye targeting specifically would involve the use of head-mounted cameras in order to compare the eye movements of Shank3 KO mice to the well-characterized movements of wild type mice during hunting (Michaiel et al. 2020). Performing these experiments in juvenile mice would furthermore contribute to an understanding of the evolution of eye tracking during development in wild type animals in an ethologically relevant complex behavior. By using this technique it should be possible to examine mouse gaze and targeting both before and during cricket approach, and to determine the distribution of the gaze azimuth during these phases of hunting. If Shank3 KO hunting deficits are the result of multisensory integration causing impaired triggering of hunting behavior, the azimuth should display a larger variance before approach. If, however, deficits result from impaired visual function then the azimuth should show a larger variance during approach as the mouse struggles to visualize the cricket.

Direct measures of homeostasis in SC may also help to determine whether altered plasticity is indeed a factor in the inability of Shank3 KO mice to efficiently learn hunting behavior observed in our experiments. Hebbian long term potentiation has been studied in excitatory neurons in the SC, including in narrow-field vertical neurons (Zhao et al. 2006) separately shown to be involved in efficient targeting behavior during prey approach (Hoy et al. 2019). While mechanisms of homeostatic plasticity are best studied in cortical circuits, it is

feasible that Shank3-dependent homeostatic plasticity mechanisms may also be present in excitatory neurons in brain regions such as the SC. By performing electrophysiological experiments in SC slices, it may be possible to gather direct evidence for such plasticity mechanisms. If these experiments show that Shank3-dependent homeostatic plasticity operates in the SC, this would supply a compelling mechanism for the origins of observed hunting differences.

Chapter 5 References

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