7-31-2017

Behavioral and Neuroanatomical Assessment of Transgenic Mouse Models of Language-related Developmental Disorders

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University of Connecticut, 2017

Language encompasses an ability to acquire and integrate complex cognitive systems in order to communicate with others, and inherited factors are thought to play a key role in modulating these emergent skills. In recent years, the role of genetics in language has gained focus and attention, based on accumulating empirical knowledge about the genes, proteins, and cellular machinery involved. In particular, disruption to various mechanisms has been shown to relate to impairments in language -- as seen in neurodevelopmental disorders such as autism, dyslexia and SLI. Mouse models can serve as a useful tool in studying the genetic modulators of related neural circuitry. The central over-arching aim of the current series of studies was to examine behavioral and neuroanatomic profiles of transgenic mice modeled on several established neurodevelopmental disorders (NDDs). We focused on five transgenic murine preparations exhibiting mutations derived from NDD populations characterized by atypical language. These include NDDs with language disability as a core feature of the disorder (e.g., specific language impairment (SLI), dyslexia), or as a sub-type (i.e., only some individuals affected; autism spectrum disorders (ASD)). The transgenic models assessed include: (1) *Cntnap2* knock-out (KO; implicated in ASD and SLI); (2) *Dyx1c1* conditional forebrain KO (implicated in dyslexia); (3) Ts2-neo model (a mutation associated with ASD); (4) *Dcdc2* KO (implicated in dyslexia); and (5) *Shank3*b KO (associated with ASD). Using these models, we assessed specific links between: (a) genetically driven alterations in neurodevelopment; and (b) anomalies in fundamental non-verbal behaviors subserving aspects of language-learning. Our novel behavioral paradigms were able to tap “intermediate” language-related behavioral phenotypes in mice.

Measures included acoustic processing of rapid and complex stimuli, visual motion perception, sensorimotor functions, social/communicative interactions, and working memory. We also quantified gross neuroanatomy to assess whether neural anomalies correlate with any atypical behaviors. Cumulative findings provide insight about the role of genes critical in the polygenic developmental cascade supporting emergent language, as well as the consequences of disruption to those pathways. Ongoing research may promote enhanced early screening of infants, as well as individualized treatment techniques for neurodevelopmental disorders that include language and communicative impairments.

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B.S., Stony Brook University, 2012

M.A., University of Connecticut, 2016

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of Doctor of Philosophy

at the

University of Connecticut

2017

APPROVAL PAGE

Doctor of Philosophy Dissertation

Behavioral and Neuroanatomical Assessment of Transgenic Mouse Models of Language-related Developmental Disorders

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Acknowledgements

This work would have not been possible without the help and contribution of my committee members, fellow lab members, and support from my family and friends.

Firstly, I would like to express my deepest gratitude to my advisor Dr. Holly Fitch for the continuous support of my scientific endeavors and for her patience, motivation, and immense knowledge. She has inspired me to be an independent thinker and encouraged me to participate in cross-disciplinary research, which is necessary to bridge the gap between human and animal studies and advance the field of neuroscience.

Thank you so much to Drs. John Salamone, Inge-Marie Eigsti, Heather Read, and Joseph LoTurco for your encouragement and your diverse perspectives and contributions. I would also like to thank them for their time and constructive critiques, the work presented here is more comprehensive and coherent because of it.

I would like to thank the Fitch lab members, past and present. Particularly, Nhu and Peter for their direct influences and significant additions to the studies but also their amazing support and friendship. I am also grateful for Michelle and Amanda for their collegial guidance and enthusiastic encouragement. Thank you to all the Fitch Lab undergraduates who have volunteered their time to assist with behavioral testing – I would have not been able to present all this data without their help.

I would like to thank all the fellow graduate students I have encountered during my time at UConn. Their endurance, dedication, and insights have been inspiring. I am grateful to Iris, Russell, Katie and Lauren for their support, motivation, intelligence and humor.

Last but not least, I would like to thank my family: my parents and my siblings, Jessica, Samantha, Billy and Ryan for their endless love and support throughout my graduate career and my life.

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Introduction

1.1 Background -- Neurogenetics of developmental language disabilities

Language use is an enormously complex process, yet surprisingly, most children acquire these skills with little to no initial instruction. By the late toddler/early pre-school years, most children have acquired a large number of words, and can assemble them into sentences following complex grammatical rules. However, not all children acquire language so effortlessly. In fact, substantial variability exists in the speed and proficiency of language acquisition, reflecting both environmental and genetic factors. Although some children grow out of their language impairments, many others have persistent difficulties with language expression and comprehension throughout life. These difficulties appear to be common, with developmental disorder of speech, language and communication accounting for 40% of referrals to pediatric services (Harel et al., 1996).

In recent years, the role of genetics in language has gained particular attention, given accumulating empirical knowledge about the genes, proteins, and cellular machinery involved in both brain development and cognitive outcomes. Disruptions to various neurodevelopmental mechanisms (e.g. neuronal migration and synaptic plasticity) have been related to impairments in language as seen in neurodevelopmental disorders (NDDs) such as dyslexia, specific language impairment (SLI), and autism spectrum disorders (ASDs). Developmental dyslexia is a learning disability characterized by unexpected difficulties in learning to read and write (Shaywitz & Shaywitz, 2005), while SLI is diagnosed when children exhibit significant delays in spoken language acquisition (Leonard, 2000). Both dyslexia and SLI employ a diagnosis of "exclusion",

meaning that observed dysfunctions cannot be explained by concomitant factors such as intellectual impairment, lack of educational opportunity, or other co-morbid neurological disorders (e.g., epilepsy, or primary sensory impairments (blindness, deafness); Leonard, 2000; Shaywitz &Shaywitz, 2005). Language impairment is also a core feature of ASD, affecting approximately 50% of all children diagnosed with autism, and can manifest in varying degrees that range from a complete absence of language to hyperlexic skills (Alarcón et al., 2008). Although these clinical populations are diverse, they do share overlapping symptomatology that is thought to contribute to the similar language deficits observed.

The language-related NDDs described above have shown to be highly heritable (indicating a strong genetic basis), with familial rates ranging from 40 to 90% (Bailey et al., 1995; Steffenburg et al., 1989; Rosenberg et al., 2009; Schumacher, Hoffmann, Schmal, Schulte-Korne, & Nothen, 2007). However, many different genes have been implicated in these language-based developmental disabilities, leading investigators to postulate a complex polygenic and environmental array of "risk factors" that may contribute to developmental disruption. This puzzling identification of hundreds of gene pathways leading to a small number of common and/or overlapping language-related phenotypes is not unprecedented, and has been seen for other genetically-mediated non-syndromic disorders such as schizophrenia and bipolar disorder (Gelernter, 2015).

1.2 Genetics of language-related disorders

The first gene identified to have an influence on speech and language was the transcription factor, forkhead box protein P2 (*FOXP2*). In 2001, *FOXP2* was discovered to be the cause of a monogenic verbal dyspraxia in the KE family (Lai et al., 2001). Not only did these family members have difficulty producing speech, they also displayed an array of linguistic

deficits affecting expressive and written language (Watkins, Dronkers & Vargha-Khadem, 2002). Although *FOXP2* is popularly designated the "language gene," this is not completely accurate, since evidence clearly shows that one gene does not mediate all aspects of language abilities. Moreover, *FOXP2* is not the only risk gene of language-related disorders. For instance, *FOXP2* directly downregulates contactin-associated-like-protein 2 (*CNTNAP2*), which has also been linked to specific language impairment, autism and dyslexia (Alarcón et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Peter et al., 2013; Vernes et al., 2008). *CNTNAP2* variants have also been specifically associated with non-word repetition as well as age at first word and phrase (Alarcon et al., 2008; Anney et al., 2012; Peter et al., 2011; Vernes et al., 2008).

Developmental dyslexia has been known to have familial clustering for well over 100 years (Hinshelwood 1902, Stephenson 1907; Thomas 1905). Specifically, a child with an affected parent has a risk of 40-60% of developing dyslexia, and this risk is increased to 76-78% when both parents are affected (Gilger et al., 1996; Olson, Forsberg & Wise, 1994; Schulte-Körne et al., 1996; Ziegler et al., 2005). To date, at least nine loci for dyslexia have been mapped (DYX_1 to DYX_9), which include candidate risk genes *DYX1C1*, *DCDC2*, *KIAA0319* and *ROBO1*. *DYX1C1* (dyslexia susceptibility 1 candidate 1) was the first dyslexia candidate risk gene identified, specifically in two Finnish families with a history of dyslexia (Nopola-Hemmi et al., 2000). Of the more recently identified candidate genes, *DCDC2* (doublecortin domain containing protein 2) and *K1AA0319* seem to be of most significance for dyslexia, with multiple reports from well-established independent samples, and including severely affected individuals with dyslexia (Schumacher et al., 2007). By contrast, *DYX1C1* and *ROBO1* have seen inconsistent findings of involvement in the development of dyslexia across various populations.

Although the genetic etiology of dyslexia is complex, understanding the genetic mechanisms underlying ASD has been one of the most puzzling biological questions of the past decade. The genetic influence in ASD is strong, with heritability rates consistently ranging from 70-80% (Bailey et al., 1995; Rosenberg et al., 2009). However, contrary to the several candidate risk genes identified for dyslexia, it is suggested that over 1,000 genes may be involved in ASD, reflecting a tremendously complex genetic architecture (De Rubeis & Buxbaum, 2015). Additionally, no one of these known genetic contributors accounts for more than 1-2% of the phenotypic variance seen in ASD, despite having strong inheritance patterns (Abrahams & Geschwind, 2008). Several dozen ASD susceptibility genes have been identified in the past decade, collectively accounting for 10–20% of ASD cases (Geschwind, 2011). Considerable insight into potential risk genes has been discovered by the molecular study of defined syndromes related to ASD, such as fragile X syndrome (*FMR1*), Rett syndrome (*MECP2*), Angelman syndrome (*UBE3A*, *GARBRB3*), Timothy syndrome (*CACNA1C*) and Phelan-McDermid syndrome (*SHANK3*). Culminating evidence is starting to link these syndromes and idiopathic autism. For instance, levels of *UBE3A* and *GABRB3* are reduced in each of Angelman syndrome, Rett syndrome and idiopathic autism (Samaco, Hogart, & LaSalle, 2005). This is also seen for other candidate genes such as *CACNA1C* and *SHANK3,* which associate with both syndromic and non-syndromic forms of ASD. Although these ASD-associated syndromes involve genes with multiple molecular functions, it seems increasingly plausible that they converge on common biological pathways and neural circuits that generate ASD phenotypes. These candidate genes provide an opportunity for insight into the biological pathways that underpin normal language acquisition. However, it is difficult to understand how a mutation in just one gene could have a profound and specific effect on the development of language (leaving

many other neural functions relatively intact), as well as how mutations in so many different genes with distinct functions could lead to a *common* phenotype of atypical language skill. An integrative approach across disciplines examining genetic contribution at multiple levels is necessary to bridge this gap between genes and language.

1.3 Neurobiological themes of language-related disorders

Language development is a biologically complicated process that involves the development and recruitment of multiple neural systems needed to process auditory, visual, sensorimotor, and memory functions in an integrated fashion for the individual to perceive, comprehend and produce language. Early disturbances in neurodevelopment may consequently disrupt fundamental processes necessary to acquire typical language abilities, and ultimately lead to language disability. To date, several neurobiological themes have been established that may account for the language impairments observed in these clinical populations.

1.3.1 Neural mechanisms underlying dyslexia

The neurobiological mechanisms underlying dyslexia/reading disability are not yet fully understood, however, most of the risk genes identified appear to converge on similar biological mechanisms (i.e., neuronal migration and cilia function; Kere, 2014). Early studies of post-mortem dyslexic brains revealed anomalies of neuronal migration (molecular layer ectopias and focal microgyri) predominantly in the left hemisphere (Galaburda et al., 1985; Galaburda & Kemper, 1979; Humphreys et al., 1990; Kaufmann & Galaburda, 1989). In parallel, neuronal migration defects have also been observed in animal models that express a downregulation of dyslexia candidate risk genes. Specifically, rat models using RNA interference to knock-down *Dcdc2*, *Dyx1c1* and *Kiaa0319* resulted in disruption of the typical neuronal migration trajectory

from the ventricular zone to the cortex, leaving cells in subcortical localizations (Meng et al., 2005; Peschansky et al., 2010; Wang et al., 2006). Furthermore, *Robo1* knockout mice display neuronal migration defects in the forebrain. Interestingly,, these neuronal migration anomalies have not yet been observed in other knockout mouse models targeting dyslexia risk genes, although signaling of primary cilia in neurons has been also associated with *DCDC2*, *DYX1C1*, and *KIAA0319* (Chandrasekar et al., 2013; Ivliev et al., 2012; Massienen et al., 2011; Tarkar et al., 2013). Overall findings are consistent with aberrant neuronal migration, since primary cilia have a critical role in cortical morphogenesis of the developing forebrain (Willaredt et al., 2008). These deviant neurodevelopmental processes could lead to abnormal cortico-cortical and cortico-thalamic circuits that affect sensorimotor, perceptual and cognitive processes critical for learning and language processing.

1.3.2 Magnocellular theory of dyslexia

Cytoarchitectonic anomalies have also been observed in the thalamus of human dyslexic brains, particularly in the lateral geniculate nucleus (LGN), where magnocellular layers were seen to be more disorganized and characterized by smaller cell bodies (Livingstone et al., 1991). This reduction in the distribution of large cells ("magnocells") versus small cells ("parvocells") was seen in both the lateral geniculate (visual), and also the medial geniculate (auditory) nuclei of dyslexic subjects (note there is no distinctive layering in the MGN; Galaburda et al., 1994). These findings, along with related neuroanatomical evidence and findings of atypical sensory processing for magnocellular-dependent tasks, led to the magnocellular theory of dyslexia. This theory posits that both visual and auditory processing dysfunction associated with reading disability can be attributed to low-level processing deficits resulting from specific disruption to the magnocellular sub-systems of thalamic nuclei, and potentially elsewhere in sub-cortical

sensory structures (Galaburda et al., 1994; Livingstone et al., 1991; see Stein, 2001, for review). It is well established that the magnocellular component of the visual system is specialized for processing rapidly-changing temporal information (e.g., motion). Further support of this theory has come from animal models displaying similar thalamic anomalies within the MGN (more small and fewer large neurons), along with rapid auditory processing deficits consistent with magnocellular processes (Fitch et al., 1994; Fitch et al., 1997; Herman et al., 1997; Peiffer et al., 2001; Peiffer et al., 2002a; Peiffer et al., 2002b). It is thought that a resulting disruption in temporal processing in both visual and auditory domains might make it difficult for individuals with dyslexia to process rapidly changing sensory input crucial to the initial establishment of critical speech sounds, as well as the later visual processing of letters (Stein & Walsh, 1997).

1.3.3 Synaptic dysfunction in ASD

Due to the numerous autism candidate susceptibility genes that are associated with synaptic structure, function and regulation, ASD is in fact referred to as a "synaptopathy" (Zoghbi, 2003; Geschwind & Levitt, 2007; Zoghbi & Bear, 2012). Many identified ASD risk genes encode synaptic scaffolding proteins, receptors, cell adhesion molecules or proteins that are involved in chromatin remodeling, transcription, protein synthesis or degradation, or actin cytoskeleton dynamics -- all which are key regulators of synaptic plasticity (see Bourgeron 2015 for review). The possibility that alteration of synaptic functions could lead to ASD was first indicated by the phenotypic overlap between autism, fragile X syndrome, and Rett syndrome (Belmonte & Bourgeron, 2006; Zoghbi, 2003). Moreover, an imbalance between inhibitory and excitatory currents in ASD was further supported by the observation that 10-30% of affected individuals have epilepsy (Canitano, 2007). Synaptic dysfunction was further implicated in ASD when mutations affecting postsynaptic cell adhesion molecules, Neuroligins (NLGN), were

identified in a subset of individuals with ASD (Jamain et al., 2003; Laumonnier et al., 2004). Mutations were also reported in other synaptic proteins such as *SHANK3*, *NRXN1*, *CNTNAP2*, *CNTN3/4*, and *PCDH9/10* (Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; Durand et al., 2007; Kim et al., 2008; Marshall et al., 2008; Morrow et al., 2008; Szatmari et al., 2007). Atypical voltage-gated calcium channel subunits (*CACNA1C*, *CACNB2*, *CACNA1I*) have also been identified in ASD populations, and these subunits are crucial for establishing synaptic homeostasis (Turrigiano, 2011). Mice carrying various mutations in these genes display ASD-like behaviors, such as repetitive grooming and deficits in social interaction and communication (see Ey, Leblond & Bourgeron, 2010 for review). Disruption of these proteins alters synaptic homeostasis and may ultimately induce atypical brain connectivity.

1.3.4 Abnormal connectivity observed in ASD

Abnormal brain growth and connectivity have also been a hypothesized cause of ASD (Courchesne & Pierce, 2005; Just et al., 2007). It is suggested that connectivity within the frontal lobe is excessive, disorganized, and inadequately selective, whereas connectivity between frontal cortex and other systems is poorly synchronized and weakly responsive (Courchesne & Peirce, 2005). In addition to these findings, evidence has shown that connections across cortical regions are often diminished in general in ASD. Conversely, functional whole-brain connectivity analyses have revealed that individuals with ASD show subcortical areas that exhibit hyperconnectivity, even though cortiocortical areas in the same subjects are predominantly hypoconnected (Di Martino et al., 2014). This developmental "disconnection" may account for clinical heterogeneity, as well as the frequent late emergence during development (around 2 yrs) seen in ASD (Belmonte et al., 2004). This is consistent with the notion that people with ASD suffer from a lack of "central coherence" -- the cognitive ability to bind together a jumble of

separate features to form a single coherent concept (Frith, 1989). Impairments in the integration of sensory information could in turn reflect diminished cross-modal white matter connectivity, as reported in some DTI/MRI studies (Maximo et al., 2013; Travers et al., 2012). The purported hypoconnectivity and multisensory integration issue may be further disrupting higher-order cognitive abilities, such as learning and social communication that are heavily dependent on multi-modal and trans-cortical integration.

1.4 Intermediate language phenotypes

Given the enormous number of identified gene risk factors underlying language-related disorders, it is crucial that we try to deconstruct complex diagnostic outcomes into simpler behavioral endophenotypes or "intermediate phenotypes" that can more easily be associated with individual genetic and neurobiological mechanisms, particularly where these features are shared across disorders. Language-related endophenotypes employed in more recent human gene wide association studies (GWAS) include scores on specific language-based tasks thought to tap specific sub-skills or domains. This contrasts more traditional gene association studies that rely on broad diagnostic categorization (i.e., GWAS using "affected" versus "unaffected" groups). More novel language-related intermediate phenotypes include measures of impairment in phonological processing (Alcántara et al., 2012; Benasich et al., 2006; Bhatara et al., 2013; Boscariol et al., 2010; McArthur &Bishop, 2005; Kovelman et al., 2012; Melby-Lervag, Lyster & Hulme, 2012; Peyrin et al., 2012), short-term and/or working memory (Beneventi et al., 2010; Gathercole et al., 2006; Menghini et al., 2010; Barendse et al., 2013) rapid auditory processing (Cohen-Mimran & Sapir, 2007; Fitch & Szalkowski, 2012; Hamalainen, Salminen & Leppanen, 2013; Bonnel et al., 2003; Järvinen-Palsey, Peppé, King-Smith & Heaton, 2008; O'Riordan & Passetti, 2006; Tomchek, & Dunn, 2007), visuospatial attention (Franceschini, 2012; Gabrieli &

Norton, 2012), and/or visual attention/perception (Galaburda and Livingstone, 1993; Stein and Walsh, 1997; Vidyasagar and Pammer, 2010; Townsend, Harris &Courchesne, 1996; Frischen, Bayliss & Tipper, 2007). Use of these more fine-grained functional measures to link genetic risks with specific behavioral traits (rather than global and highly variable diagnostic categories) permits a much more focused analysis of how genes are specifically contributing to behavioral or functional components within a broader clinical domain.

1.4.1 Intermediate language phenotypes - Auditory processing

Deficits in auditory temporal processing are commonly shared across SLI, dyslexia, and autism, suggesting that auditory processing may be a core skill for the development of language, as well as subsequent reading ability. For example, work has shown that language impaired populations exhibit difficulties in detecting and discriminating rapidly changing acoustic stimuli (i.e., in the range of tens of milliseconds), regardless of whether stimuli comprise verbal or nonverbal information (Cohen-Mimran & Sapir, 2007; Tallal & Piercy, 1973; Tallal et al., 1993; Tallal & Newcombe, 1978; Vandermosten et al., 2011). Most of these studies have reported that when stimulus durations were longer, language impaired individuals were comparable to typical controls in effectively detecting differences in auditory information. Therefore the deficits appear to be specific to rapidly changing sounds, and *not* whether stimuli are verbal or nonverbal in nature (Tallal, 1980; Vandermosten et al., 2011). Related research has described evidence of longitudinal prediction for future language impairments, based on evidence of abnormal non-lingual rapid auditory processing measured during infancy (Benasich & Tallal, 2002; Benasich et al., 2006; Choudhury et al., 2007). Interestingly, these same studies showed that early acoustic processing indices were effective predictors of long-term language outcomes not only in at-risk samples (i.e., infants with affected family members), but also in typically developing infants.

Thus overall evidence strongly supports the view that intact auditory processing ability is crucial to early language development, including the ability to discriminate and categorize sounds for the formation of phonological categories, which in turn become the building blocks for words.

In the context of autism, anomalies in auditory processing can also relate to deficits in language abilities, but in this case it appears that enhancements in processing low-level detailed acoustic information (e.g., frequency information) may also undermine subsequent global processing required for effective language skills (e.g., formation of phoneme categories, processing of words and sentences; Eigsti & Fein, 2013; Bonnel et al. 2010, 2003; Heaton and Heaton 2003, 2005; Jones et al. 2009; O'Riordan & Plaisted 2001; Stewart, Griffiths, Grube, 2015). These theories are supported by robust evidence that individuals with ASD display superior pitch discrimination, and enhanced ability to detect and discriminate low-level auditory information (Hyde et al., 2011; Hertrich et al., 2013). Moreover, the incidence of low-level acoustic enhancements appears to relate specifically to the incidence of speech onset delays, meaning that acoustic superiorities may be specifically associated with -- not merely co-occurring with -- language deficits in ASD populations (Eigsti & Fein, 2013; Mottron et al., 2014).

1.4.2 Intermediate language phenotypes - Visual processing

Visual attention and perception have also shown to impact language development. Specifically, atypical or impaired visual perception of motion has been strongly implicated in dyslexia, and may be a one causal factor underlying slow reading speeds (Lyon et al., 2003; Nicholson and Fawcett, 2007). Children with dyslexia are reported to have some combination of spatial (Lovegrove et al., 1980; Cornelissen et al., 1995; Stein and Walsh, 1997; Lawton, 2000, 2007, 2008, 2011; Talcott et al., 2000; Hansen et al., 2001; Stein, 2001) and/or temporal (Stanley

and Hall, 1973; Bradley and Bryant, 1983; Tallal et al., 1993; Temple et al., 2003) visual sequencing deficits. These impairments could relate to reports from dyslexics that letters in words and/or words on a page appear distorted, displaced, or crowded together (Atkinson, 1991). Moreover, visual processing deficits are prominent in dyslexic samples when images are rapidly presented or moving – findings that support speculation about neural timing deficits associated with sluggish magnocellular neurons in dyslexia (Livingstone et al., 1991; Stein and Walsh, 1997; Vidyasagar, 1999, 2001, 2012; Lawton, 2000, 2007, 2008, 2011; Stein, 2001; Vidyasagar and Pammer, 2010; Boets et al., 2011). In fact, early evidence pointed to a selective deficit of the magnocellular-dorsal system as measured both by atypical cellular morphology in thalamic nuclei (Galaburda and Kemper, 1979; Galabur

da et al., 1985; Livingstone et al., 1991), as well as electrophysiological profiles during motion-related magnocellular processing tasks (Lovegrove et al., 1980; Cornelissen et al., 1995; Stein and Walsh, 1997; Demb et al., 1998; Slaghuis and Ryan, 1999, 2006). Importantly, these deficits in motion perception can be detected earlier than language impairments, and are also predictive of language outcome, making motion perception another useful "intermediate" and pre-lingual phenotype for early diagnosis and intervention (Boets et al., 2011).

1.4.3 Intermediate language phenotypes – Working memory

It has also been suggested that working memory may be disrupted in autism, as well as dyslexia (Barendse et al., 2013; Williams et al., 2005; Jeffries & Everatt, 2004; Smith-Spark & Fisk, 2007). Working memory involves the temporary storage and manipulation of information that is required for a wide range of complex cognitive tasks, including language. Therefore, disruptions in working memory may consequently constrain language development in children (Gathercole & Baddeley, 1993; Bishop et al., 1996; Baddeley, 2003). It has been postulated that

impairments in phonological memory in particular may influence the efficiency and accuracy with which stable long-term memory phonological representations can be established. Such deficits would negatively impact the ability to acquire grammatical constructs, as well as new words (Speidel and Herreshoff, 1989; Baddeley et al., 1998; Gathercole and Baddeley, 1990). Therefore, disruptions in working memory could also fundamentally alter language development.

Overall, language requires stability and integration from multiple systems that depend on intact auditory processing, visual attention/perception, and working memory. Importantly, all of these fundamental processes can be measured during early development -- well before the onset of language and reading. Yet, these indices also appear to be predictive of later language outcomes, and may provide particularly useful screening tools for at-risk infants. Additionally, auditory processing, visual perception, and working memory can be reliably assessed in mouse models, using various carefully designed behavioral paradigms. Finally, a focus on intermediate processing skills crucial to language development opens a dramatic new domain for the use of animal models in studying NDDs.

1.5 Dissertation Purpose

The purpose of the current thesis was to target specific genes that have been implicated in language disorders, and assess the behavioral and neuroanatomical consequences of manipulating the protein products associated with the genes. To accomplish this, we used a variety of transgenic mouse models that capitalize on recent developments in the capacity to turn genes down or off ("knock-outs"), turn genes "up" (using over-expressive mutations such as copy number variants), or alter the functionality of gene protein products through insertion of more precise mutations (e.g., SNP and point mutation "knock-ins"). Using such manipulations, we can further explore gene-brain-behavior relationships to examine how each targeted gene

mediates core behavioral endophenotypes associated with language and reading-related dysfunction. In the current dissertation, we focused on five transgenic preparations exhibiting mutations derived from NDD populations with atypical language. These include NDDs with language disability as a core feature of the disorder (specific language impairment (SLI), dyslexia), or as a sub-type (some individuals affected; autism spectrum disorders (ASD)). Transgenic strains assessed include: (1) *Cntnap2* knock-out (KO; implicated in ASD and SLI); (2) *Dyx1c1* conditional forebrain KO (implicated in dyslexia); (3) Ts2-neo model (a mutation associated with ASD); (4) *Dcdc2* KO (implicated in dyslexia); and (5) *Shank3b* KO (associated with ASD). In all cases, mutants were compared to same-sex and age wild-type (WT) mice. Using these models, we assessed specific links between: (a) genetically driven alterations in neurodevelopment; and (b) anomalies in fundamental non-verbal behaviors subserving aspects of language-learning. Our novel behavioral paradigms were able to tap into "intermediate" language-related behavioral phenotypes in these mice. Behavioral measures included acoustic processing of rapid and complex stimuli, visual motion perception, sensorimotor functions, social/communicative interactions, and working memory. We also quantified gross neuroanatomy to assess whether neural anomalies correlate with any atypical behaviors. In closing, ongoing research can provide valuable insight to our understanding of the biological substrates of atypical language development. Future applications of data could allow for more precise diagnosis, and advanced early screening tools as well as more targeted interventions using these genetic and anatomical markers.

Chapter 2

Learning delays in a mouse model of autism spectrum disorder

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Published: *Brain Behavioral Research*, 2016, doi: 10.1016/j.bbr.2016.02.006

2.1 Abstract

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder with core symptoms of atypical social interactions and repetitive behaviors. It has also been reported that individuals with ASD have difficulty with multisensory integration, and this may disrupt higher-order cognitive abilities such as learning and social communication. Impairments in the integration of sensory information could in turn reflect diminished cross-modal white matter connectivity. Moreover, the genetic contribution in ASD appears to be strong, with heritability estimates as high as 90%. However, no single gene has been identified, and over 1,000 risk genes have been reported. One of these genes -- contactin-associated-like-protein 2 (*CNTNAP2*) -- was first associated with Specific Language Impairment, and more recently has been linked to ASD. *CNTNAP2* encodes a cell adhesion protein regulating synaptic signal transmission. To better understand the behavioral and biological underlying mechanisms of ASD, a transgenic mouse model was created with a genetic knockout (KO) of the rodent homolog *Cntnap2*. Initial studies on this mouse revealed poor social interactions, behavioral perseveration, and reduced vocalizations -- all strongly resembling human ASD symptoms. *Cntnap2* KO mice also show abnormalities in myelin formation, consistent with a hypo-connectivity model of ASD. The current study was designed to further assess the behavioral phenotype of this mouse model, with a focus on learning and memory. *Cntnap2* KO and wild-type mice were tested on a 4/8 radial arm water maze for 14 consecutive days. Error scores (total, working memory, reference memory, initial and repeated reference memory), latency and average turn angle were independently assessed using a 2 x 14 repeated measures ANOVA. Results showed that *Cntnap2* KO mice exhibited significant deficits in working and reference memory during the acquisition period of the task. During the retention period (i.e., after asymptote in errors), *Cntnap2* KO mice

performed comparably to wild-type mice. These findings suggest that *CNTNAP2* may influence the development of neural systems important to learning and cross-modal integration, and that disruption of this function could be associated with delayed learning in ASD.

2.2 Introduction

Autism Spectrum Disorder (ASD) is a set of neurodevelopmental disorders characterized by a complex behavioral phenotype, encompassing deficits in both social and cognitive domains. Accepted core symptoms are heterogeneous ranging from atypical social interactions and language impairments to repetitive behaviors. Accordingly, individual cases vary substantially in severity and presentation of symptoms. The current estimated prevalence for ASD in the United States is 1 in 68, and is consistently more prevalent in boys than girls (1 in 42 boys versus 1 in 189 girls) (Baio, 2012; Elsabbagh et al., 2012). To date, causal mechanisms underlying ASD remain poorly understood, but likely include a complex combination of polygenic and environmental risk factors (Moreno-De-Luca, 2013).

Ongoing ASD research has focused on the genetic and neurobiological mechanisms of ASD, based on the notion that characterization of the varied neurogenetic features of ASD could provide insight to the diverse behavioral symptoms. The genetic contribution in ASD appears to be strong; for example, monozygotic twin studies estimate the concordance rates are as high as 70% - 90% (Bailey et al., 1995; Steffenburg et al., 1989; Rosenberg et al., 2009). However, the relative proportion of ASD that can be accounted for by either rare or common genetic variation remains to be determined, and no single gene has been identified as a major cause. In fact, over 1,000 risk genes have been reported, pointing to a very complex genetic etiology (De Rubeis & Buxbaum, 2015).

One of the autism susceptibility candidate genes -- contactin-associated-like-protein 2 (*CNTNAP2*) -- was first linked to Specific Language Impairment, and more recently has been linked to ASD (Alarcón et al., 2008; Arking et al., 2008). *CNTNAP2* has also been linked to other complex neurological disorders such as schizophrenia, dyslexia and depression in genome-wide association studies (Vernes et al., 2008; Newbury et al., 2011; Peter et al., 2011; Ji et al., 2013). Thus *CNTNAP2* mutations could underlie similar endophenotypes across various disorders. In clinically language-impaired populations, *CNTNAP2* variants have been associated with difficulties with non-word repetition -- a measure of working memory that critically underlies language and social cognition (Vernes et al., 2008; Peter et al., 2011). Further studies have highlighted significant association between specific SNPs in *CNTNAP2* and language endophenotypes of ASD including age at first word (Alarcon et al., 2008) and age at first phrase (Anney et al., 2012).

CNTNAP2 is located on chromosome 7, and is responsible for encoding a cell adhesion protein regulating synaptic signal transmission (Alarcón et al., 2008).To better understand the behavioral and biological underlying mechanisms of ASD, a transgenic mouse model was created with a genetic knockout (KO) of the rodent homolog *Cntnap2 (*Poliak et al., 2003). Initial behavioral studies of this mouse revealed poor social interactions, perseveration, and reduced pup vocalizations -- all strongly resembling human ASD symptoms (Peñagarikano et al., 2011; Penagarikano & Geschwind 2012). *CNTNAP2*'s role in neurodevelopment has been further studied using this mouse model, revealing that *Cntnap2* KO mice show abnormalities in myelin formation -- consistent with a hypo-connectivity model of ASD (Poliak et al., 2003). These mice also exhibit abnormal cortical neural synchrony (i.e., enhanced asynchrony), fewer inter-neurons (which are mostly inhibitory), and atypical neuronal migration (Peñagarikano et al., 2011). All of

these cellular anomalies can be linked to current biological theories for mechanisms of ASD. More recent studies from our lab revealed that the KO mice exhibit unexpected enhancements in acoustic frequency processing, despite impairments on more complex silent gap detection tasks (Truong et al., 2015). The latter results have been linked with anomalies at the level of the thalamus, and also could also reflect atypical patterns of cortical connectivity.

The current study was designed to further assess the behavioral phenotype of the *Cntnap2* KO mouse model, with a focus on putative anomalies in spatial learning and memory. Specifically, impairments in working memory have been noted in individuals with ASD, and these deficits are more pronounced when the task load is high (Barendse et al., 2013). It is also important to note that although most of these working memory impairments in ASD are found in the spatial domain (e.g. Lind et al., 2013), they have also been observed in complex verbal working memory tasks (Schuh & Eigsti, 2012; Steele et al., 2007; Luna et al., 2007; Williams, Goldstein, & Minshew, 2005; Willims et al., 2005). Previous studies investigating the *Cntnap2* KO mice, however, found similar learning rates on the Morris Water maze task for KOs versus WT controls. This result suggests a *lack* of spatial learning and memory impairments (Peñagarikano et al., 2011). However, when presented with a maze reversal task, *Cntnap2* KOs *did* show significant impairments in learning the new platform location (Peñagarikano et al., 2011). These results reinforce the notion that difficulty of task may play a role in inconsistent findings for memory deficits associated with ASD. Our goal was to further assess *Cntnap2* KOs spatial memory ability utilizing a more difficult 4/8 arm radial water maze task. This task also allows for the analysis of *both* reference and working memory, while introducing a higher cognitive load (compared to Morris Water maze, with only one platform). Finally, this task

generates a more extended learning curve, allowing us to adequately evaluate performance during acquisition and retention periods separately.

2.3 Methods and Materials

2.3.1 Subjects

10 *Cntnap2* KO mice (B6.129(Cg)-*Cntnap2*^{tm1Pele}*/J*; stock number 017482) and 11 wild type (WT) controls (C57BL/6J; stock number 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME)[1]. [1]Subjects were delivered to the University of Connecticut, Department of Psychology at 7 weeks of age. Upon arrival, subjects were single housed in standard plexiglass laboratory cages (12:12 light/dark cycle) with food and water available *ad lib*. Only male subjects were used for testing, based on evidence of a higher incidence of ASD and developmental language impairments in males as compared to females (Baio, 2012). Maze testing began when the animals were around 24 weeks of age, and occurred during the subjects' light cycle. All procedures were performed blind to subject genotype and were conducted in compliance with the National Institutes of Health and approved by the University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

2.3.2 Water maze assessment – Visible platform and 4/8 radial water maze

Subjects were initially tested on a visible platform control task (also known as "water escape") prior to the 4/8 radial water maze task, to evaluate any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm x 55.5 cm) filled with room temperature water, and given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) located at the opposite end of the tub. Latencies to the

[1] Jax guarantees "rigorous genetic quality control and mutant gene genotyping programs" for mouse strains with identified molecular mutations (see Terms of Sale).

visual platform were recorded for assessment. None of the subjects displayed any impairments, and there were no observed differences between genotypes on this task. We therefore proceeded to testing on the water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998).

The 4/8 radial arm water maze assesses spatial reference and working memory abilities simultaneously, using a standard 8 arm radial maze with 4 arms containing a submerged goal (escape) platform, and 4 open arms that never contain a platform (Fig. 2.1). Configuration of goal arms were counterbalanced between subjects, but remained fixed for each subject across all test sessions. Additionally, high contrast extra maze cues were present in the room, and the locations of these remained static for the entire experiment.

The day prior to testing (Day 1), subjects were given a training session where all arms that would never contain a platform were blocked, forcing the animals to only enter arms containing a platform. Subjects were placed in the middle of the maze and were given 120 seconds to locate a platform. Every subject completed 4 training trials. Each time they found a platform, the recently located platform was removed, and the entrance to that arm was blocked. This ensured that the subject could no longer enter this arm for the remainder of the training session. If the subject failed to find a platform in this time-period, they were guided to the nearest available goal. Once on the platform, subjects remained for 20 seconds and then were removed to their home cage (30 second inter-trial interval; ITI).

Testing began on Day 2 and continued for 14 consecutive days. The testing session followed training procedures, except instead of blocking the goal arm of the most recently located platform, the platform was simply removed during the 30 second ITI. This arm remained open for the remainder of the test session, but contained no escape. Test sessions were recorded

using a Sony camera, integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). An arm entry was counted for a subject when all four paws entered an arm. Three types of errors were quantified for analysis: 1) Working memory errors (the number of initial and repeat entries into arms from which a platform had been removed during a testing session on a given day); 2) Initial reference memory errors (the total number of first entries into arms that never contained a goal platform) and; 3) Repeat reference memory incorrect errors (the total number of repeat entries (following the initial entry) into arms that never contained escape platforms). Total errors per test session in each category were tabulated, averaged within Genotype, and used for analysis across days of testing.

Finally, in order to determine whether subjects utilized a spatial or chaining (swimming to successive adjacent arms) strategy to solve the water maze, angles of arm choices were derived and analyzed. Specifically, video tracking data obtained from the SMART system was reviewed, and turn angle entry was calculated to determine the average turn angle utilized across sessions. Lower turn-angle averages (closer to 45°) suggest that subjects preferred adjacent arm choices to solve the maze. Alternatively, higher averages (around 90° and greater) suggest a preference for more spatial strategies to solve the maze.

2.3.3 Statistical Analysis

A univariate ANOVA was conducted to compare latencies as a function of Genotype. Average total, working memory, total reference memory, initial reference memory, and repeated reference memory errors, and average turn angle, were independently assessed using a 2 x 14 repeated measures ANOVA. Genotype (2 levels: WT and *Cntnap2* KO) as the between measure, and Days (14 levels) served as the within measure. A one-way ANOVA was also conducted to assess significance between genotypes of each day of testing for all error types. Some analyses

also were performed as a function of test periods, as defined by Acquisition (days 1-7) and Retention (days 8-14) portions of the learning curve.

2.4 Results

2.4.1 Water maze assessment

2.4.1.1 Water Escape

A univariate ANOVA found no main effect of Genotype [$F(1,19)=.915$, N.S.]. Thus no subjects showed any impairment that might confound a swim-task, and all 10 *Cntnap2* KO and 11 WT mice advanced to the testing sessions (Fig. 2.2).

2.4.1.2 Total errors

The 4/8 radial arm water maze was used to simultaneously measure spatial working and reference memory performance. Analysis of the average number of total errors (working memory, initial reference, and repeated reference memory errors) revealed a significant difference between WT and *Cntnap2* KO groups [$F(1,19)=4.791$, $p<0.05$] via repeated measures ANOVA, with *Cntnap2* KOs making significantly more errors than WTs. A main effect of Day [$F(13,247)=4.036$, $p<.001$] was also observed, confirming that both groups reduced errors across days (i.e., showed learning). Within test session an analysis of total errors across days revealed a Day × Genotype interaction [$F(13,247)=1.886$, $p<0.05$], with *Cntnap2* KOs making significantly more errors during the Acquisition period of testing (days 1-7 of testing) [$F(1,19)=5.332$, $p<.05$], but performing comparably to WTs during the Retention period (days 8 – 14 of testing) [$F(1,19)=1.846$, N.S.] (Fig. 2.3a). A one-way ANOVA was conducted to look at differences between genotypes at each day [Day 1: $F(1,19)=.001$, $p>0.05$; Day 2: $F(1,19)=1.620$, $p>0.05$; Day 3: $F(1,19)=4.374$, $p<0.05$; Day 4: $F(1,19)=.056$, $p>0.05$; Day 5: $F(1,19)=5.498$, $p<0.05$; Day 6: $F(1,19)=9.537$, $p<0.05$; Day 7: $F(1,19)=1.372$, $p>0.05$; Day 8: $F(1,19)=7.374$, $p<0.05$;

Day 9: $F(1,19)=.003$, $p>0.05$; Day 10: $F(1,19)=.177$, $p>0.05$; Day 11: $F(1,19)=.118$, $p>0.05$; Day 12: $F(1,19)=1.276$, $p>0.05$; Day 13: $F(1,19)=.074$, $p>0.05$; Day 14: $F(1,19)=.588$, $p>0.05$].

2.4.1.3 Reference Memory Errors

We examined the group differences for four different performance error types including working memory, initial reference memory, repeated reference memory, and total repeated reference memories (METHODS, Fig. 2.1). A repeated measures ANOVA on total reference memory errors (across Days) revealed that *Cntnap2 KOs* did in fact make significantly more errors than WT subjects [$F(1,19)=4.514$, $p<0.05$]. As seen with total errors, there was also a Day x Genotype interaction [$F(1,19)=4.514$, $p<.05$], wherein the *Cntnap2* KOs made significantly more errors during the Acquisition period [$F(1,19) = 3.305$, $p<0.05$], but performed comparably to the WTs during the Retention period [$F(1,19)=2.902$, $p>0.05$] (Fig. 2.3b). A one-way ANOVA was conducted to look at differences between genotypes at each day [Day 1: $F(1,19)=.139$, $p>0.05$; Day 2: $F(1,19)=1.878$, $p>0.05$; Day 3: $F(1,19)=1.451$, $p>0.05$; Day 4: $F(1,19)=.160$, $p>0.05$; Day 5: $F(1,19)=6.713$, $p<0.05$; Day 6: $F(1,19)=7.680$, $p<0.05$; Day 7: $F(1,19)=1.421$, $p>0.05$; Day 8: $F(1,19)=8.352$, $p<0.05$; Day 9: $F(1,19)=.003$, $p >0.05$; Day 10: $F(1,19)=.003$, $p>0.05$; Day 11: $F(1,19)=.318$, $p>0.05$; Day 12: $F(1,19)=4.494$, $p<0.05$; Day 13: $F(1,19)=.048$, $p>0.05$; Day 14: $F(1,19)=.880$, $p>0.05$].

Further analysis of reference memory error type also revealed that *Cntnap2* KOs made significantly more initial reference memory errors [$F(1,19)=5.522$, $p<.05$] (Fig. 2.3c). A one-way ANOVA revealed the following statistics at each day [Day 1: $F(1,19)=.158$, $p>0.05$; Day 2: $F(1,19)=5.600$, $p<0.05$; Day 3: $F(1,19)=1.947$, $p >0.05$; Day 4: $F(1,19)=.033$, $p>0.05$; Day 5: $F(1,19)=8.913$, $p<0.05$; Day 6: $F(1,19)=7.902$, $p<0.05$; Day 7: $F(1,19)=2.533$, $p>0.05$; Day 8: $F(1,19) = 18.736$, $p<0.05$; Day 9: $F(1,19)=.195$, $p>0.05$; Day 10: $F(1,19)=.032$, $p >0.05$; Day

11: $F(1,19)=.068$, $p>0.05$; Day 12: $F(1,19)=2.987$, $p>0.05$; Day 13: $F(1,19)=.051$, $p>0.05$; Day 14: $F(1,19)=.766$, $p>0.05$].

Cntnap2 KOs also made more repeated reference memory errors across the 14 days of testing, but there was no significant main effect of Genotype [$F(1,19) = 3.040$, N.S] (Fig. 2.3d). A one-way ANOVA was conducted to look at differences between genotypes at each day [Day 1: $F(1,19)=.102$, $p>0.05$; Day 2: $F(1,19)=.793$, $p>0.05$; Day 3: $F(1,19)=.820$, $p>0.05$; Day 4: $F(1,19)=.287$, $p>0.05$; Day 5: $F(1,19)=4.131$, $p<0.10$; Day 6: $F(1,19)=4.935$, $p<0.05$; Day 7: $F(1,19)=.635$, $p>0.05$; Day 8: $F(1,19)=2.327$, $p>0.05$; Day 9: $F(1,19)=.248$, $p>0.05$; Day 10: $F(1,19)=.002$, $p>0.05$; Day 11: $F(1,19)=.554$, $p>0.05$; Day 12: $F(1,19)=3.822$, $p<0.10$; Day 13: $F(1,19)=.022$, $p>0.05$; Day 14: $F(1,19)=.529$, $p>0.05$].

2.4.1.4 Working memory errors

A repeated measures ANOVA on working memory errors revealed that *Cntnap2* KOs made significantly more working memory errors, specifically during the Acquisition period [$F(1,19)=4.560$, $p<.05$]. However, they performed comparably to WTs during the Retention period of the task [$F(1,19)=.257$, $p>0.05$] (Fig. 2.3e). Another one-way ANOVA was conducted to look at differences between genotypes at each day [Day 1: $F(1,19)=.249$, $p>0.05$; Day 2: $F(1,19)=.573$, $p>0.05$; Day 3: $F(1,19)=2.556$, $p>0.05$; Day 4: $F(1,19)=.782$, $p>0.05$; Day 5: $F(1,19)=3.243$, $p<0.10$; Day 6: $F(1,19)=5.202$, $p<0.05$; Day 7: $F(1,19)=1.003$, $p>0.05$; Day 8: $F(1,19)=3.403$, $p<0.10$; Day 9: $F(1,19)=.019$, $p>0.05$; Day 10: $F(1,19)=.417$, $p>0.05$; Day 11: $F(1,19)=.860$, $p>0.05$; Day 12: $F(1,19)=.001$, $p>0.05$; Day 13: $F(1,19)=.310$, $p>0.05$; Day 14: $F(1,19)=.139$, $p>0.05$].

2.4.1.5 Latency

Total latency across the 4 trials was computed, and a repeated measures ANOVA was performed to analyze Genotype and Day differences (as above). This revealed no significant difference of total latency to the platform during testing sessions, when comparing *Cntnap2* KOs and WTs [$F(1,19) = 2.842$, $p>0.05$]. There was, however, a main effect of Day, indicating both groups were completing the task more quickly as testing progressed (Fig. 2.4a).

2.4.1.6 Average turn angle

Average turn angle per testing session was recorded and analyzed to assess possible differences in strategies used to complete the task. A repeated measures ANOVA revealed no main effect of Genotype [$F(1,19)=.343$, $p>0.05$], but did reveal a significant Day effect [$F(13,246)=2.856$, $p<.05$]. Overall, subjects used shorter turn angles during the beginning of testing, but as testing continued, subjects used wider turn angles (indicating more selective arm choices; Fig. 2.4b).

2.5 Discussion

Cntnap2 KO and wild-type mice were tested on a 4/8 radial arm water maze for 14 consecutive days. Results showed that *Cntnap2* KO mice exhibited significant deficits in spatial working and reference memory as indicated by higher numbers of errors, specifically during the acquisition period of the task. However, during the retention period (i.e., after an asymptote in errors), *Cntnap2* KO mice performed comparably to wild-type mice. These findings indicate that the mutant animals are able to learn, but exhibit delayed learning -- resulting in a different learning curve. It is important to note the differences between *Cntnap2* KOs and WTs are particularly robust on days 5 through 8. This likely reflects the floor effect that occurs the first few days of testing, when none of the animals perform substantially better than chance. During day 5-8, however (when WTs begin to learn and improve on the task), *Cntnap2* KOs do not

appear to learn as quickly as WTs, and continue to exhibit relatively high number of errors during this time period. Importantly, KOs do display some improvement, but not as robust as WTs. This would suggest that once the *Cntnap2* KOs begin to learn the platform location they are perseverating on these locations within a testing session. As *Cntnap2* KOs are learning the platform locations, they struggling learning which arms were already visited during the testing session. Furthermore, *Cntnap2* KO mice and WT mice displayed similar turn angles throughout testing, suggesting they used similar strategies to complete the maze. That is, as testing proceeded, wider turn angles were noted, indicating subjects used more of a spatial strategy and less chaining to find a platform. These findings were likely due to the difficulty of the task used in this study, based on prior findings that failed to show a *Cntnap2* deficit when compared to WTs on a simple MWM learning task (Peñagarikano et al., 2011).

Current findings are consistent with deficits in executive learning as demonstrated in ASD (Ozonoff, Pennington & Rogers, 1991; Rosenberg et al., 2009; Hill, 2004). Moreover, our findings may further explain the dyad of core symptoms, given the central role of executive processing in both higher and lower levels of processing. That is, the global connectivity deficiency seen in ASD could contribute to the spatial working memory and learning impairments observed here. This pattern has also been seen in neuroimaging studies with high functioning ASD participants (Di Martino et al., 2014). This disconnection may result in problems with sensory integration, and therefore thereby learning. This could explain why *Cntnap2* KOs require more experience to effectively learn the maze, as compared to their WT controls.

The impairments observed in the current study also may be explained by the abnormal myelin formation seen in this transgenic mouse model, consistent with the hypo-connectivity

theory of the neurobiology of ASD, as well as the spatial learning deficits seen in ASD. Future studies are planned to look into neuroanatomical differences in white matter tracks spanning cortical regions, and correlate these measures to the cognitive differences seen here (using anatomy from these same subjects). Overall, these behavioral findings suggest that *CNTNAP2* plays a clear underlying role in the development of neural systems important to learning and cross-modal integration, and disruption of this function could be associated with delayed learning observed in individuals with ASD. Future studies should investigate heterozygous mice performance as well, since this would be relevant due to the presence of heterozygous mutations in the clinical population.

Acknowledgments

This work was supported in part by NSF IGERT Grant 1144399 (J. Magnuson, PI) and by the National Institutes of Health grant P01HD057853 to R.H. Fitch.

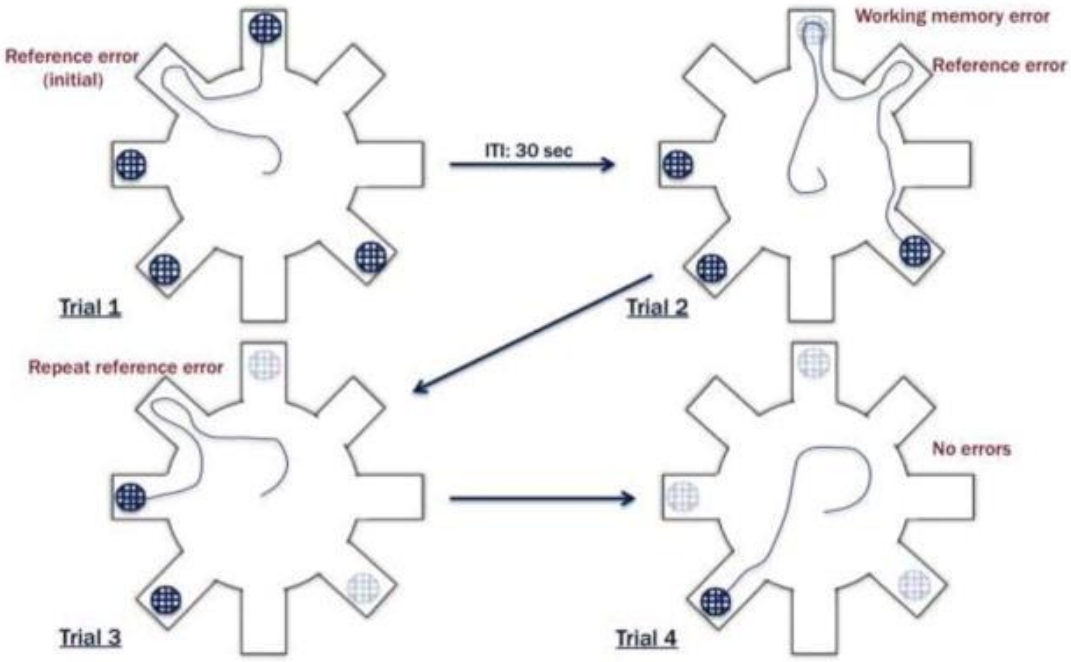

Figure 2.1. A schematic of the 4/8 radial arm maze and the categorization of memory errors used to evaluate all subjects.

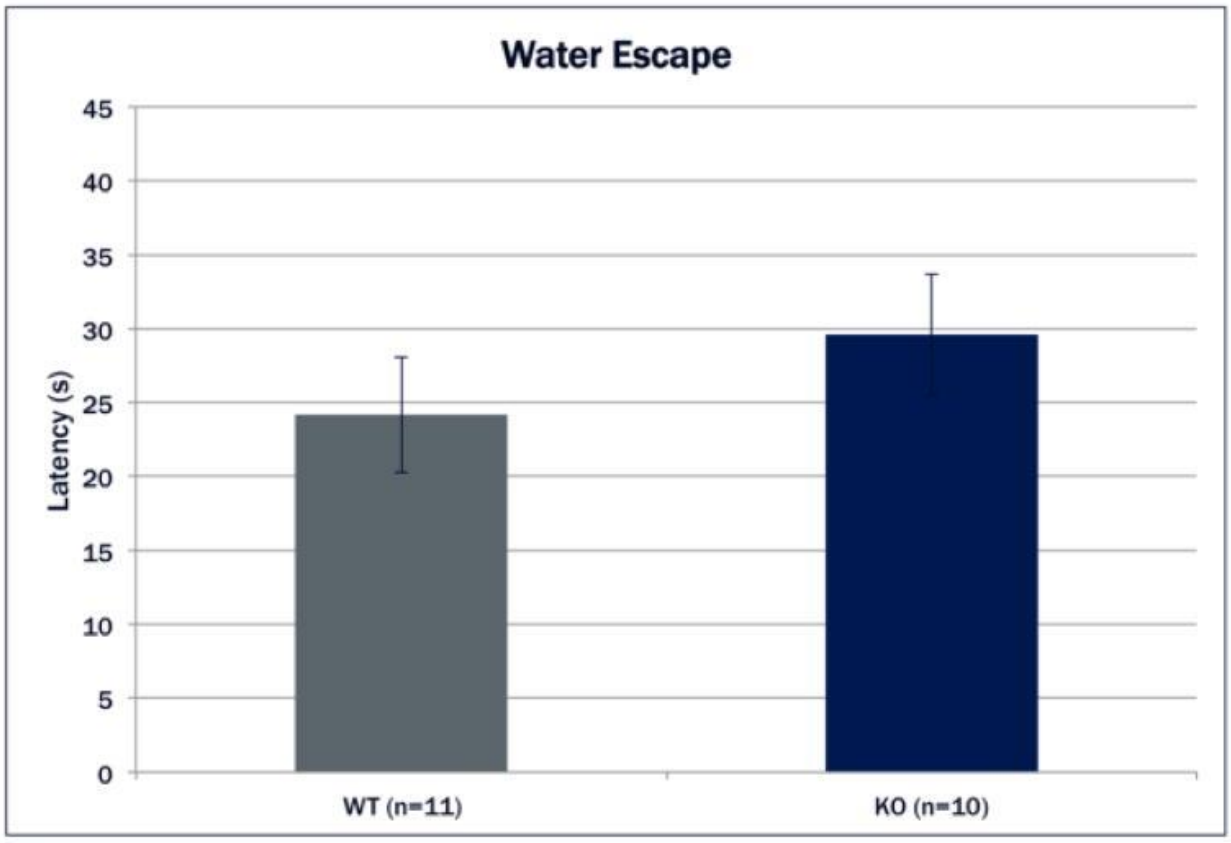

Figure 2.2. Water escape performance in Cntnap2 KO mice. Mean latency to platform in the water escape task (+SEM). There were no significant differences between Genotypes on latency to platform indicating no underlying motor or visual impairments.

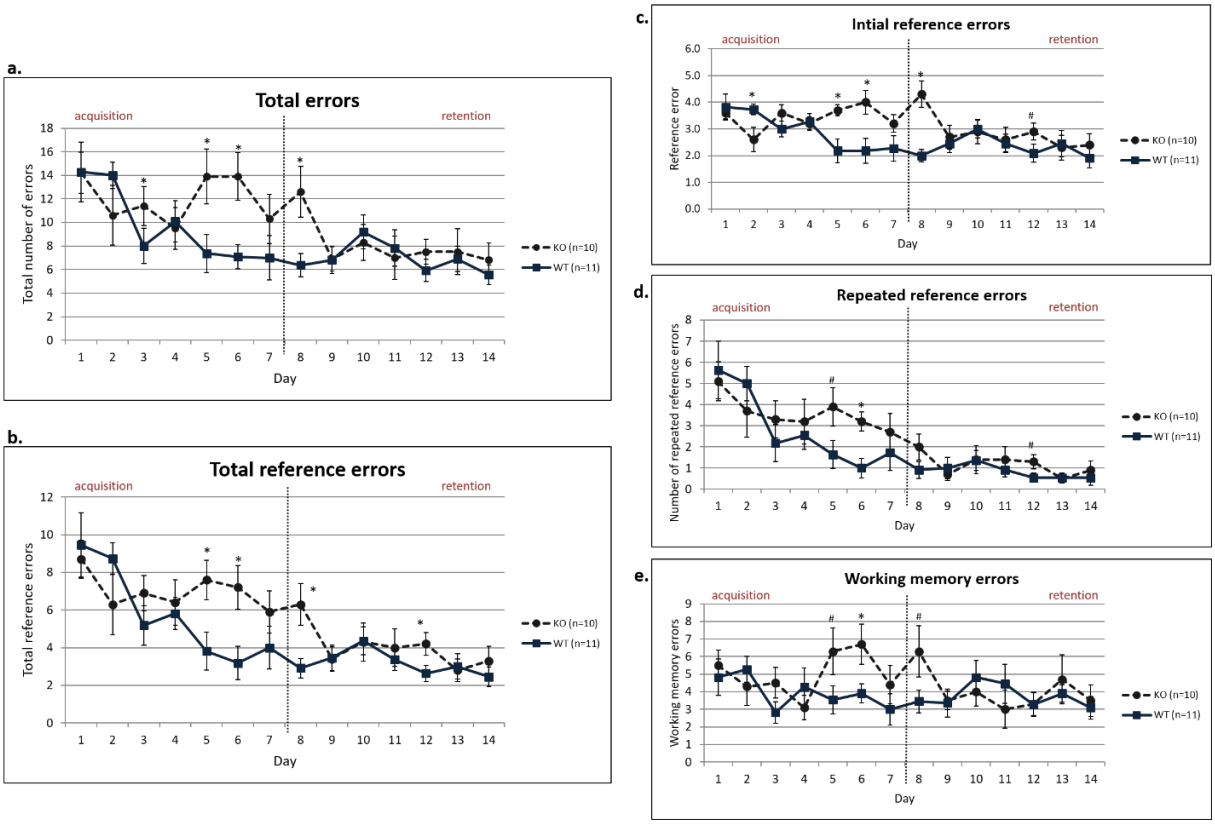

Figure 2.3 4/8 radial arm maze performance in *Cntnap2* KO mice. (**A**) Total number of errors in the 4/8 arm radial water maze task over 14 days of testing (+SEM). Analysis of the average number of total errors (working memory, initial reference, and repeated reference memory errors) revealed a significant difference between WT and *Cntnap2* KO groups, with *Cntnap2* KOs making more errors. (**B**) Total number of reference memory errors (+SEM) in the 4/8 arm radial water maze task over 14 days of testing. Analyses revealed that *Cntnap2* KOs did make significantly more errors than WT subjects. (**C**) Total number of initial reference memory errors (+SEM) in the 4/8 arm radial water maze task over 14 days of testing. *Cntnap2* KOs made significantly more initial reference memory errors. (**D**) Total number of repeated reference memory errors (+SEM) in the 4/8 arm radial water maze task over 14 days of testing. *Cntnap2* KOs made more repeated reference memory errors across the 14 days of testing but overall

performance was comparable between KOs and WTs. (**E**) Total number of working memory errors (+SEM) in the 4/8 arm radial water maze task over 14 days of testing. *Cntnap2* KOs made significantly more working memory errors specifically during the Acquisition period. * $p<.05$, # $p<.10$.

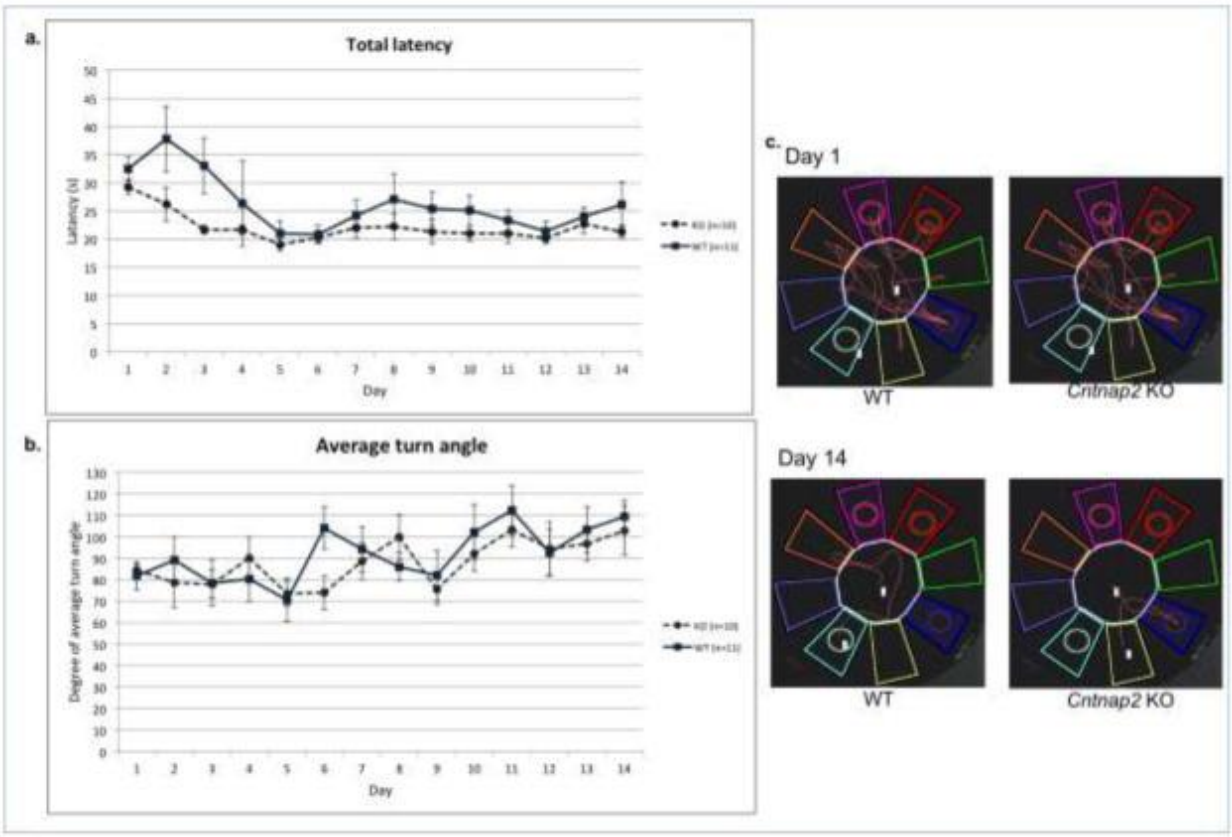

Figure 2.4. Latency and turn angles on 4/8 radial arm maze in *Cntnap2* KO mice. (**A**) Total latency (+SEM) over testing sessions in the 4/8 arm radial water maze task over 14 days of testing. There was no significant difference of total latency to the platform during testing sessions, when comparing *Cntnap2* KOs and WTs. (**B**) Average turn angle (+SEM) over testing sessions in the 4/8 arm radial water maze task over 14 days of testing. There were no significant differences between Genotypes. (**C**) Examples of swimming tracks from a WT and *Cntnap2* KO mouse on testing day 1 and 14.

Chapter 2 Addendum

As our initial aim, we sought to behaviorally characterize *Cntnap2* KO mice on various tasks with a primary focus on learning and memory. Here, we present additional data that was not included in the manuscript. *Cntnap2* KO mice were also evaluated on rotarod, novel object recognition, social dominance tube task as well as vocalizations during male-female interactions. A repeated measures ANOVA did not reveal a main effect of Genotype on rotarod [$F(1,19) = 2.141$, $p>.05$], indicating that there was no overall difference in performance on the rotarod (and hence sensorimotor function) between the wild-type and mutant groups. Analysis of novel object recognition was conducted using a univariate ANOVA. This revealed no main effect of Genotype [$F(1,19) = .269$, $p> .05$] with exploratory preference and object recognition being comparable between *Cntnap2* KOs and WTs. Social dominance (tube task) was conducted to evaluate social dominance. Number of wins was calculated as a percentage of the 4 trials. A univariate ANOVA revealed there was a main effect of Genotype [$F(1,19) = 48.245$, $p<.01$]. *Cntnap2* KO mice had significantly more losses on this task compared to their wild-type controls, suggesting they are more passive. A univariate ANOVA was conducted to evaluate time spent vocalizing for each minute spent interacting with a female mouse and revealed no main effect of Genotype [$F(1,14) = .409$, $p>.05$]. We did not observe any differences on sensorimotor motor coordination as well as novel object recognition or vocalizations. We did, however, observe differences in a social dominance task where *Cntnap2* KO mice expressed more passive behavior. This more extensive behavioral characterization increases the scope of our conclusions on the role of *CNTNAP2* in behavior and cognition.

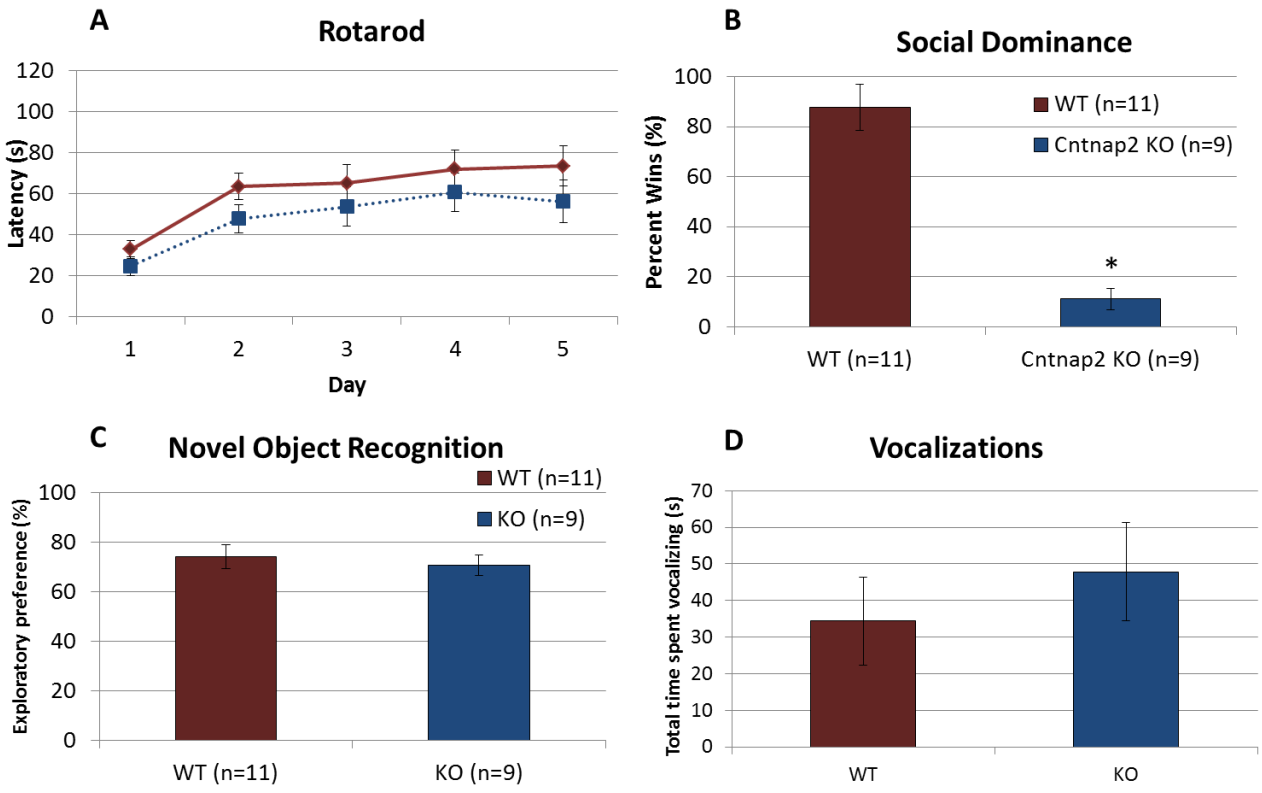

Figure 2.1.Supplemental – Additional behavioral tasks evaluating *Cntnap2* KO mice. (**A**) Rotarod performance across 5 days. (**B**) Percent wins on social dominance tube task across 4 trials. (**C**) Novel object recognition after a 5-minute delay. (**D**) Total time spent vocalizing during a 5-minute male-female interaction.

CHAPTER 3

Deficits in learning and memory in mice with a mutation of the candidate dyslexia susceptibility gene *Dyx1c1*

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Published: Brain and Language (2015), doi: 10.1016/j.bandl.2015.04.008

3.1 Abstract

Dyslexia is a learning disability characterized by difficulty learning to read and write. The underlying biological and genetic etiology remains poorly understood. One candidate gene, dyslexia susceptibility 1 candidate 1 (*DYX1C1),* has been shown to be associated with deficits in short-term memory in dyslexic populations. The purpose of the current study was to examine the behavioral phenotype of a mouse model with a homozygous conditional (forebrain) knockout of the rodent homolog *Dyx1c1*. Twelve *Dyx1c1* conditional homozygous knockouts, 7 *Dyx1c1* conditional heterozygous knockouts and 6 wild-type controls were behaviorally assessed. Mice with the homozygous *Dyx1c1* knockout showed deficits on memory and learning, but not on auditory or motor tasks. These findings affirm existing evidence that *DYX1C1* may play an underlying role in the development of neural systems important to learning and memory, and disruption of this function could contribute to the learning deficits seen in individuals with dyslexia.

3.2 Introduction

Developmental dyslexia is a heritable learning disability defined by difficulties in learning to read and write that cannot be explained by factors such as intellectual impairment, lack of educational opportunity or other co-morbid neurological disorders (e.g., epilepsy, or primary sensory impairments (blindness, deafness)). Dyslexia can be deconstructed into underlying core components, known as "intermediate phenotypes". These include deficits in phonological processing (Kovelman et al., 2012; Melby-Larvag, Lyster & Hulme, 2012; Peyrin et al., 2012), short-term and/or working memory (Beneventi et al., 2010; Gathercole et al., 2006; Menghini et al., 2010), visuospatial attention (Franceschini, 2012; Gabrieli & Norton, 2012), and rapid auditory processing (Cohen-Mimran & Sapir, 2007; Fitch & Szalkowski, 2012; Hamalainen, Salminen & Leppanen, 2013). Other behavioral deficits that have been specifically associated with dyslexia include naming speed, motor functioning and visual processing of motion (Denckla & Rudel, 1976; Liao et al., 2015; Capellini, Coppede & Valle, 2010; Olulade, Napeliello & Eden, 2013). Dyslexia is also encompassed by the term "specific developmental reading disability," although reading disability is generally regarded as a more inclusive term, capturing comprehension deficits that would not usually be classified as dyslexia (Snowling & Hulme, 2012). The symptomology for both dyslexia and reading disability is heterogeneous, and the biological mechanisms underlying associated intermediate phenotypes remain poorly understood.

What we do know of the biological and genetic etiology of dyslexia/reading disability indicates strong but complex genetic and environmental influences, with heritability estimates ranging from 40% to as high as 80% (Schumacher, Hoffmann, Schmal, Schulte-Korne, & Nothen, 2007). Not surprisingly, multiple genes have been implicated as contributing to this

disorder, much as seen for other complex disorders such as autism and schizophrenia (Gelernter, 2015). Although the neurobiological mechanisms underlying dyslexia/reading disability are not yet fully understood, most of the risk genes identified to date appear to be involved in surprisingly similar biological mechanisms (i.e., neuronal migration and cilia function; Kere, 2014). These identified genetic and neurobiological mechanisms in turn contribute to establishing the complex neurocircuitry that may subserve abilities such as phonological and visual processing, as well as learning. Disruptions in this neurocircuitry could result in impairments that are associated disorders of language and reading functions.

The first candidate risk gene to be reported was dyslexia susceptibility 1 candidate 1 (*DYX1C1*), a gene identified in two Finnish families with a history of dyslexia (Nopola-Hemmi et al., 2000). *DYX1C1* was further supported as a candidate risk gene in 2003 (Taipale et al.,2003), although there have been inconsistent findings in clinical populations (Cope et al., 2005; Marino et al., 2005; and Meng et al., 2005). Importantly, some reports have shown that *DYX1C1* variants are specifically associated with core component features of dyslexia, including deficits in verbal short-term memory (Marino et al. 2007), short-term memory (Dahdouh, 2009), and orthographic choice tasks and non-word reading (Bates, 2009). These previous studies have provided evidence of *DYX1C1* variants being explicitly linked to memory deficits in some language-impaired populations.

With regard to the biological role of *DYX1C1,* this gene has been shown to be active in neuronal migration in the developing cortex, as well as more generalized cilia function (Wang et al., 2006; Tarkar et al., 2013). These appear to be recurrent biological "themes" in the etiology of dyslexia. For example, animal models using *in utero* RNA interference (RNAi) against the rat homolog *Dyx1c1* showed disruptions of neuronal migration in the developing neocortex, thus

supporting a role for *DYX1C1* in neuronal migration (Wang et al., 2006). Migration anomalies in the neocortex have also been associated with dyslexia in clinical populations, as evidenced by cortical malformations indicative of early migration disturbances (Galaburda et al., 1985, Chang et al., 2005). Rodent models for these types of developmental cortical malformations have been used to evaluate rapid auditory processing and working memory, since these are considered intermediate phenotypes of developmental dyslexia, and yet are accessible to rodent evaluations. Researchers found deficits in both rats with induced cortical malformations, and also rats with knockdown of *Dyx1c1*, in complex acoustic processing (Threlkeld *et al.* 2007; Threlkeld et al., 2009). Working memory abilities were also assessed in the *Dyx1c1* RNAi model, and significant impairments were noted for this core phenotype as well (Szalkowski et al., 2011). More recently, a mouse knockout model of *Dyx1c1* was generated that demonstrated cilia defects, as well as severe embryonic lethality of approximately two-thirds of homozygous mutants. Homozygous constitutive mutants that survived after birth developed severe hydrocephalus by postnatal day P16, and died by P21 (Tarkar et al., 2013). This mouse model obviously could not be used for extensive behavioral profiling, and a conditional forebrain *Dyx1c1* knockout model was developed.

The current study was designed to further examine the behavioral features of this conditional preparation, using male mice with both homozygous and heterozygous conditional (forebrain) knockout of rodent homolog *Dyx1c1*. A forebrain conditional knockout model was chosen in part based on evidence of malformations in neocortex as well as hippocampus in the RNAi *Dyx1c1* model (Rosen et al., 2007). These abnormalities in the neocortical and hippocampal regions resulted from disruption of neuronal migration, and have been observed in individuals with dyslexia (Galaburda et al., 1985). We hypothesized these differences in

underlying neuroanatomy may contribute to deficits in learning and memory reported in individuals with dyslexia. A forebrain conditional was also selected to avoid the lethal hydrocephaly observed in the systemic KO. Adult male littermates (generated through het x het breedings) were assessed on various behavioral paradigms that have been validated in our lab as effectively tapping core functions implicated in language and reading impairments. These tasks include auditory processing, and working and reference memory. Based on previous research and clinical evidence, we hypothesized that animals with homozygous conditional knockout of *Dyx1c1* would show specific acoustic processing and/or memory impairments, while performance on other behavioral assessments (e.g., gross motor learning, pre-pulse inhibition, water escape) would not differ from matched wild-types.

3.3 Methods and Materials

3.3.1 Subjects

Mice carrying the *loxP*– exon 2–4–*loxP* conditional allele of *Dyx1c1* (*Dyx1c1*^{flox}) were generated by the University of Connecticut Health Center Gene Targeting and Transgenic Facility, as described previously (Tarkar et al., 2013). Briefly, embryonic stem cells harboring a *loxP*-flanked allele of exons 2–4 of *Dyx1c1* were produced by electroporating mouse embryonic stem (ES) cells (129S6) with a targeting construct designed to replace exons 2–4 and flanking intronic sequence through homologous recombination. After PCR screening of the ES cell clones for correctly targeted colonies, a single positive colony was expanded, and chimeric mice were generated by embryo reaggregation. The animals were crossed with C57BL/6J mice and transmitted the targeted allele to the offspring through germ line. These mice were subsequently crossed with *129S4/SvJaeSor-Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$*/J* mice (The Jackson Laboratory) to remove the *PGK-Neo* cassette in the targeting construct and the offspring thus produced were

used to generate *Dyx1c1*$^{flox/flox}$ mice colony. These mice were genotyped by PCR using three pairs of primers (PL452F-5'-CGAAGTTATTAGGTCCCTCG-3' and loxgtR 5'-TGAGCACCCTGCTTCTACCT-3'; loxwtF 5'- AAAACCAACCATCCAACCAA-3' and loxgtR 5'-TGAGCACCCTGCTTCTACCT-3'; FrtgtF 5'-TAGGGATTCACCGTCACACA-3' and FrtgtR 5'-AACCAAGTCCAAGGCCTTCT-3').

To generate the conditional forebrain knockout (*Dyx1c1*$^{flox/flox}$*/Emx-Cre*$^{+/+}$*),* -- mice with a deletion of exons 2-4 only in the forebrain -- we crossed *Dyx1c1*$^{flox/flox}$ mice with Emx1-IRES-Cre knockin mice, B6.129-*Emx1*$^{tm1(cre)Krj}$/J (The Jackson Laboratory).). Emx1-IRES-Cre strain expresses Cre recombinase enzyme from the endogenous *Emx1* locus, and when crossed with loxP-site containing *Dyx1c1* sequence, leads to recombination in approximately 88% of neocortical neurons as well as hippocampus. The heterozygous offspring (*Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$*)* generated from this cross were then inbred to create the experimental forebrain conditional knockout (*Dyx1c1*$^{flox/flox}$*/Emx-Cre*$^{+/+}$*)* mice and the control (*Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$*, Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/+}$*, Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/+}$ and *Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/-}$*)* mice. The knockout (KO) group was comprised of 12 animals with the genotype *Dyx1c1*$^{flox/flox}$*/Emx-Cre,*$^{+/+}$ while the heterozygous (HT) group was comprised of 6 subjects with either *Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/-}$ or *Dyx1c1*$^{flox/wt}$*/Emx-Cre*$^{+/+}$genotypes. Lastly, the wild type (WT) group consisted of 7 subjects with *Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/+}$*or Dyx1c1*$^{wt/wt}$*/Emx-Cre*$^{+/-}$ genotypes. Genotyping was performed by PCR for *Dyx1c1*$^{flox/flox}$ using the primers described above. The mice were genotyped for EMX-Cre using two pairs of primers *(*oIMR1084 5'- GCG GTC TGG CAG TAA AAA CTA TC-3'and oIMR085 5'- GTG AAA CAG CAT TGC TGT CAC TT-3'; oIMR4170 5'- AAG GTG TGG TTC CAG AAT CG-3' and oIMR4171 5'- CTC TCC ACC AGA AGG CTG AG-3').

All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water *ad libitum,* and all behavioral testing occurred during the light cycle. At the start of testing, animals were between the ages of postnatal day (P) 61 - P75. Procedures were performed blind to Genotype (ascertained at weaning by tail-snip PCR), and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

3.3.2 Anatomy

Finally, it is important to note that in contrast to the systemic KO preparation that exhibited lethal hydrocephaly (Tarkar et al., 2013), the forebrain conditional preparation has been shown *not* to exhibit any gross neurologic abnormalities in the cortex or hippocampus (homozygous or heterozygous), nor to exhibit any gross behavioral anomalies. To confirm these and other prior characterizations of the conditional model, a western blot was performed to examine the *Dyx1c1* protein expression in the forebrain KO and the control conditions. Additionally, we included the constitutive KO and WT protein lysates as a control for the experiment. We further investigated the lamination of the cortex obtained from the forebrain knockout using the neocortical layer marker Ctip2 and Cux1. We measured the depth of each layer of the cortex marked by Ctip2 and Cux1, and we further normalized the measurement with the total cortical depth to compare the thickness of the Ctip2 and Cux1 positive neuron-containing cortex.

3.3.3 Rotarod (P124 to P129)

All subjects were assessed at age P124 for sensorimotor ability and motor learning using the rotarod task. Subjects were placed on a rotating cylindrical drum that gradually accelerated from 4 to 40 rotations per minute across a span of 2 minutes. Four trials were administered per test

day, across four consecutive days. For analysis, latency to fall from the rotating drum was measured and averaged across the four trials for each day.

3.3.4 Auditory Processing (P68 to P98)

All subjects were assessed for auditory processing ability using a modified pre-pulse inhibition paradigm (see Fitch et al., 2008 for review). Subjects were placed on individual load-cell platforms (Med Associates, St. Albans, VT) and presented with auditory stimuli generated using RPvdsEx on a Dell Pentium D PC and RZ6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Sounds were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The acoustic startle reflex (ASR; a reflexive response elicited by an unexpected, intense stimulus) was recorded by an iMac 7.1 running Acknowledge 4.1, and obtained via the voltage output from each load cell platform through a linear amplifier (PHM-250U; Med Associates, St. Albans, VT) connected to a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA). The modified pre-pulse inhibition paradigm measured differences in ASR to a loud startle-eliciting stimulus (SES; 105dB, 50 ms, broadband white noise burst (1kHz-10kHz)) when presented with or without a preceding acoustic cue. The ASR difference on cued versus uncued trials provided a measure of cue detection and/or discrimination. If the auditory cue was detected, a reduction (attenuation) in the ASR was expected relative to the ASR elicited when the auditory cue was not present (or not detected). This phenomenon was quantified using an "attenuation score" (ATT) that compared the average amplitude of the ASR from the cued trial to the average ASR of the uncued trial ([average cued ASR/average uncued ASR]*100).

3.3.4.1 Normal Single Tone P68

Prior to more complex auditory testing, all animals were tested on a Normal Single Tone (NST) to measure baseline pre-pulse inhibition and auditory ability (P68). This auditory PPI control task was used to establish whether subjects exhibited hearing deficits and/or impaired gross motor reflexes that could confound other auditory PPI tests, and provided an index of baseline auditory pre-pulse inhibition ability across test groups. Testing sessions consisted of 104 pseudorandomly presented cued and uncued trials at inter-trial intervals (ITI) of varying durations (16–24 s). The task comprised of a silent background and a simple single tone cue (50 ms, 75 dB, 8,000 Hz tone) presented 50 ms prior to the 50 ms, 105 dB. All subjects were able to perform this task and therefore were used for further behavioral evaluation.

3.3.4.2 Embedded Tone (P71 to P83)

First, the variable duration Embedded Tone (EBT) task was administered (with 300 sequential pseudorandom trials). This task assessed ability to detect a change in tone frequency relative to a standard background tone (cue was a variable duration 5.6 kHz pure tone embedded in a 10.5 kHz background pure tone). On cued trials, the cue was presented 100 ms before the SES, while uncued trials used a "cue" of 0 ms. Two EBT tasks were used in this study – a long-duration EBT (0 ms to 100 ms), and a short-duration EBT (0 ms to 10 ms). A range of cue durations were used to evaluate specific thresholds for performance differences between the various genotypes, based on a hypothesis that subjects may perform comparably on longer durations yet may differ on shorter durations (which are more difficult to detect). Using a range of cue durations enables ascertainment of stimulus features that all animals can discriminate (ceiling), that no animals can discriminate (basement), as well as any group differences in the mid-range. Both EBT tasks were administered for five consecutive days, starting at P71 until P83.

3.3.4.3 Silent Gap (P86 to P98)

Next, a Silent Gap (SG) task was used to assess the ability to detect silent breaks in continuous white noise (P86 to P98). A session included 300 trials with a continuous 75 dB broadband white noise background. Cued and uncued trials occurred pseudorandomly. On cued trials, a silent gap of variable duration (0-100 ms) was presented 100 ms before the SES, with "0 ms" trials serving as the uncued condition. Subjects were tested on the Silent Gap task for five consecutive days, for each version of the task.

3.3.5 Water Maze Assessment

3.3.5.1 Water Escape (P132)

Subjects were initially tested on a water escape task prior to the 4/8 arm radial water maze task, to evaluate the presence of any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm x 55.5 cm) filled with room temperature water, and were given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) located at the opposite end of the tub. Latencies to reach the visible platform were recorded for assessment. None of the subjects displayed any impairments on this task (the subject code was used to analyze this data by an investigator who was not conducting testing). Therefore, we proceeded to implement a water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998).

3.3.5.2 4/8-arm radial water maze (P133 to P148)

This task was used to assess spatial reference and working memory ability simultaneously, using a standard 8 arm radial maze with 4 arms baited (i.e., containing a submerged goal platform), and 4 arms open but never baited with a platform. Configuration of goal arms were counterbalanced between subjects but remained fixed per subject across all test sessions. Additionally, high

contrast extra-maze cues were present, and the locations of these remained static for the entire experiment.

The day prior to testing (Day 1), subjects were given a training session where all arms that would not contain a platform were blocked, forcing the animals to only enter arms containing a platform. Subjects were placed in the start arm and given 120 seconds to locate a platform. Every subject completed 4 training trials, and each time they found a platform, that platform was removed and the entrance to that arm was blocked. This ensured that the subject could no longer enter this arm for the remainder of the training session. If the subject failed to find a platform in this window, they were guided to the nearest available goal. Once on the platform, subjects remained for 20 seconds and then were removed from the maze to their home cage (30 second inter-trial interval; ITI).

Testing began on Day 2 and continued for an additional 14 consecutive days. Here, instead of blocking the goal arm of the most recently located platform, the platform was simply removed during the 30 second ITI, but the arm remained open and unbaited for the remainder of the test session. Animals were required to locate all 4 platforms, and thus received 4 test trials per day. Test sessions were recorded using a Sony camera integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). Latency and path distance were recorded and in addition, subjects were given a point for each completed trial (i.e., successful location of a platform in 120 sec), with a maximum of 4 points per test session. All scores were recorded and used for further analysis.

3.3.6 Novel Object Recognition (P162)

One day prior to testing, all subjects were habituated to the testing chamber (40 cm × 24 cm × 20 cm plexiglas tub with opaque walls) for 10 minutes. On testing day, each subject underwent a

habituation phase of 5 minutes to reduce stress and the chance of a neophobic response, and to promote exploratory activity in the test phase. The subject was given a 1-minute resting period in their home cage, and was then exposed to two identical Lego configurations for 5 minutes. This constituted the "familiarization" session. Afterwards, the subjects were given a short delay period of 5 minutes (resting in their home cage). Then, they were introduced to the testing chamber containing a new object and one familiar object (different Lego configurations) for 3 minutes ("test" session). Anytime a mouse sniffed the object or touched the object while looking at it (i.e., when the distance between the nose and the object was less than 2 cm) this behavior was scored as exploratory. Total time spent exploring, and percent time spent with the familiar vs. novel object, were evaluated and analyzed.

3.3.7 Modified T-Maze (P225 to P268)

Subjects were gradually food restricted to 80%-85% of their baseline body weight (3 weeks). During the last week before training, subjects were given a sample of the food reward in their home cage, to habituate them to its taste and eliminate hyponeophagia. Animals were then introduced to the modified T-maze (30 cm x 10 cm start arm; 30 cm x 10 cm goal arm). This configuration included curved arms, to eliminate visual or olfactory cues of the food reward at the end of the arm. The left and right arms were high contrast colors - - the left arm was black, and the right arm was white. Prior to rewarded alternation testing, animals were placed in the start arm with both arms opened and containing food wells (with food reward) for about 3 min. Any time an animal consumed the reward, a food well was replenished. This was performed four times, with intervals between exposures of at least 10 min.

During the training period, a subject was placed in the start arm with one of the goal arms blocked while the other arm remained opened, forcing the subject to enter the open arm. Multiple

trials were administered in a daily session, with equal numbers of left and right arms serving as the open arm. When the animal completed a forced trial by entering the open arm and consuming the reward, the blocked arm's door was removed, and the animal was placed back into the start arm. This process trained the animal to learn that once when it entered an arm and consumed the reward, there would no longer be a reward at that location, so they must visit a new arm for reward. Now the animal was placed again in the start arm facing away from the two goal arms, and was permitted to make a choice between the two opened arms. If the animal chose correctly, they were allowed time to consume the reward. If the subject chose incorrectly, they were removed (after the experimenter ensured the subject adequately explored the empty well).

Once the animals were habituated and trained, ten trials were given in a daily session. Each one of a squad of approximately 8 mice received a trial in succession before the first animal started its next trial. The identity of the sample goal arm for each trial was determined by random sequence. The maximum number of consecutive identical arms was three, as a precaution against development of temporary position habits or reinforced perservation. A percentage reflecting correct trials completed per animal was calculated and evaluated (Deacons & Rawlins, 2006).

3.3.8 Statistical analysis

All behavioral data was analyzed using a mixed factorial design. All subjects were used for analysis (WT, n = 7; HT, n = 6; KO, n = 12). Group differences in rotarod performance were analyzed using a repeated measures ANOVA with Genotype (3 levels: WT, HT and KO) as the between measure, and Day (4 levels) as the within measure. A one-way between subjects ANOVA was conducted to compare the effect of Genotype on average Attenuation Scores on all auditory processing tasks. This included: EBT 0-100 ms, EBT 0-10 ms, SG 0-300 ms and SG 0-

100 ms. Total number of completed trials on the 4/8 radial arm maze were independently examined using a 3 x 14 repeated measures ANOVA, with Genotype (3 levels: WT, HT and KO) as the between measure, and Day (14 levels) as the within measure. Finally, a univariate ANOVA was performed to analyze group differences on total time of exploration on the novel object recognition task, as well as accuracy (% trials correct) on the modified T-maze task. All statistical analyses were conducted using SPSS 19 with an alpha criterion of 0.05, two-tailed.

3.4 Results

3.4.1 Anatomy

Western blot results showed that *Dyx1c1* protein expression was reduced by 77% in the forebrain KO condition as compared to the control condition (Fig. 3.1). Since we know that Emx1-IRES-Cre strain expresses Cre recombinase enzyme from the endogenous *Emx1* locus that (when crossed with loxP-site containing *Dyx1c1* sequence) leads to recombination in approximately 88% of neocortical neurons as well as hippocampus, the slightly higher *Dyx1c1* expression observed in the forebrain knockout could be attributed to the other cell types in the neocortex and hippocampus (e.g. interneurons (20%)), as well as vasculature. We also investigated the motility of the cilia lining the lateral ventricles of the forebrain KO and the control using videomicroscopy, and found that the KO cilia were immotile -- consistent with the previously identified phenotype of ciliary immotility in the *Dyx1c1* ubiquitous KO. Finally, we found no significant differences in the cortical lamination patterning, as evident from the Cux1 and Ctip2 staining in the forebrain conditional knockout compared to controls. We also found no observable anomalies in the cortex of the conditional forebrain knockout animals compared to controls (Fig. 3.2).

3.4.2 Auditory Processing

All subjects were initially tested on a normal single tone (NST) task, to establish baseline hearing and PPI ability. None of the subjects showed impairments on NST, nor was there a main effect of Genotype [$F(2,22) = 2.196$, N.S.]. Therefore subjects were advanced to subsequent levels of more complex acoustic tasks. On the embedded tone (EBT) 0-100 ms task, we found no main effect of Genotype [$F(1,22) = .419$, N.S.]. Moreover, all subjects were able to discriminate the stimuli, based on cued/uncued amplitude comparisons. Subjects were then tested on a more difficult embedded tone task, using cue durations ranging from 0 to 10 ms (where 0 ms is the uncued condition). Again, we found no main effect of Genotype [$F(1,22) = 3.110$, N.S.], and all subjects showed significant discrimination of the cues (particularly longer gaps). Next, auditory processing ability was evaluated on a silent gap detection task. First, we administered the silent gap 0-300 ms task, and again saw no main effect of Genotype [$F(1,22) = .532$, N.S.]. Subjects overall performed well on the task, and therefore we advanced them to the more difficult version of the task with silent gap durations ranging from 0 to 100 ms. Overall performance was poor on the silent gap 0-100 ms task and there was again no main effect of Genotype [$F(1,22) = .591$, N.S.].

Finally, for each of the auditory tasks, we found that all 3 groups of animals showed significant discrimination of the cue, based on t-test comparisons between mean cued and uncued values within groups. The exception was on the hardest task (silent gap 0-100 ms), where *all* animals performed poorly (mean attenuation scores approximately 85%, and as high as 100% for the 2 ms cue). Comparing across groups, we found no significant effects of Genotype on any task, confirming that subjects performed comparably on all tasks (Fig. 3.4).

3.4.3 Water Maze Assessment

Prior to spatial water maze testing, a visible platform control task was conducted to assess for underlying impairments that could confound subsequent water maze performance (e.g., impairments in swimming ability, visual acuity, or motivation). A univariate ANOVA on latencies found no main effect of Genotype [$F(2,22)=.126$, N.S.], indicating that genetically modified groups had no impairments on underlying aspects of the water task (e.g., swimming) (Fig. 3.5).

The 4/8 radial-arm water maze was then used to assess spatial working and reference memory ability. Animals were scored on errors and latencies, as well as their ability to complete this task (i.e., successfully locate a platform). Thus for every trial successfully completed, subjects received a point. We did find a main effect of Genotype on the number of trials successfully completed [$F(2,22)=7.518$, $p<.01$]. We also found a significant Day by Genotype interaction [$F(26,286)=4.887$, $p<.001$], with KOs continuing to drop in number of trials successfully completed as testing progressed (Fig. 3.6). Specifically, KOs performed comparably to WTs and HTs during the first few days of testing, with significant differences emerging by Day 5 [$F(2,22)=4.148$, $p<.05$].

3.4.4 Novel Object Recognition

Subjects were tested on a novel object recognition task, to assess both exploration and short-term memory abilities. We found a main effect of Genotype on total time of exploration, with KO animals exploring substantially less [$F(2,22) = 8.613$, $p<.01$]. As a result, KO animals did not meet criteria to evaluate novel object recognition ability (Fig. 3.7).

3.4.5 Modified T-maze

Lastly all subjects were evaluated on a modified T-maze involving a rewarded alternation task. However, 3 subjects were dropped from the analysis (1 WT, 1 HT and 1 KO) since they did not perform the task during the testing period. The percentage of correct responses as a function of total trials completed was calculated for each subject, and a univariate ANOVA did reveal significant group differences. There was a main effect of Genotype on this measure, [$F(2,19) = 5.453$, $p<.05$] with KOs performing the worst (Fig 3.8). Furthermore, a t-test was performed specifically comparing KOs versus WTs performance, which confirmed this significant difference between the two groups on accuracy of an win-shift task using a T-maze [$t(15) = -2.229$, $p< .05$].

3.5 Discussion

The purpose of this study was to evaluate conditional forebrain knockout mice on motor learning, auditory processing, and working and reference memory tasks. Results showed that disrupting the function of *Dyx1c1 does* impair memory performance, but does *not* negatively impact motor learning and auditory processing abilities. These results further validate a role for *Dyx1c1* in learning and memory capabilities, and support prior animal model work with this gene. For example, research on the relative behavioral impact of embryonic transfection of RNAi for the dyslexia risk homologs *Kiaa0319* versus *Dyx1c1* in rats revealed: (1) deficits in the discrimination of rapidly changing acoustic stimuli but *not* working memory when *Kiaa0319* was knocked-down (Szalkowski et al., 2012); but (2) acoustic processing deficits for complex stimuli (e.g., FM sweeps) but not rapidly changing stimuli, coupled with *robust* deficits on a working memory task, when *Dyx1c1* was knocked-down (Threkeld et al., 2007; Szalkowski et al., 2011; Szalkowski et al., 2013). Unfortunately, the current study was not able to dissociate potential complex acoustic processing deficits in the conditional *Dyx1c1* KO model, because we

have found that mice are not capable of performing the same complex FM sweep and tone-pair discrimination tasks that can be effectively used in rats. Nonetheless, these cumulative results are intriguing in light of human evidence that has associated mutations in the *KIAA0319/DCDC2* region of chromosome 6 with anomalies in mismatch negativity (MMN) for acoustic phonologic stimuli (Czamara, 2011), while *DYX1C1* has been more closely associated with deficits in working memory and visual attention (Wigg et al., 2004; Marino et al. 2007; Dahdough et al., 2009; Bates et al., 2010; Lim et al., 2011; Mascheretti et al., 2013). Taken together, these findings suggest that different dyslexia risk genes may contribute more or less to different underlying intermediate phenotypes of dyslexia. If true, then early screening could provide indications for optimal intervention strategies on an individual basis.

Overall, our results add support to a putative role for *DYX1C1* in learning/memory components of language, based on the clear inability of conditional KO *Dyx1c1* mice to learn a radial arm maze. However, we acknowledge that the deficits seen in the current study could be interpreted as reflecting motivational or attentional problems. In fact, significant differences comparing the KO group to the WT and HT group emerged on day 5 of testing. Specifically, within the first few days of testing, all animals were learning the task and making a high number of errors. The KO animals continued to make a high number of errors, and then appeared to become unmotivated -- possibly due to the level of task difficulty. At this point, they simply stopped performing. At the same time, the WT and HT group continued to perform the task and make decreasing numbers of errors. Unfortunately, we could not quantify KO animals' errors during this later portion of testing, since they "timed out" and did not successfully complete the testing trials. Another interpretation may be that KO subjects performed poorly on this task because they did not attend to the paradigm and constraints, and therefore, did not successfully

complete as many trials. This hypothesis is in fact consistent with a lack of initial exploration by KOs in the acclimation phase of the Novel Object task. In that paradigm, intact mice typically explore a novel object, and when presented with that same object and a similar but new one, attend more to the new as compared to the familiar object. In the current study, the KO mice did not attend to the initial novel object for an adequate duration to proceed with testing. This could reflect a lack of attention, which might also impact on the radial arm maze. (It is important to again emphasize that the failure to explore in the Novel Object task, and fewer trials successfully completed in the radial arm maze, are not likely a reflection of motor impairments in the KOs, given that our rotarod and visible platform water escape tasks showed no group differences). Importantly, a counter to this argument to the core "failure to perform" as an explanation for deficits is the significantly worse % correct scores seen on T-maze learning for KO mice. Here all the mice performed the task, yet KOs performed significantly more poorly than comparison wild-types.

Finally, in interpreting these findings, it is important to note that the conditional forebrain *Dyx1c1* KO model specifically targets gene function in the cortex and hippocampus, but *not* subcortical structures. Specifically, Emx1- Cre recombinase activity is reported in neurons of the neocortex and hippocampus, and in the glial cells of the pallium. This conditional KO was created to avoid the lethal hydrocephalous observed with a systemic KO. While some very early physiology data could be obtained from the systemic KO preparation, subjects did not survive to an age that would allow behavioral testing. The forebrain conditional *Dyx1c1* preparation, in contrast, has no associated lethality. It would be interesting to assess the effects of a KO preparation that extended into some of the sub-cortical structures not affected here (for example

the cochlear nucleus -- particularly with respect to our acoustic findings). This is an unfortunate limitation of the current model that may be overcome with future technologies.

In closing, the conditional forebrain *Dyx1c1* knockout mouse model examined here displayed learning and memory deficits, consistent with previous animal research using a *Dyx1c1* RNAi knockdown model, and with human findings linking *DYX1C1* to working memory performance. Future research, preferably using a larger sample size, will be needed to assess additional variables including effects in female mice, as well as relationships between aberrant behaviors and underlying changes in neural function and circuitry.

Acknowledgements

This research is supported by the National Institutes of Health grant P01HD057853. AR received support from an NSF IGERT Grant 1144399 (J. Magnuson, PI). HC received an NSF Bridge to the Doctorate grant 1400382. The authors report no biomedical financial interests or conflicts of interest.

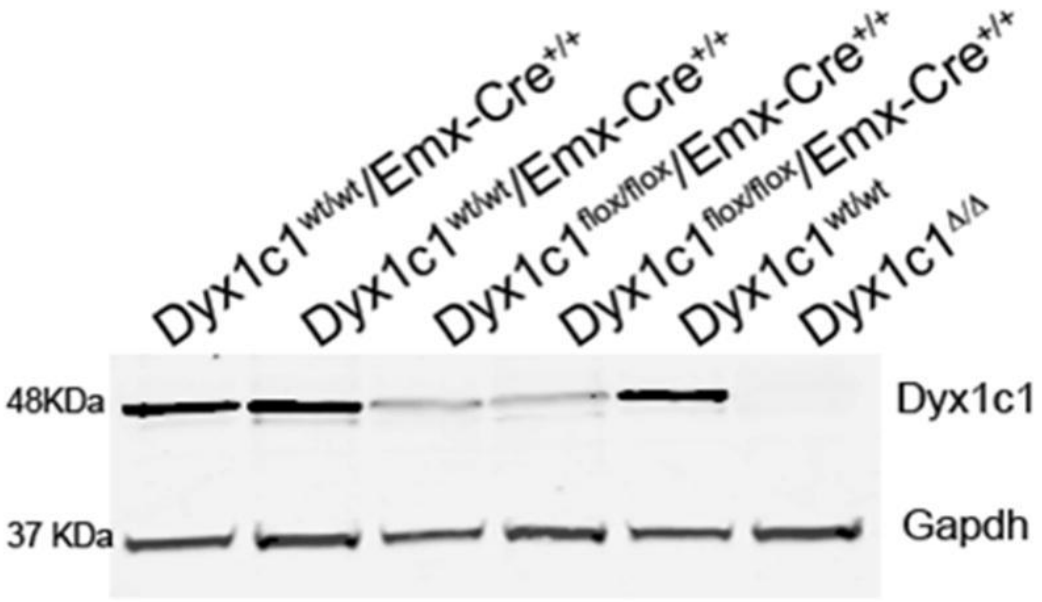

Figure 3.1. Western blot results of *Dyx1c1* protein expression. This blot shows the *Dyx1c1* protein expression in the forebrain KO and the control conditions. We have also included the constitutive KO and WT protein lysates as a control for the experiment. *Dyx1c1* protein expression was reduced by 77% in the forebrain KO condition as compared to the control condition.

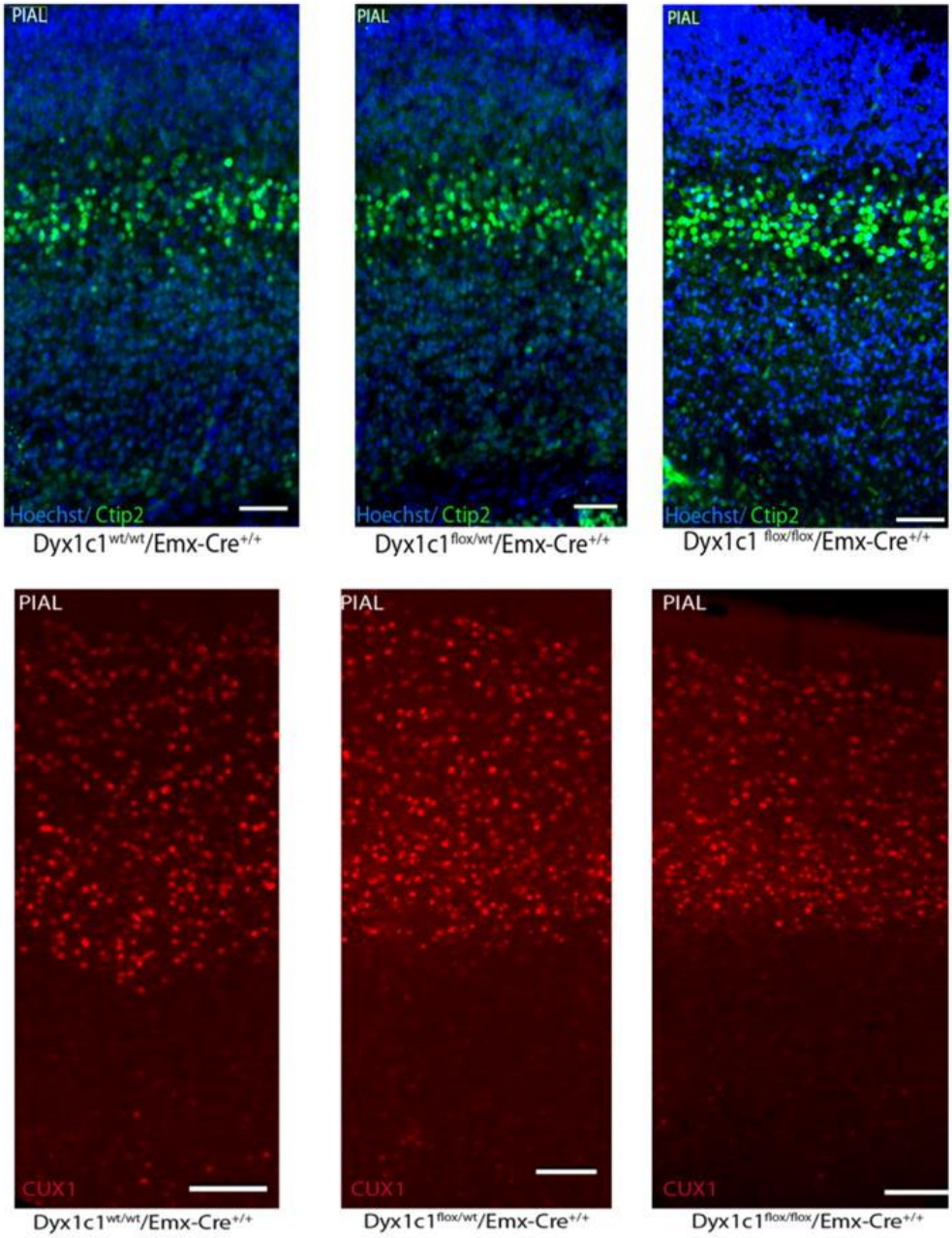

Figure 3.2. **Cortical lamination patterning in *Dyx1c1* conditional KO mice**. There were no significant differences in the cortical lamination patterning as evident from the Cux1 and Ctip2 staining in the forebrain conditional knockout compared to the control. We found no observable anomalies in the cortex of the conditional forebrain knockout animals compared to the controls

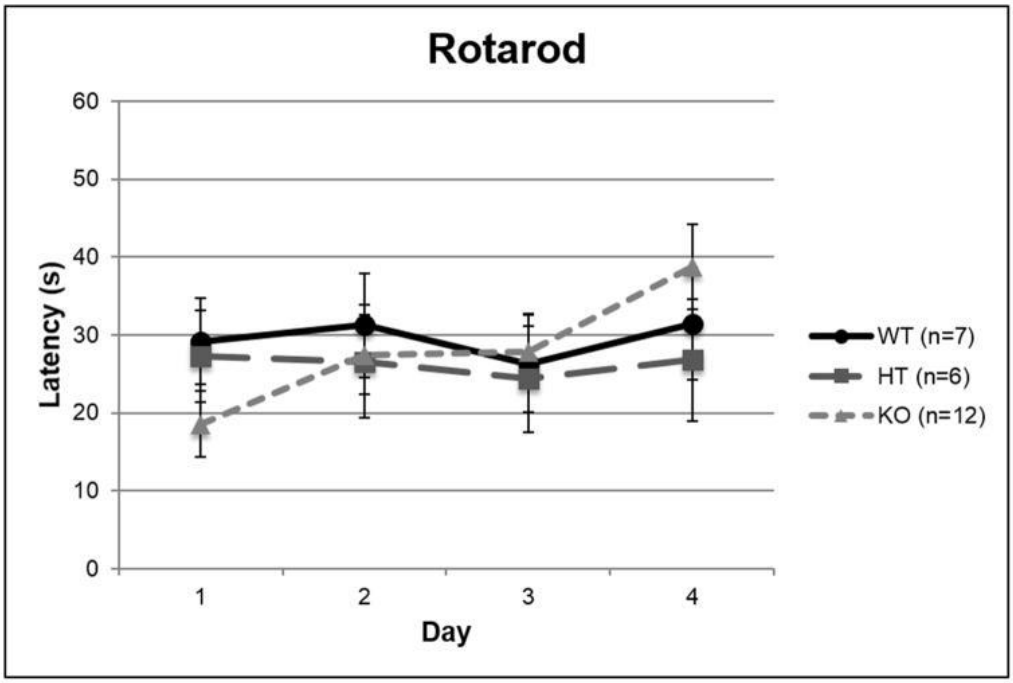

Figure 3.3. Sensorimotor ability in *Dyx1c1* conditional knockout mice. No differences in sensorimotor performance between Genotype were observed on the rotarod task. Both groups were comparable in their latency to remain on the rotating cylinder across four days of testing.

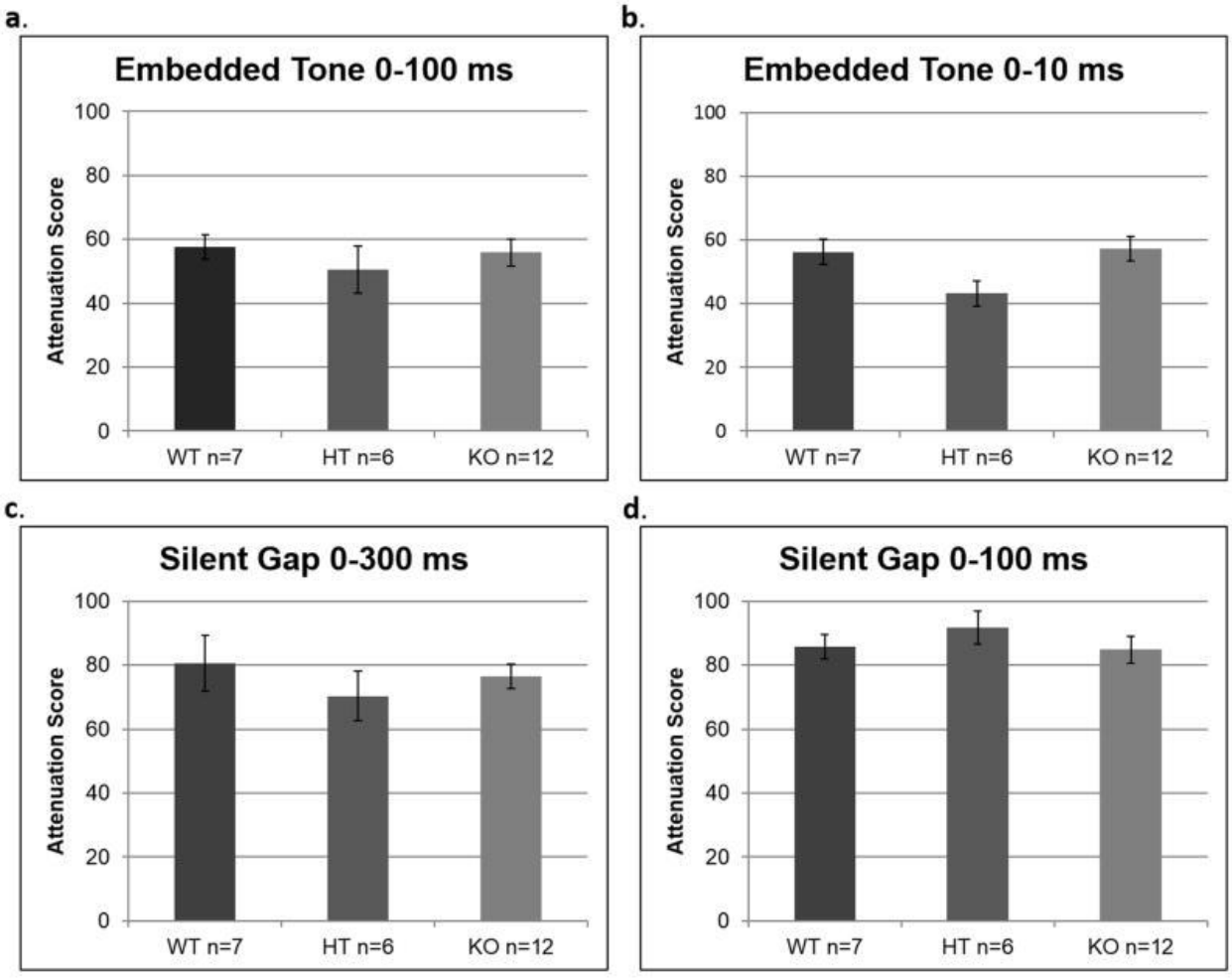

Figure 3.4. Auditory processing on *Dyx1c1* conditional KO mice. Homozygous conditional *Dyx1c1* knockout mice displayed comparable auditory processing abilities on the following tasks (a.) Embedded Tone 0-100 ms, (b.) Embedded Tone 0-10 ms, (c.) Silent Gap 0-300 ms and (d.) Silent Gap 0-100ms.

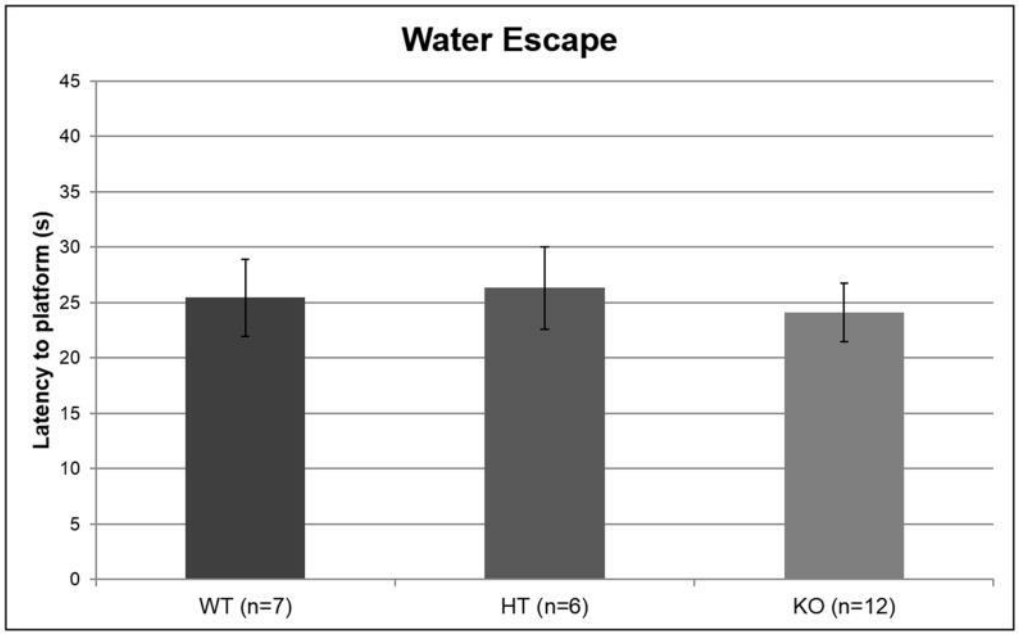

Figure 3.5. Water escape performance of *Dyx1c1* conditional KO mice. No significant differences between Genotypes on latency to swim to platform indicating no underlying motor or visual impairments.

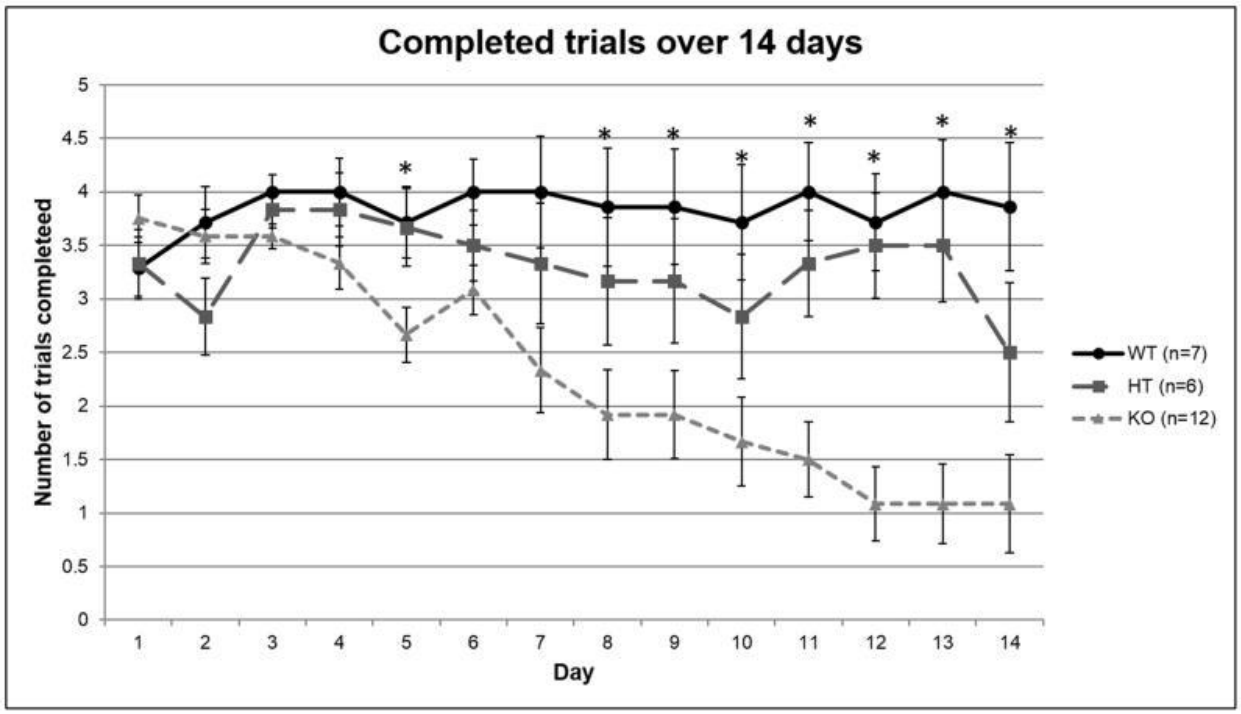

Figure 3.6. 4/8 radial arm maze performance of *Dyx1c1* conditional KO mice. General learning and memory impairment in mice with homozygous conditional *Dyx1c1* knockout mice on a 4/8 radial arm water maze. Analysis of total completed trials over 14 days reveal significant differences between Genotypes (*p <.05), as well as, significant Genotype x Day interaction (p<.001) revealing KOs drop in successfully completing trials as testing progresses.

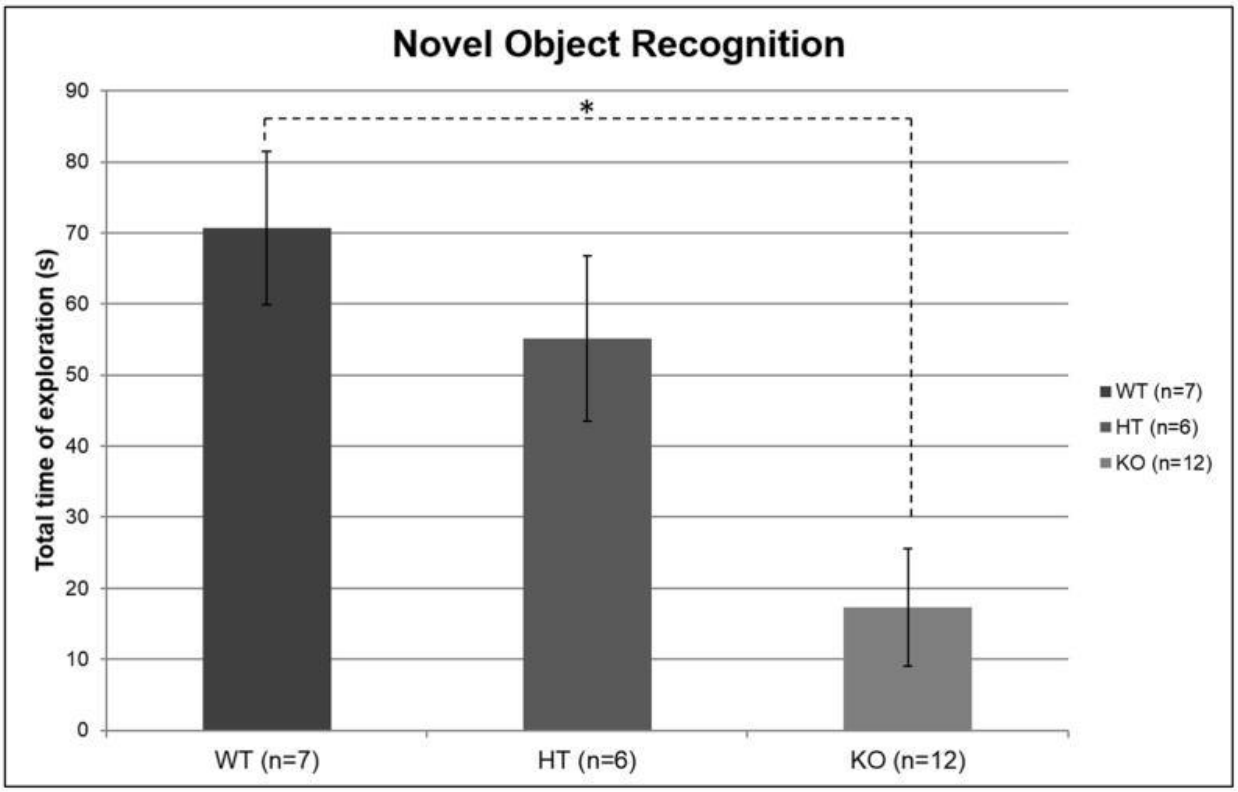

Figure 3.7. Novel object performance of *Dyx1c1* conditional KO mice. Lack of exploration during the novel object recognition task in homozygous conditional *Dyx1c1* knockout mice (*p<.01).

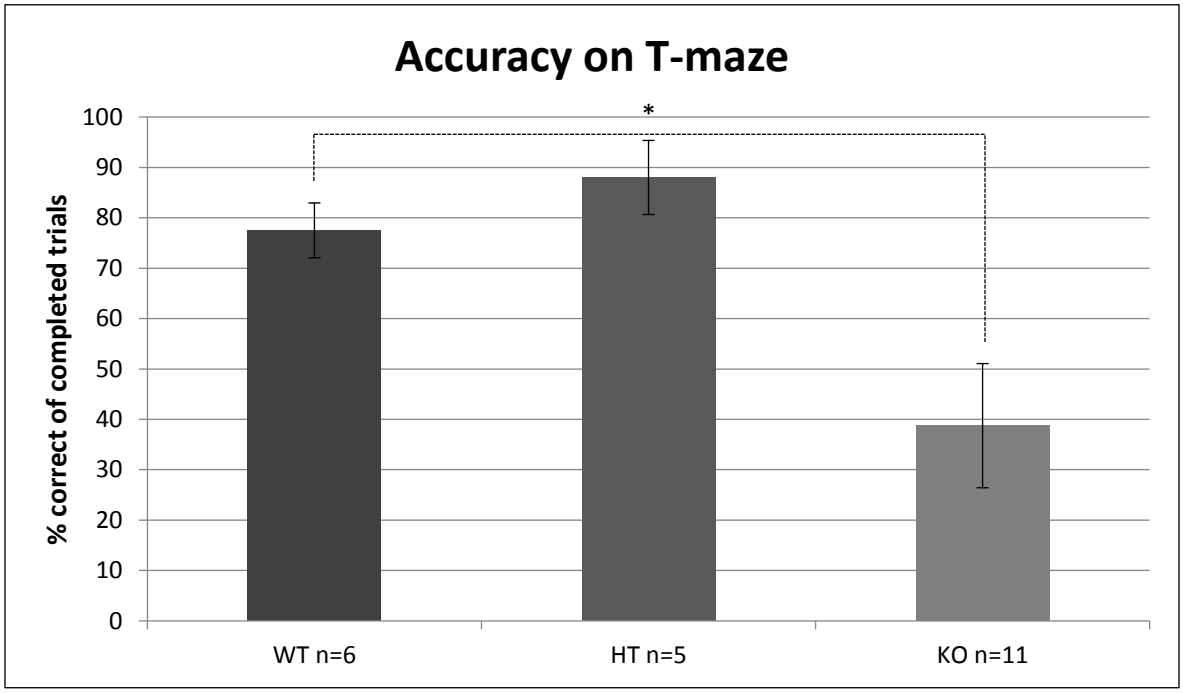

Figure 3.8. T-maze performance of *Dyx1c1* conditional KO mice. Accuracy on T-maze task. Homozygous conditional *Dyx1c1* knockout mice exhibited significantly poorer performance on the T-maze (*p<.05) with accuracy around chance levels.

CHAPTER 4

Auditory processing enhancements in the TS2-neo mouse model of Timothy Syndrome, a rare genetic disorder associated with autism spectrum disorders

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Published: Advances in Neurodevelopmental Disorders (2017), doi: 10.1007/s41252-017-0029-1

4.1. Abstract

Timothy syndrome (TS) is a rare genetic disorder caused by a single de novo missense mutation to the 8A exon of CACNA1C gene, which codes for the voltage-gated L-type Ca2+ channel (Cav1.2). TS is strongly associated with cardiac arrthytmias, autism spectrum disorders (ASDs), and neurological dysfunction such as language impairments, seizures, and intellectual disability. A genetically engineered knock-in mouse with a heterogeneous TS2 (G406R) mutation in the L-type calcium channel containing a neomycin resistance cassette was developed to study ASD-like behaviors. This mouse model (TS2-neo) provides us with a platform to investigate the role of calcium channel inactivation and calcium signaling related to brain development and ASD. The purpose of the current study was to behaviorally characterize TS2-neo mice by assessing their performance on a wide variety of behavioral paradigms, including replication of findings that support the TS2-neo as a valid platform for studying ASD-like behavior. In addition to examining core social and repetitive anomalies, we sought to focus on basic perceptual processing in the auditory domain. Results indicate that the loss of Cav1.2 inactivation in this mouse model results in deviant social and repetitive behaviors as well as poor sensorimotor learning. TS2-neo mice display superior performance on both an embedded tone and silent gap discrimination task for short-duration acoustic stimuli. These findings parallel the low-level auditory enhancements observed within the ASD clinical population. Additionally, structural anomalies were seen for mutant mice in some white matter tracts and in the medial geniculate nucleus (MGN). Co-occurrence of these findings suggests that aberrant MGN morphology may be related to enhanced auditory processing phenotype as seen in ASD.

4.2 Introduction

Autism Spectrum Disorder (ASD) is a set of neurodevelopmental disorders characterized by a complex behavioral phenotype, encompassing deficits in both social and cognitive domains. The core symptoms are heterogeneous, and range from atypical social interactions and language impairments to repetitive behaviors (American Psychiatric Association, 2013). To date, causal mechanisms underlying ASD remain poorly understood, but likely include a complex combination of polygenic and environmental risk factors (Moreno-De-Luca, 2013).

There is a strong genetic influence in ASD, with heritability rates ranging from 70-90% (Bailey et al., 1995; Rosenberg et al., 2009; Steffenburg et al., 1989). However, much is still unknown about the genetic contribution. It is suggested that over a 1,000 genes are involved in ASD, reflecting a complex genetic architecture (DeRubeis et al., 2014). Notably, most of the genes identified have been shown to play a critical role in neurodevelopment, and in fact converge onto several core functional pathways. These can be roughly divided into two major categories: synaptic transmission and excitation/inhibition imbalance; and gene expression involved in transcription/translation (Bourgeron, 2015, for review; Ey, Leblond & Bourgeron, 2011). This is consistent with evidence that genetic mutations associated with ASD influence the structure and the turnover of synapses at different levels, including increasing or decreasing synaptic strength or numbers. Disruption of synapses and signal transmission that alters neuronal connectivity in the brain could in turn mediate functional changes associated with ASD (Auerbach, Osterweil & Bear, 2011; Hahamy, Behrmann, & Malach, 2015). Recent pathway network analyses, coupled with genome-wide association studies of autism, reveal the calcium signaling pathway to be the most affected, suggesting that it is highly involved in the molecular basis of ASD (Skafidas et al., 2014; Wen, Alshikho & Herbert, 2016; Wittkoski et al., 2014).

Genes associated with calcium channels modulate neuronal function by mediating influx of calcium into neurons (and thus neurotransmitter release), intracellular signaling, and gene transcription. Disruption to any of these can interfere with the neurodevelopmental trajectory.

Among identified ASD risk genes, calcium voltage-gated channel subunit alpha1 C (*CACNA1C*) has been associated with disorders such as bipolar disorder, schizophrenia, major depression, and more recently ASD (Bhat et al., 2012, for review; Li et al., 2015). A single *de novo* missense mutation to the 8A exon of *CACNA1C* gene (which codes for the voltage-gated L-type Ca2+ channel (Cav1.2)) results in a rare multisystem disorder known as Timothy syndrome (TS) (Splawski et al. 2004). This mutation sharply reduces calcium channel inactivation, which may lead to heightened Ca2+ influx (Barrett & Tsien, 2008; Splawski et al., 2004). TS is strongly associated with cardiac arrhythmias, ASD, and neurological dysfunctions that include language impairments, seizures and intellectual disability. All individuals with TS exhibit proarrhythmic prolongation of the cardiac action potential, which generally results in sudden cardiac death at a young age (Splawski et al. 2004). Therefore, it has been challenging to study the basic mechanism of TS in humans, including how this mutation leads to a high co-morbidity with ASD. A genetically engineered knock-in mouse with a heterogeneous TS2 (G406R) mutation in the L-type calcium channel containing a neomycin resistance cassette was developed to study this condition (Bader et al., 2011). The resulting mouse model (TS2-neo knock-in) provides a platform to investigate the role of calcium channel inactivation and calcium signaling in atypical brain development, and in the expression of ASD-like behaviors. Previous behavioral studies on TS2-neo mice found that these animals' exhibit normal general health and anxiety levels, but display a strong autistic phenotype indicated by restricted and repetitive behaviors, altered social behavior, and decreased ultrasonic vocalizations (Bader et al., 2011; Bett et al.,

2012).

Understanding the causes of ASD will allow for earlier detection and more refined intervention. However, efforts have been hindered by the heterogeneity and complicated genetic and environmental influences implicated. The study of transgenic mouse models allows us to assess the role of individual genes in modulating biological and behaviorally phenotypes relevant to ASD. Numerous mouse models targeting ASD risk genes have been used to behaviorally phenotype core symptoms of ASD, and particularly repetitive and abnormal social behaviors. One area that has *not* been well explored involves "splinter skills," or enhanced discrimination of local details within perceptual information among individuals with ASD (Bertone et al. 2005; Plaisted et al. 2003). Superior performance in ASD individuals has been shown in low-level visual perceptual tasks such as visual search (O'Riordan, Plaisted, Driver, & Baron-Cohen, 2001; Plaisted, O'Riordan, & BaronCohen, 1998) and discrimination tasks (Plaisted et al., 1998). Also seen are specific enhancements in pitch discrimination among those with ASD (Bonnel et al. 2010, 2003; Eigsti & Fein, 2013; Heaton and Heaton 2003, 2005; Jones et al. 2009; O'Riordan & Plaisted 2001). A more recent study also found superior auditory performance on detecting perceptual features of pitch and timing in individuals with autistic traits (Stewart, Griffiths, Grube, 2015). These aberrant perceptual processing may relate to the core features of ASD, for example social and communication deficits. However, little animal research has focused on the low-level perceptual enhancements seen in ASD, even though they may impact higher-level cognition and behavior.

The purpose of the current study was to behaviorally characterize TS2-neo mice by assessing their performance on a wide variety of behavioral paradigms, including replication of findings that support the TS2-neo as a valid platform for studying ASD-like behavior. In addition to

examining core social and repetitive anomalies, we sought to focus on basic perceptual processing in the auditory domain. All subjects underwent various tasks evaluating their motor coordination, auditory processing, social behaviors, and learning and memory. Following behavioral assessment subjects underwent neuroanatomical analysis of white matter tracts, as well as the medial geniculate nucleus (MGN), since these structures are known to be critical to many behaviors that are affected in ASD.

4.3 Methods and Materials

4.3.1 Subjects

Twelve male TS2-neo mice (B6.Cg-Cacna1ct^{m2Itl}/J; stock number 019547) and 12 matched male wild type (WT) controls (C57BL/6J; stock number 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME). The TS2-neo mouse model has the G406R mutation associated with severe Timothy Syndrome (TS2) and an inverted neomycin resistance cassette, all inserted at the end of exon 8 of the CaV1.2 L-type calcium channel locus (*Cacna1c*) (for more detail on the development of the TS2-neo mouse model see Bader et al., 2011; Bett et al., 2012). The TS2-neo mice do not display any gross behavioral abnormalities in vision, olfaction, or motor strength, thus replicating reports by Bett el al., (2012). Subjects were delivered to the University of Connecticut, Department of Psychology in two separate cohorts (Cohort 1: 6 TS2-neo, 6 WT mice; Cohort 2: 6 TS2-neo, 6 WT mice, all mice received at 7 weeks). All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water *ad libitum.* All behavioral testing occurred during the light cycle. At the start of testing, animals were between the ages of postnatal day (P) 55 – P57. Procedures were performed blind to Genotype, and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

4.3.2 Rotarod P57-P61

Subjects were assessed at age P57 for sensorimotor ability and motor learning using a rotarod task. All mice were habituated to the rotarod a day prior to testing, where they were placed on a rotating cylindrical drum that was held at a constant speed of 4 rotations per minute. Subjects underwent 4 trials that maxed out at 2 minutes. Testing began the following day with subjects placed on a rotating cylindrical drum that accelerated from 4 to 40 rotations per minute across 2 minutes. Four trials were administered per day, across five consecutive days. Latency to fall from the rotating drum was averaged across the four trials for each day.

4.3.3 Auditory Processing P70-P105

Subjects then advanced to auditory processing testing, which utilizes a modified pre-pulse inhibition paradigm (see Fitch et al., 2008 for review). Subjects were placed on individual load-cell platforms (Med Associates, St. Albans, VT) and presented with auditory stimuli generated using RPvdsEx on a Dell Pentium D PC and RZ6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Sounds were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The acoustic startle reflex (ASR; a reflexive response elicited by an unexpected, intense stimulus) was recorded by an iMac 7.1 running Acknowledge 4.1, and obtained via the voltage output from each load cell platform through a linear amplifier (PHM-250U; Med Associates, St. Albans, VT) connected to a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA). The modified pre-pulse inhibition paradigm measured differences in ASR to a loud startle-eliciting stimulus (SES; 105dB, 50 ms, broadband white noise burst (1kHz-10kHz)) when presented with or without a preceding acoustic cue. The ASR difference on cued versus uncued trials provided a measure of cue detection and/or

discrimination. If the auditory cue was detected, a reduction (attenuation) in the ASR was expected relative to the ASR elicited when the auditory cue was not present (or not detected). This phenomenon was quantified using an “attenuation score” (ATT) that compared the average amplitude of the ASR from the cued trial to the average ASR of the uncued trial ([average cued ASR/average uncued ASR]*100).

4.3.3.1 Normal Single Tone P70

Animals were initially tested on Normal Single Tone (NST) to measure baseline pre-pulse inhibition and auditory ability (P70). This auditory PPI control task was used to establish whether subjects exhibited hearing deficits and/or impaired gross motor reflexes that could confound other auditory PPI tests, and provided an index of baseline auditory pre-pulse inhibition ability across test groups. Testing sessions consisted of 104 pseudorandomly presented cued and uncued trials at inter-trial intervals (ITI) of varying duration (16–24 s). The task comprised a silent background and a simple single tone cue (50 ms, 75 dB, 8,000 Hz tone) presented 50 ms prior to the 50 ms, 105 dB SES. All subjects were able to perform this task, and therefore were used for further auditory processing evaluation.

4.3.3.2 Embedded Tone P71-P83

The variable duration Embedded Tone (EBT) task (300 sequential pseudorandom trials) assessed ability to detect a change in tone frequency relative to a standard background tone (cue was a variable duration 5.6 kHz pure tone embedded in a 10.5 kHz background pure tone). On cued trials, the cue was presented 100 ms before the SES, while uncued trials used a "cue" of 0 ms. Two EBT tasks were used in this study – a long-duration EBT (0 ms to 100 ms), and a short-duration EBT (0 ms to 10 ms). A range of cue durations were used to evaluate specific thresholds for performance differences between the genotypes, since groups might perform comparably on

longer durations yet differ on shorter durations (which are more difficult to detect). Using a range of cue durations enables ascertainment of stimulus features that all animals can discriminate (ceiling), that no animals can discriminate (basement), as well as any group differences in the mid-range. Both EBT tasks were administered for five consecutive days (P71-P83).

4.3.3.3 Silent Gap P86-P98

A Silent Gap (SG) task was used to assess ability to detect silent breaks in continuous white noise (P86 to P98). A session included 300 trials with a continuous 75 dB broadband white noise background, with pseudorandomized cued and uncued trials. On cued trials, a silent gap of variable duration (0-100 ms or 0-10 ms) was presented 100 ms before the SES, while a "0 ms" trial served as the uncued condition. Subjects were tested on the Silent Gap task for five consecutive days using both versions of the task.

4.3.3.4 Pitch Discrimination P101-P105

Pitch discrimination testing also took place across five consecutive days of testing (300 trials/day). This task assessed ability to detect very small changes in pitch embedded in a background tone. A variable ITI (16–24s) was used, and the cue was a 300 ms, 75 dB tone of variable frequency embedded in a standard 75 dB, 10500 Hz background pure tone (2 ms up/down linear frequency ramp) prior to the SES. The experimental frequencies used for the pitch discrimination task deviated from the standard background frequency by as much as 75 Hz, to as little as 5 Hz. Uncued trials did not include a frequency deviant prior to the SES.

4.3.4 Three-Chamber Social Interaction P107-P110

The Three-Chamber test was used to assess general sociability as well as social recognition. This test derives from observations that healthy wild-type mice typically prefer to spend time

with a conspecific (social stimulus) rather than an object (non-social stimulus). After a 5 min habituation period, the subjects were allowed to freely explore three chambers, with one containing another "stranger" mouse, and another chamber on the opposite side containing a novel object. The subject was placed into the middle (empty) chamber and was able to freely explore all the chambers for 10 min. Next we placed an unfamiliar conspecific mouse ("stranger 2") where the novel object was previously located, and subjects were given another 10 min to explore the chambers. The percent time spent interacting with the mouse during the social preference phase, and percent time spent interacting with the novel mouse during the social recognition phase, were recorded and analyzed.

4.3.5 Social Dominance – Tube Test P113-P116

The Tube Test was administered to evaluate social dominance/aggression. The tube used for this task was a clear plexiglas tube (length 30.5cm; outer diameter 4.5cm; inner diameter 3.5cm). This narrow space is just sufficient for a mouse to walk through without being able to reverse direction. Mice were trained to walk through the tube before testing. A WT and a mutant mouse were randomly paired on different sides of the tube (balanced), and released at the same time into the tube. The mouse that forced the other mouse to back out of the tube was considered the "winner" of the trial (recorded for analysis). Each mouse underwent 4 trials paired with a different randomly assigned subject and the percentage of wins was calculated and analyzed. Mice were not paired within Genotype since by definition this would yield a score of 50%.

4.3.6 Marble Burying P115-P118

Subjects were placed in a standard polycarbonate cage (26 cm x 48 cm x 20 cm) filled with fresh mouse bedding (5cm deep) for the marble burying test. Standard glass toy marbles (assorted styles and colors, 15 mm diameter, 5.2 g in weight) were placed on the surface of the bedding in

3 rows of 7 marbles. A marble was considered buried if at the end of the 45 minute session the marble was more than half-way covered by bedding. Subjects were given 45 minutes to explore the area; number of marbles buried were reported and used for analysis.

4.3.7 Vocalizations during male-female interactions P122-P126

Adult male vocalizations were recorded during individual male-female pair social interactions. Male mice produce ultrasonic vocalizations when they are in the presence of a female (and particularly during estrus), or when they detect a female's urinary estrus pheromones. We measured the vocalization emission of all subjects when exposed to accumulated seven-day dirty bedding obtained from mature, age-matched C57 female. Bedding from seven-days was used to ensure inclusion of estrus phase (4 day cycle). That same female was free moving in the cage with the male subject during recording. On P122-126 male subjects were individually placed in a standard laboratory cage filled with bedding, dirty bedding and a freely moving female. Only WT females were used to avoid confounds. Vocalizations were recorded for 5 minutes using a 1/4 inch condenser microphone (Brüel & Kjær type 4136, Nærum, Denmark) suspended 10 cm above the test subject. The microphone signal was preamplified with a Bru¨el & Kjær type 2619 preamplifier and then amplified using a Brüel & Kjær type 2636 amplifier (Bru¨el & Kjær, Nærum, Denmark). The signal was digitized at a sampling rate of 200 kHz using a Tucker Davis Technologies (Alachua, FL) multifunction processor (RZ6) and saved as a .wav file using a custom MATLAB program (MathWorks, Natick, MA) on a Dell Pentium IV PC. Recorded sound waveforms were visualized and assessed using Adobe Audition (Adobe, San Jose, CA). Total time spent vocalizing was calculated by extracting vocalization intervals (continuous vocalization epochs, 200 ms apart) from periods of silence (no vocalizations). Time spent vocalizing was binned into minute periods across the 5 minute male-female interaction. Since

females vocalize little in the presence of males, all recorded vocalizations were presumed to reflect the male subject (D'Amato & Moles, 2001; Moles et al., 2007; Wang et al., 2008).

4.3.6 Water Maze Assessment P127-P150

Subjects were initially tested on a water escape to assess any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm x 55.5 cm) filled with room temperature water, and given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) at the opposite end of the tub. Latencies to reach the visible platform were recorded. None of the subjects displayed any impairment on this task, and thus all proceeded to Morris water maze testing.

The Morris water maze is a behavioral task commonly used to assess spatial learning and memory, and specifically the ability to locate the position of a submerged escape platform using extra maze cues. Beginning on P129, subjects were tested on the Morris water maze over a span of five consecutive test days (sessions). During each test session, subjects were given four trials to locate the submerged platform. For each trial, the subject starting location was selected pseudorandomly at one of the four compass locations (i.e. north, south, east, and west), with each location used once per test session. Subjects were allowed 45 seconds to complete the trial and find the escape platform. If the platform was not located at 45-seconds, subjects were gently guided to the goal before removal from the maze. The position of the hidden platform remained static throughout all five test sessions. Latency to the escape platform was measured and recorded using a Sony camera integrated with a SMART video-tracking program (Panlab, Barcelona, Spain).

Subjects then progressed to a water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998). This task was used to assess spatial reference and working memory ability simultaneously, using a standard 8 arm radial maze with 4 arms baited (i.e., containing a submerged goal platform), and 4 arms open but never baited. Configuration of goal arms were counterbalanced between subjects but remained fixed per subject across all sessions. Additionally, high contrast extra-maze cues were present, and the locations of these remained static for the experiment.

The day prior to testing (Day 1), subjects were given a training session where all arms that would not contain a platform were blocked, forcing the animals to only enter arms containing a platform. Subjects were placed in the start arm and given 120 seconds to locate a platform. Every subject completed 4 training trials, and each time they found a platform, that platform was removed and the entrance to that arm was blocked. This ensured that the subject could no longer enter this arm for the remainder of the training session. If the subject failed to find a platform in this window, they were guided to the nearest available goal. Once on the platform, subjects remained for 20 seconds and then were removed from the maze to their home cage (30 second inter-trial interval; ITI).

Testing began on Day 2 and continued for an additional 14 consecutive days. Here, instead of blocking the goal arm of the most recently located platform, the platform was removed during the 30 second ITI. However, the arm remained open and unbaited for the remainder of the test session. Animals were required to locate all 4 platforms, and thus received 4 test trials per day. Test sessions were recorded using a Sony camera integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). Total number of errors were recorded and used for analysis.

4.3.7 Perfusion and histology P191

At the completion of testing, all 24 subjects were weighed, anesthetized using ketamine (100mg/kg) and xylazine (15 mg/kg), and transcardially perfused using a .9% saline solution with formalin as the fixative. Brains were extracted and stored in formalin to postfix at 4°C. Sixteen tissue samples (8 TS2-neo, 8 WT) were stored long-term to await neuroanatomic assessment using a Nissl stain (with remaining tissue used in other immunohistochemical analyses).

Formalin-fixed brains were serially sectioned in the coronal plane at 60 μm using a vibratome (Leica VT1000 S). Every second section was mounted on gelatin-subbed slides and stained for Nissl bodies using cresyl violet (coverslipped with DPX mounting medium). Volumetric measures of white matter structures were assessed, including corpus callosum, cingulum, external and internal capsule, fornix, and anterior commissure. All prepared samples were analyzed using the Stereo Investigator System (MBF Biosciences, Williston, VT, USA) integrated with a Zeiss Axio Imager A2 microscope (Carl Zeiss, Thornwood, NY). For all structures, volumes were reconstructed from serial area scores using the Cavalieri Estimator probe in StereoInvesigator. Neuronal cell populations were estimated using the Optical Fractionator probe, with cross sectional neuronal cell area estimated concurrently using the Nucleator probe. Neurons were only counted for analysis if the nucleolus was in focus and within the appropriate boundaries of the active counting frame and dissector depth. A standard stereotaxic atlas was used to determine the borders of the MGN for quantification (Lein et al., 2007; Paxinos & Watson, 1986). An average of 8 to 10 sections per brain was used for white matter tract analysis while an average of 6 sections per brain was used for MGN analysis. Volumetric measurements as well as contours of the MGN were drawn at 2.5X magnification.

Cell size measurements and counts were assessed at 100X magnification. A sampling grid size of 200 µm × 200 µm and a 30 µm × 30 µm counting frame were used for stereological examination. Estimates for both neuronal cell population and neuronal cell area were obtained for left MGN, right MGN, and total (left + right) MGN for each subject. All measurements were performed blind to genotype.

4.3.8 Data Analyses

All subjects were used for analysis (WT, n = 12; TS2-neo, n = 12). Group differences on rotarod performance were analyzed using a 2 (Genotype; TS2-neo and WT) x 5 (Day) repeated measures analysis of variance (ANOVA). Normal Single Tone (baseline control) data was examined using a univariate ANOVA comparing TS2-neo and WT attenuation scores. Although, there were no significant differences in performance on NST, NST attenuation scores were used as a covariate in subsequent statistical analysis to control for individual variations in baseline auditory prepulse inhibition (PPI). Differences in attenuation (ATT) scores during all auditory tasks (embedded tone, silent gap and pitch discrimination) were examined using a 2 × 5 × 9 repeated measures ANOVA with Genotype (2 levels: TS2-neo and WT) as the between-subjects variable, and Day (5 levels) and cue (9 levels) as the within-subjects variable. A univariate ANOVA was performed to analyze differences between Genotypes on the following tasks, social interaction, social dominance tube task, marble burying, vocalizations and water escape. A repeated measures ANOVA was performed to analyze latency to platform in the Morris water maze with Day (5 levels) being the within-subject variable and Genotype being the between subjects variable. Average total errors were examined for the 4/8 radial water maze, using a 2 x 14 repeated measures ANOVA, with Genotype (2 levels: TS2-neo and WT) as the between measure and Days (14 levels) as the within measure. For neuroanatomical measures, subjects in the Nissl

stained group (n=16, 8 TS2-neo/8 WT) were used for analysis. Group differences in regional volume and cellular measurements (mean cell count and size within defined boundaries) were assessed using a univariate ANOVAs. Examination of cell size distribution was conducted using a cumulative percent distribution. To examine group differences in cell size distribution, non–parametric analyses with Kolmogorov Smirnov (K-S) tests were conducted on the cumulative percent distributions of each Genotype. A bivariate correlation was used to examine the relationship between number of neurons and auditory processing performance. All statistical analyses were conducted using SPSS 19 with an alpha criterion of 0.05, two-tailed.

4.4 Results

4.4.1 Rotarod

A repeated measures ANOVA examining average rotarod latency across 5 days of testing revealed a main effect of Genotype [$F(1,22) = 14.037$, $p<.05$], as well as a Genotype x Day interaction [$F(4,88) = 8.251$, $p<.001$]. TS2-neo mice showed impaired sensorimotor/motor ability, and the lack of improvement across days indicates they failed to show motor learning on this task (Fig. 4.2a).

4.4.2 Auditory Processing

All subjects were initially tested on a normal single tone (NST) task, to establish baseline hearing and PPI ability. None of the subjects showed impairments on NST, nor was there a main effect of Genotype [$F(1,22) = .392$, $p>.05$]. We did however controlled for individual variations in baseline auditory prepulse inhibition (PPI) performance using NST attenuation scores as a covariate. On the embedded tone (EBT) 0-100 ms task, we found no main effect of Genotype [$F(1,21) =1.394$, $p>.05$] (Fig 4.3a), and all subjects were able to discriminate the stimuli based on cued/uncued amplitude comparisons. On the more difficult embedded tone task (cue durations

ranging from 0 to 10 ms, where 0 ms is the uncued condition) we found a main effect of Genotype [$F(1,21) = 5.388$, $p<.05$], with TS2-neo mice showing enhanced detection of the embedded tone cue compared to WTs (Fig. 4.3b). For silent gap detection we assessed performance on the 0-300 ms task, and again saw no main effect of Genotype [$F(1,21) = .004$, $p>.05$] (Fig. 4.3c). On the silent gap 0-100 ms task, we found overall worse performance (since the task was harder), and also a main effect of Genotype [$F(1,21) = 7.369$, $p<.05$], with TS2-neo mice again demonstrating superior performance on gap detection (Fig. 4.3d). On the pitch discrimination task, we did not find a main effect of Genotype [$F(1,21) = .731$, $p>.05$].

4.4.3 Three-Chamber Social Interactions

On the three-chamber social interaction tasks we did not find a main effect of Genotype, either in the social preference phase [$F(1,22) = 1.426$, $p>.05$], nor the social recognition phase [$F(1,22) = .001$, $p>.05$]. On all tasks, WTs and TS2-neo mice spent a comparable percentage of time exploring the social stimuli (Fig. 4.1d).

4.4.4 Social Dominance

On the social dominance (tube task) the number of wins was calculated as a percentage of the 4 trials. We found a main effect of Genotype [$F(1,22) = 12.138$, $p<.01$], with TS2-neo mice showing more "wins" compared to WT controls. These findings indicate TS2-neo's were more aggressive (Fig. 4.1a).

4.4.5 Marble Burying

A univariate ANOVA revealed a main effect of Genotype [$F(1,220 = 10.662$, $p<.01$] on the marble burying task with TS2-neo mice burying significantly more marbles compared to WTs. This indicates the mutants expressed more stereotyped and repetitive behaviors (Fig. 4.1c).

4.4.6 Vocalizations

A univariate ANOVA was conducted to evaluate time spent vocalizing for each minute spent interacting with a female mouse. Analysis revealed that TS2-neo mice vocalized significantly less during male-female interactions after the initial two minutes, [Minute 1: $F(1,22) = .187$, $p>.05$; Minute 2: $F(1,22) = .180$, $p>.05$; Minute 3: $F(1,22) = 4.803$, $p<.05$; Minute 4: $F(1,22) = 4.058$, $p<.10$; Minute 5: $F(1,22) = 6.498$, $p<.05$] (Fig. 4.1b).

4.4.7 Water Maze Assessment

Prior to spatial water maze testing, a visible platform control task was conducted to assess any underlying impairments that could confound subsequent water maze performance (e.g., impairments in swimming ability, visual acuity, or motivation). A univariate ANOVA on latencies found no main effect of Genotype [$F(1,22) = .037$, $p>.05$] indicating that genetically modified groups had no impairments on underlying aspects of the water task (e.g., swimming) On the Morris Water Maze task we found no main effect of Genotype [$F(1,22) = .013$, $p>.05$], but did find a main effect of Day [$F(4,88) = 6.313$, $p<.01$], with all subjects showing decreased latencies as testing progressed (indicating learning; Fig. 4.2b). This indicated learning for all subjects.

The 4/8 radial arm water maze was used to simultaneously measure spatial working and reference memory performance. A repeated-measures analysis of the average number of total errors (working memory, initial reference, and repeated reference memory errors) revealed no significant difference between WT and TS2-neo groups [$F(1,22)=.016$, $p>.05$] (Fig. 4.2c). A main effect of Day [$F(13,286)=5.348$, $p<.01$] was observed, confirming that both groups reduced errors across days (i.e., showed learning). We also examined group differences in error types including working memory, initial reference memory, and repeated reference memory. Again repeated measures ANOVA reveal no effect of Genotype on any error types (working memory

[F(1,22)=.098, p=.757], initial reference memory [Genotype: F(1,22)=.00, p>.05] and repeated reference memory [Genotype: F(1,22)=.574 p>.05]).

4.4.8 White Matter Tract Volumes

Initial analysis of white matter tract volumes (Nissl stained samples, n=16 (8 TS2-neo/8 WT)), revealed a main effect of Genotype for volume of external capsule volume, with TS2-neo mice showing significantly smaller external capsule volumes compared to WTs[F(1,16)=6.417, p<.05]. A subsequent examination of hemisphere-dependent effects showed that this decrease was localized to the right external capsule [F(1,16)=10.737 p<.01]. Analysis of total fornix volume also showed a trend towards reduced volume in the TS2-neo group [F(1,16)=4.209, p<.1] (Fig. 4.4). No Genotype differences were seen for other white matter structures assessed.

4.4.9 Medial Geniculate Nucleus

A univariate ANOVA comparing MGN volume revealed a marginal main effect of Genotype, with a smaller MGN volume in TS2-neo mice compared to WTs [F(1,14) = 4.250, p=.058] (Fig. 4.5a). Examination of mean cell size in the MGN found no main effect of Genotype [F(1,14) = .384, p>.05]. However, comparisons of cumulative percent distributions between TS2-neo and WT controls using a Kolmogorov Smirnov test revealed a significant K-S statistic (p<.05; Fig. 4.5b). Analysis of cell size distribution in TS2-neo and WT brains revealed that TS2-neo brains contain more small and fewer large MGN cells than controls. A univariate ANOVA comparing MGN neuronal population revealed a main effect of Genotype, specifically for the right MGN, with TS2-neo mice exhibiting fewer neurons compared to WTs [total: F(1,14) = 2.489, p>.05; left: F(1,14) = .180, p>.05; right: F(1,14) = .045, p<.05] (Fig.4.5c). A bivariate correlation revealed a significant positive relationship between neuronal population in the MGN and average attenuation scores on EBT 0-10 in TS2-neo mice only (r = .792 n = 8, p<.05) (Fig. 4.5d).

Specifically, an increase in the number of neurons in the MGN was correlated with an increase in attenuation score (worse performance) on the EBT 0-10 task. This relationship was not seen in the WTs ($r = -.269$, $n= 8$, $p>.05$) or on any other auditory processing task.

4.5 Discussion

The purpose of this study was to behaviorally evaluate TS2-neo mice on motor learning, auditory processing, social and repetitive behaviors, as well as, on learning and memory tasks. Results showed that the loss of Ca.V1.2 inactivation in this mouse model significantly affected motor learning, auditory processing, and social and repetitive behaviors, but did not impact on spatial learning and memory. Additionally, structural anomalies were seen for mutant mice in some white matter tracts, and MGN.

4.5.1 TS2-neo serves as a model of ASD

The results presented here reaffirm that when TS mutant channels are expressed at reduced levels (but low enough to avoid fatality), effects include behaviors consistent with symptoms observed in the clinical ASD population. Specifically, we found that the TS2-neo mice displayed deviant social behaviors, as well as repetitive behaviors replicating what has previously been reported in TS2-neo mice (Bader et al., 2011). Effects were also seen on a social dominance task, with mutants winning significantly more trials compared to their WT controls (thus showing more aggressive behavior). However, we did not observe any differences in social preference or recognition on the three-chamber task. This is actually consistent with Bader et al., (2011), where no differences in sociability measures were seen between TS2-neo and WT mice during the first 10 min of the task. In fact, Bader et al., (2011) only found differences in social behavior by testing over an unusually extensive period (a few hours, which is non-standard for the 3-chamber task). Also, our subjects were given the option to explore a novel inanimate

object, rather than an empty chamber (as used by Bader et al.). These discrepancies in protocols may explain why we did not observe Genotype differences in social behavior on this task. However, we found that TS2-neo subjects spent significantly less time vocalizing to a female in a vocalization task. Importantly, Bader et al., (2011) previously observed reduced vocalizations in pups, but not adults. Our findings show that aberrant social and communicative behaviors persist into adulthood, and across different types of social interactions. Lastly, subjects underwent a marble burying task, which we showed that TS2-neo mice buried twice as many marbles compared to their controls. This excessive burying is further indicative of repetitive, restricted and perseverative behavior -- another core symptom of ASD. Overall, these findings replicate prior reports on the TS2-neo model (Bader at al., 2011), and affirm that this mouse model exhibits core ASD-like traits such as repetitive behaviors and altered social behavior and ultrasonic vocalizations.

4.5.2 Motor and Spatial Learning

In addition to phenotyping core behavioral symptoms of ASD, we evaluated the TS2-neo mice on both motor and spatial learning. Although not a core criterion, motor and procedural learning deficits have been noted in individuals with ASD and it is thought that deficits in procedural learning may contribute to the cognitive and behavioral phenotype of autism. Here, the TS2-neo mice showed deficits in motor coordination and motor learning, as indicated by a lack of improvement across days on the Rotarod task. There have been parallel reports of impairments in motor coordination in ASD populations across a wide range of behaviors (Fournier et al., 2010). Furthermore, it has also been reported that children with ASD demonstrate diffusely decreased connectivity across the motor execution network relative to control children (Mostosky et al., 2009). This cortico-cerebellar connectivity dysfunction is also considered a core characteristic of

ASD, and is thought to contribute to anomalies in both sensory-motor control, and higher function such as social cognition and emotion (Crippa et al., 2016).

Finally, for spatial learning and memory in TS2-neo mice, we found no differences between Genotypes on either the Morris water maze or the 4/8 radial arm water maze. Similarly, Bader et al, (2011) evaluated TS2-neo mice on the Morris water maze and found no differences in learning. However, they did report that TS2-neo mice perseverated to the previous learned quadrant during a reversal task, suggesting that TS2-neo mice display comparable initial learning but are cognitively inflexible when given a reversal task. Our results here replicate initial findings as well as exemplify that TS2-neo mice can perform adequately on a complex spatial reference and working memory task as seen on the 4/8 radial arm water maze.

4.5.3 Auditory Processing Enhancements

The current study revealed novel findings that TS2-neo mice displayed superior performance on both the embedded tone and silent gap task for short cue durations. Enhanced low-level perceptual discrimination has been reported in individuals with ASD for visual and auditory stimuli (Bertone et al., 2005; Mottron et al., 2006; Plaisted et al., 2003) , and this is the second ASD-like mouse model our lab has reported to display enhanced performance on an auditory processing task. Specifically, we previously found that *Cntnap2* KO mice displayed superior discrimination on an embedded tone and pitch discrimination task (Truong et al., 2015). Interestingly, this type of superiority in auditory processing is not seen in other mutant rodent models, for example knock-outs using candidate susceptibility dyslexia risk genes (Rendall et al., 2015; Truong et al., 2014; Szalkowksi et al., 2013; Szalkowksi et al., 2012). Thus although low-level superiority in auditory processing may be related to language deficits seen in ASD (Eigsti & Fein, 2013), these same superiorities do not seem to occur in other language-specific

developmental disorders (e.g., dyslexia and specific language impairment). As such, low-level auditory enhancements form a particularly interesting aspect of the ASD-like animal model profile. An additional new finding --that better performance on the EBT 0-10 task (on which we have reported a similar atypical superiority in another ASD mouse model, *Cntnap2*; Truong et al., 2015) is actually correlated to fewer neurons in the MGN (which has generally been associated with deficits including language anomalies; Galaburda, Menard, & Rosen, 1994) – may further support the notion that low-level sensory enhancements are directly related to auditory-based language impairments in at least a subset of individuals with ASD. Further research should investigate other well-established mouse models of ASD by focusing on auditory processing patterns, to see if this trend of superior auditory processing is ubiquitous across multiple ASD mouse models beyond the TS2-neo and *Cntnap2* KO mice, and whether the relationship with anomalous MGN cellular morphology can be replicated.

Genetic, neurodevelopmental and behavioral exploration of these enhancements is important because low-level perceptual abilities are particularly important for the development of language, and differences in processing may be contributing to core features of autism such as language delay and aberrant social skills. In fact, atypical auditory processing in children with autism may be key to parsing different etiologies of autism, establishing interventions, and ameliorating overwhelming auditory sensory input to facilitate language development.

4.5.4 Enhanced perceptual functioning and cortico-cortical disconnection theories

The behavioral phenotype validated in the TS2-neo mouse model also aligns with the enhanced perceptual functioning theory of ASD put forth by Mottron et al. (2006). These authors contend that the paradoxical co-occurrence in ASD of enhanced sensory perceptual abilities and compromised global sensory integration (including social and language deficits) might be

explained by a developmental re-orientation of cortical functional patterns. Specifically, locally oriented "low-level" processing mechanisms may be enhanced and favored over more complex, integrative strategies that engage global and long-range processing mechanisms. This may further relate neurocognitively to regional hyper-connectivity and long-range hypo-connectivity (discussed further below).

4.5.5 Atypical white matter development in the TS2-neo

A growing body of neuroimaging studies identifies abnormal development of white matter tracts and organization of grey matter structures as markers of an ASD neurostructural phenotype, which we saw reflected in our own results. In the current study, we report reductions in volumes of the external capsule and fornix, two major white matter tracts. The external capsule contains cortico-cortical association fibers that form the basis of intercortical communication between the basal forebrain and other regions of the cerebral cortex, while the fornix is a bundle of commissural fibers connecting the hippocampus, mammillary bodies, and thalamus between the two hemispheres of the brain. Our findings of reductions in the fornix parallel reported results from neuroanatomical studies of the BTBR and NL3 mouse models of ASD, which showed decreases in fornix volume and integrity (Ellegood et al., 2015). Collectively, these results contribute to the larger body of work assessing ASD as a disorder of hypo-connectivity and disconnection between fronto-cortical and cortico-cortical networks. These pathways are critical for the integration of information that promotes normative socio-communicative development and deficits in connection integrity and structure are correlated with ASD symptom severity (Alexander et al., 2007; Poustka et al., 2012). This pattern of aberrant white matter development in ASD has become so consistent that some suggest that with further research it could become a unifying neuro-endophenotype for ASD.

4.5.6 TS2-neo mice display alterations within the MGN

Stereological analysis of the MGN revealed a reduction in MGN volume and number of neurons as well as neuronal size distribution shift toward more small neurons in TS2-neo mice relative to WTs. Furthermore, a significant correlation was observed between number of neurons and mean EBT10 performance in TS2-neo mice only. Co-occurrence of these findings suggests that aberrant MGN morphology is related to enhanced auditory processing phenotype, as observed in TS2-neo mice on EBT 0-10. These findings are consistent with in vivo neuroimaging studies showing fundamental differences in the thalamus of ASD individuals, including reduced volume (Tamura et al., 2010; Tsatsanis et al., 2003), altered neurochemical composition (Friedman et al., 2003), and abnormal thalamocortical connectivity (Chen et al., 2016; Cheon et al., 2011; Chugani et al., 1997; Mizuno, Villalobos, Davies, Dahl, & Muller, 2006; Muller et al., 1998; Nair et al., 2013). This collective evidence suggests that the thalamus may play a particular role in the etiology of ASD symptoms. TS2-neo neuroanatomical differences appear to be more robust within the right hemisphere, which corresponds to prior reports that TS2-neo brains are significantly more asymmetric than littermates (Bett et al., 2012). Further research is necessary to understand how alterations within the MGN may be contributing to the auditory enhancements observed, and in particular, why and how a reduction in the number of neurons and neuronal size could be advantageous to auditory processing but detrimental to language development.

Transgenic mouse models serve as a critical tool in evaluate the role of individual genes in the complex and polygenic cascade underlying neurodevelopment, and can further help to reveal how single-gene mutations can disrupt this process and result in neurodevelopment disorders (e.g., ASD). Among ASD genes identified, ~ 200 have been used to create engineered

mouse models, and associated phenotyping studies have revealed atypical social and perseverative/repetitive behaviors (3-chamber task, marble burying, alternating choice, task reversal) that can be linked to various disruptions in specific gene function (see Crawley, 2004 for review). Although major strides have been made in mapping the genetic etiology of ASD-like deficits in the social and repetitive domains, few studies have used transgenic mice to delineate some of the low-level enhancements associated with ASD. Yet these features may prove to be critical in unravelling neurogenetic influences on the higher-order language and communication deficits associated with ASD. Indeed, while several prominent research groups in the field have successfully associated ASD risk gene mutations with decreases in vocal calls and/or atypical call acoustics (Ey et al., 2013, Lai et al., 2014, Michetti, Ricceri & Scattoni, 2012; Penagarikano et al., 2011; Penagarikano& Geschwind, 2012), little animal work has focused on core perceptual and sensory processing features that may contribute to higher order anomalies. Here we have demonstrated an association between enhancements in low-level acoustic processing and a TS2-neo mutation associated with an ASD profile, bolstering prior data showing similar features in a *Cntnap2* KO mouse model of ASD. Ongoing research to examine perceptual processing, including such enhancements, in ASD models will continue to benefit our understanding of the neurogenetic etiology of this complex disorder.

Acknowledgements

This research was supported by NSF IGERT Grant 1144399 to the University of Connecticut (J. Magnuson, PI), by the Murine Behavioral Neurogentic Facility (MBNF) at the University of Connecticut and by the National Institutes of Health grant P01HD057853 to R.H. Fitch.

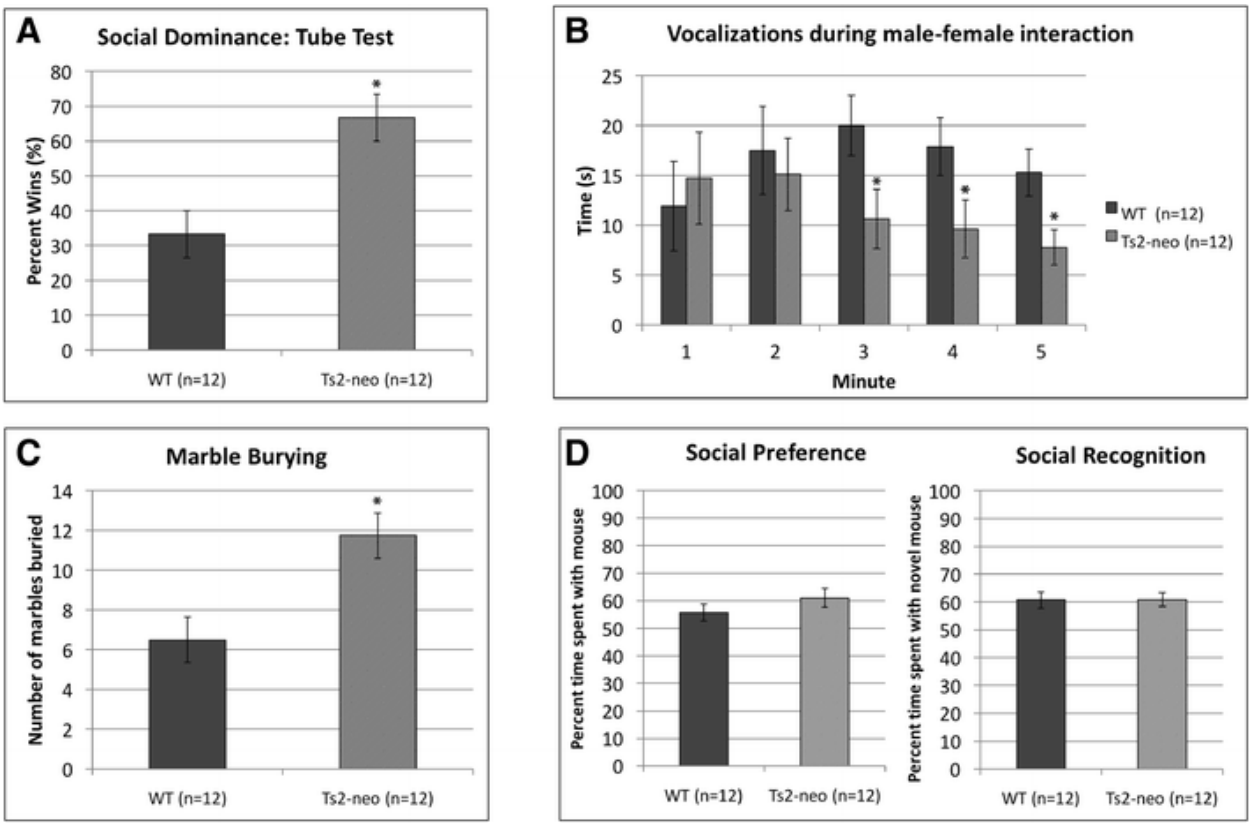

Figure 4.1 - TS2-neo mice display "ASD-like" behavioral profiles. (a.) Social Dominance: TS2-neo mice won significantly more trials on a tube test. (b.) Ultrasonic vocalizations during male-female interactions: TS2-neo mice vocalized significantly less during the last 3 minutes of interaction (c.) Marble burying: TS2-neo mice buried significantly more marbles.*p<.05

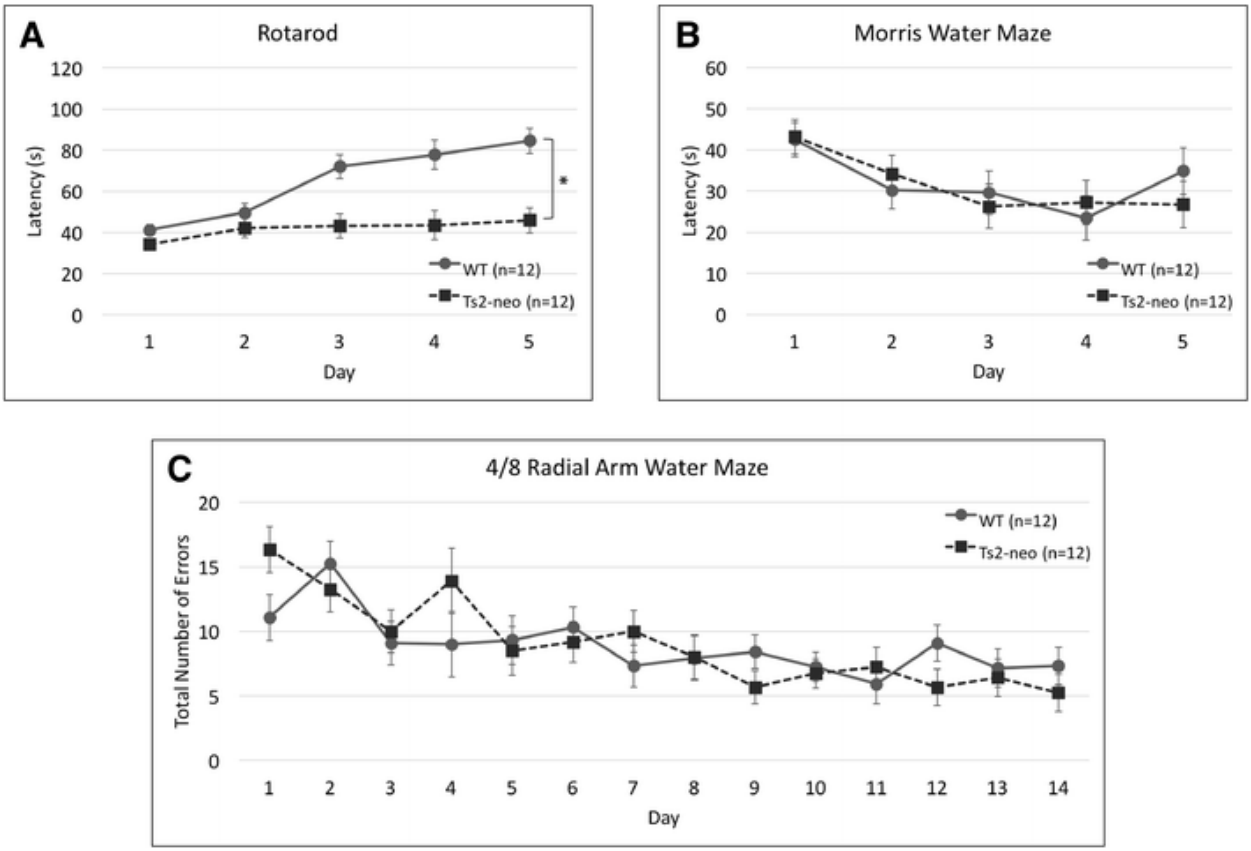

Figure 4.2 - TS2-neo mice show deficits in motor learning but not spatial learning. (a.) Rotarod: TS2-neo mice displayed a lack of motor learning. (b.) Morris Water Maze: TS2-neo mice and WTs performed comparably. (c.) 4/8 Radial Arm Water Maze: TS2-neo mice and WTs displayed similar learning curves on this complex spatial task.*p<.05

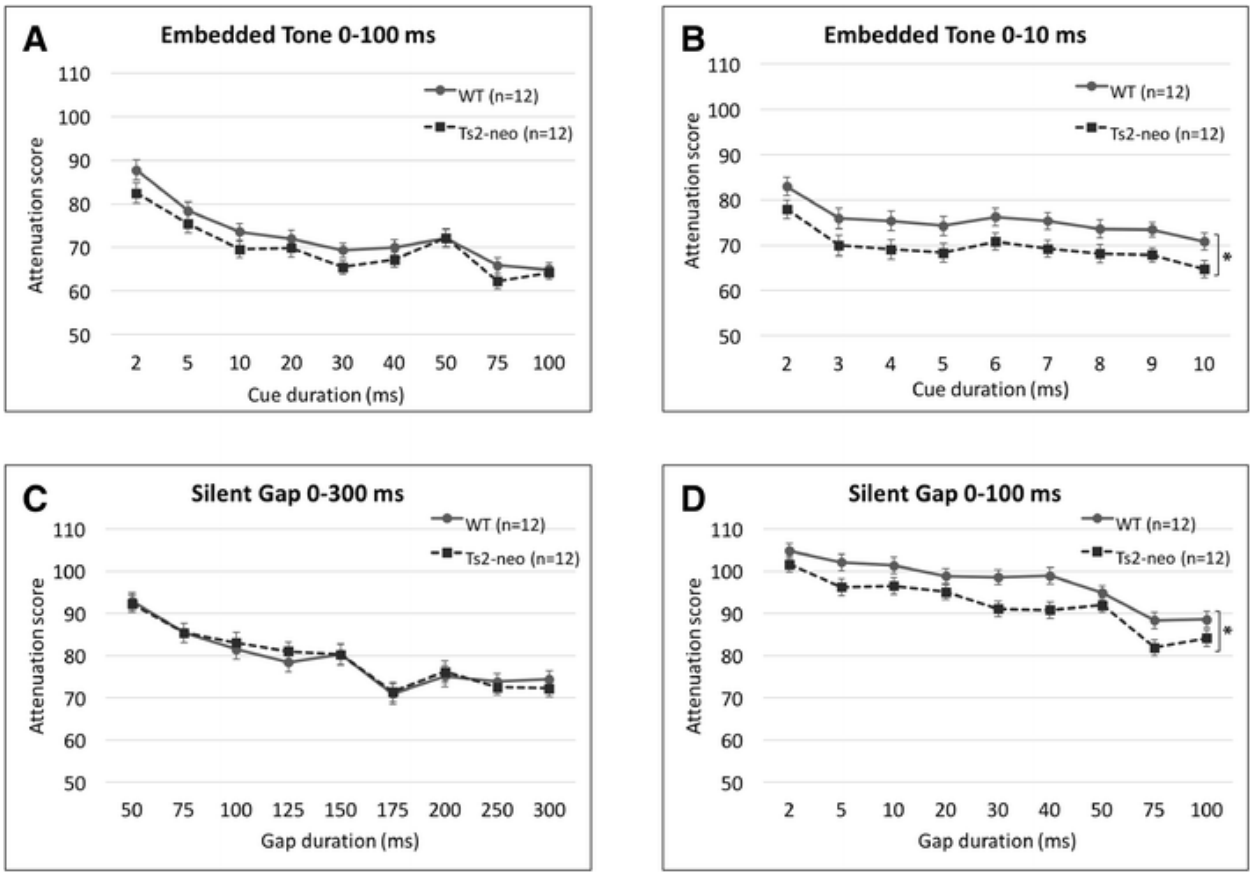

Figure 4.3 - TS2-neo mice exhibit superior processing of short-duration acoustic stimuli.

(a.) Embedded Tone 0-100 ms: TS2-neo mice and WTs showed comparable performance. (b.) Embedded Tone 0-10ms: TS2-neo mice had significantly lower (better) attenuation scores. (c.) Silent Gap 0-300 ms: TS2-neo mice and WTs had comparable performance. (d.) Silent Gap 0-100 ms: TS2-neo mice had significantly lower (better) attenuation scores.*p<.05

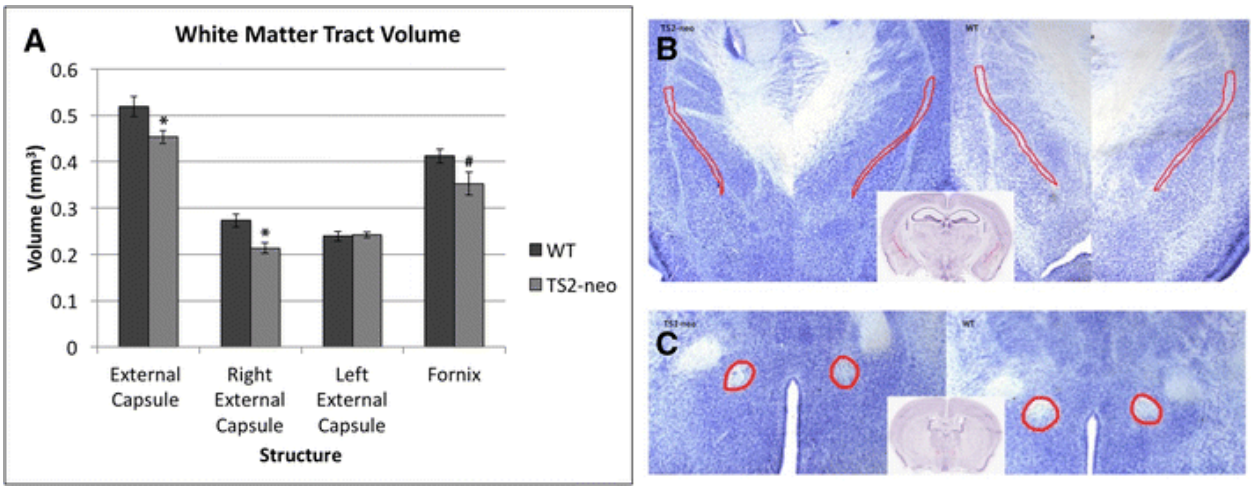

Figure 4.4 - TS2-neo mice show significant decrease in white matter structures. (a.) TS2-neo mice displayed a significant decrease in volume of the external capsule, specifically right external capsule, and a marginal decrease in the fornix. (b.) Location and size of external capsule (outlined in red) for representative TS2-neo and WT samples. (c.) Location and size of fornix (outlined in red) for representative TS2-neo and WT samples.* $p<.05$, # $p=.059$

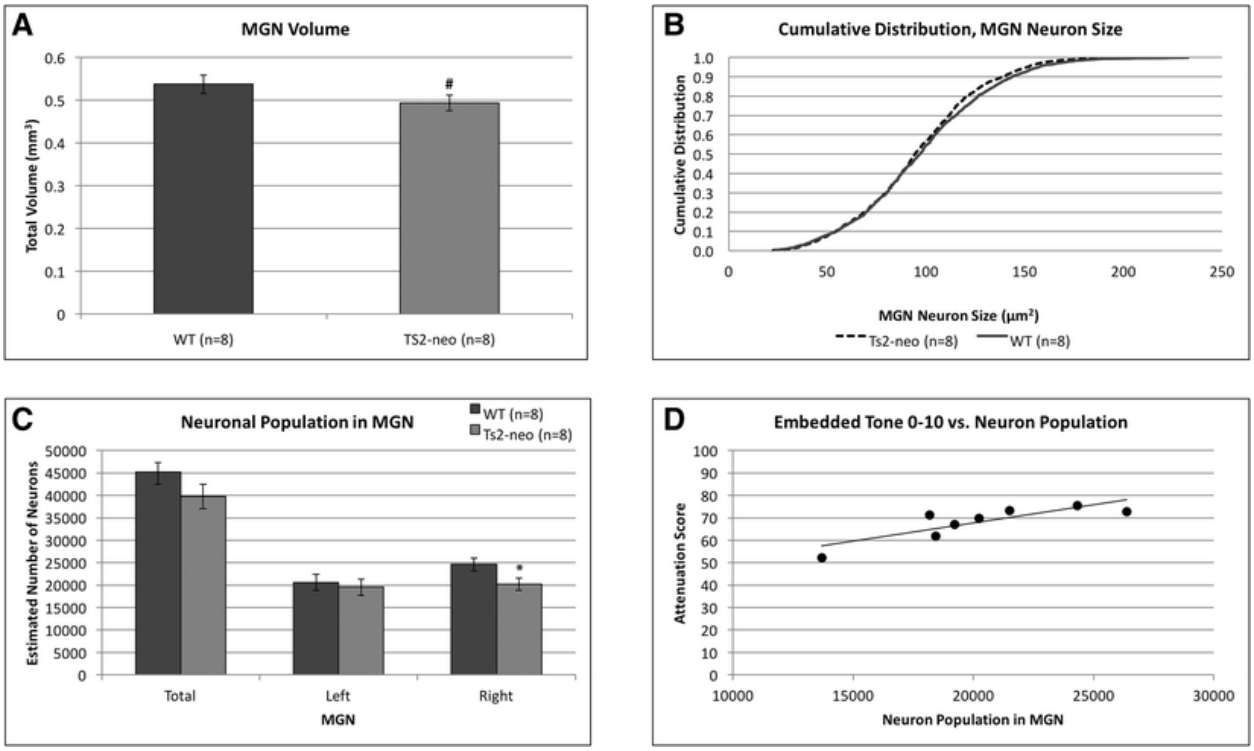

Figure 4.5- TS2-neo mice display anomalies in the medial geniculate nucleus. (a.) TS2-neo mice exhibit a marginally significant reduction in MGN volume. (b.) TS2-neo display a significant shift in cumulative MGN cell size distribution, with mutants showing more small and fewer large MGN cells compared to WTs. (c.) TS2-neo display reductions in number of MGN neurons, specifically within the right MGN. (d.) A significant positive correlation was revealed between EBT10 attenuation scores and number of neurons in MGN.* $p<.05$, # $p=.058$

Chapter 5

Evaluation of visual motion perception ability in mice with knockout of the dyslexia candidate susceptibility gene *Dcdc2*

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Submitted: Genes, Brain and Behavior (2017)

5.1 Abstract

Developmental dyslexia is a heritable disability characterized by difficulties in learning to read and write. The neurobiological and genetic mechanisms underlying dyslexia remain poorly understood; however, several dyslexia candidate risk genes have been identified. One of these candidate risk genes - doublecortin domain containing 2 (*DCDC2*) - has been shown to play a role in neuronal migration and cilia function. At a behavioral level, variants of *DCDC2* have been associated with impairments in phonological processing and working memory. More recently, a specific mutation in *DCDC2* has been strongly linked to deficits in motion perception - a skill subserving reading abilities. To further explore the relationship between *DCDC2* and dyslexia, a genetic knockout (KO) of the rodent homolog of *DCDC2* (*Dcdc2*) was created. Initial studies showed that *Dcdc2* KOs display deficits in auditory processing and working memory. Since motion perception skills have not yet been assessed in the *Dcdc2* KO mouse model, the current study was designed to evaluate the association between *DCDC2* and motion perception. We developed a novel motion perception task, utilizing touchscreen technology and operant conditioning. *Dcdc2* KOs displayed deficits on the Pairwise Discrimination task specifically as motion was added to visual stimuli. Following behavioral assessment, brains were histologically prepared for neuroanatomical analysis of the lateral geniculate nucleus (LGN). The cumulative distribution revealed that *Dcdc2* KOs exhibited more small neurons and fewer larger neurons in the LGN. Results compliment findings that *DCDC2* genetic alteration results in anomalies in visual motion pathways in a subpopulation of dyslexic patients.

5.2 Introduction

Developmental dyslexia is a heritable disability characterized by difficulties in learning to read and write that cannot be explained by comorbid factors such as intellectual impairment, lack of educational opportunity, or other neurological disorders (e.g. epilepsy or primary sensory impairments (blindness, deafness)). Dyslexia is considered a common neurodevelopmental disorder, affecting 5%-12% of the population (Lyon, Shaywitz & Shaywitz, 2003; Peterson & Pennington, 2012). It is also a highly heterogeneous disorder, with varied intermediate phenotypes, that include visual and cross-modal integration deficits. Dyslexia can also be deconstructed into core impairments that include problems with phonological processing (Kovelman et al., 2012; Melby-Lervag, Lyster & Hulme, 2012; Peyrin et al., 2012), short-term and/or working memory (Beneventi et al., 2010; Gathercole et al., 2006; Menghini et al., 2010), rapid auditory processing (Cohen-Mimran & Sapir, 2007; Fitch & Szalkowski, 2012; Hamalainen, Salminen & Leppanen, 2013), visuospatial attention (Franceschini, 2012; Gabrieli & Norton, 2012), and/or visual attention/perception (Galaburda and Livingstone, 1993; Stein and Walsh, 1997; Vidyasagar and Pammer, 2010).

With regards to visual deficits associated with dyslexia, atypical or impaired visual perception of motion may be one factor underlying slow reading speed (Lyon et al., 2003; Nicholson and Fawcett, 2007). For example, children with dyslexia are reported to have some combination of spatial (Cornelissen et al., 1995; Hansen et al., 2001; Lawton, 2000, 2007, 2008, 2011; Lovegrove et al., 1980; Stein, 2001; Stein and Walsh, 1997; Talcott et al., 2000) and/or temporal (Bradley and Bryant, 1983; Stanley and Hall, 1973; Tallal et al., 1993; Temple et al., 2003) visual sequencing deficits. These impairments could relate to reports from dyslexics that

letters within words, and words on a page, often appear distorted, displaced, or crowded together (Atkinson, 1991).

The biological mechanisms underlying intermediate dyslexia phenotypes remain poorly understood. However, heritability rates ranging from 40% to as high as 80% indicate a strong genetic basis (Schumacher et al., 2007). To date, several dyslexia candidate risk genes have been identified, including *DYX1C1, DCDC2, KIAA0319* and *ROBO1.* One of these candidate risk genes - doublecortin domain containing 2 (*DCDC2*) - has been shown to play a major role in neuronal migration during early development, as well as cilia function throughout life (Meng et al., 2005; Burbridge et al., 2008; Lee & Gleeson, 2010; Massinen et al., 2011; Szalkowski et al., 2012). At a behavioral level, variants of *DCDC2* have been associated not only with dyslexia per se, but also intermediate phenotypes such as impairments in phonological processing and working memory (Berninger et al., 2008; Marino et al., 2011). One specific mutation in *DCDC2* has been strongly linked to deficits in motion perception - a skill important to reading ability (Cicchini et al., 2015; Gori et al., 2015). This finding is consistent with evidence that visual processing deficits are more prominent in dyslexic samples when images are rapidly presented or moving, and associated theories of magnocellular neural timing deficits in dyslexia (Boets et al., 2011; Lawton, 2000, 2007, 2008, 2011; Livingstone et al., 1991; Stein, 2001; Stein and Walsh, 1997; Vidyasagar, 1999, 2001, 2012; Vidyasagar and Pammer, 2010;). Importantly, deficits in motion perception can be detected well before impairments in reading or even language, and visual motion thresholds are predictive of language outcome, making motion perception a useful intermediate phenotype for early diagnosis and intervention (Boets et al., 2011).

Motion processing deficits may reflect vulnerability of the magnocellular-dorsal system, which is a reoccurring biological theme in the study of dyslexia (Danelli et al., 2013; Gori et al.,

2015; Schulte-Körne and Bruder, 2010; Stein 2001). In fact, early evidence pointed to a selective deficit of the magnocellular-dorsal system as measured both by atypical cellular morphology in thalamic nuclei (Galaburda and Kemper, 1979; Galaburda et al., 1985; Galaburda & Livingstone, 1993; Livingstone et al., 1991), as well as electrophysiological profiles during motion-related magnocellular processing tasks (Cornelissen et al., 1995; Demb et al., 1998; Lovegrove et al., 1980; Slaghuis and Ryan, 1999, 2006; Stein and Walsh, 1997)The most direct evidence of aberrations in the visual magnocellular system came from post mortem brains of dyslexics, where the magnocellular layers of the lateral geniculate nucleus (LGN) were found to be disordered, and the neurons were 30% smaller compared to control brains (Galaburda and Livingstone, 1993; Livingstone et al., 1991).

To further explore the relationship between *DCDC2* and dyslexia, a genetic knockout (KO) of the rodent homolog of *DCDC2* (*Dcdc2*) was created. Initial behavioral studies showed *Dcdc2* KOs mice displayed persistent visuo-spatial memory deficits, as well as visual discrimination and long-term and working memory deficits on cognitively demanding tasks (Gabel et al., 2011; Truong et al., 2014). Further assessments revealed deficits in rapid auditory processing as well as disrupted ability to identify speech sounds in cortical knockdown (RNAi) *Dcdc2* rats (Centanni et al., 2016; Truong et al., 2014). Electrophysiological studies found that cortical pyramidal neurons from *Dcdc2* KO mice exhibited increased excitability and decreased temporal firing precision, which could explain a link between *DCDC2* and motion perception deficits (Che et al., 2016). Since visual motion perception skills have not yet been assessed in the *Dcdc2* KO mouse model, the current study was designed to evaluate this association. Specifically, we developed a novel motion perception task based on published human behavioral paradigms and stimuli used with dyslexic samples. To test our mice we used touchscreen

technology and operant conditioning in a modified Pairwise Discrimination task. Following shaping, subjects were exposed to visual stimuli known as Gabors (adjusted for mouse vision to provide higher contrast and longer display) adapted from those previously used to assess individuals with dyslexia (Cicchini et al., 2015). Following behavioral assessment, brains were histologically prepared for neuroanatomical analysis of the LGN. This was based on the relevance of the LGN in the magnocellular-dorsal system and evidence in dyslexic post mortem brains. Our overall goal was to replicate and extend the clinical finding that *DCDC2* influences not only auditory processing and working memory, but also visual motion perception.

5.3 Materials and Methods

5.3.1 Subjects

Dcdc2 knockout (KO) mice (*Dcdc2*del2/del2) carried a constitutive homozygous deletion of exon 2 (del2) within the *Dcdc2* gene region of a 129SJ x C57BL/6J hybrid background backcrossed to C57BL/6J for 10 generations (see Wang et al., 2011 for details). Our subjects were generated from the *Dcdc2* colony maintained by JJL at the University of Connecticut, using a heterozygous-heterozygous (*Dcdc2*wt/del2 × *Dcdc2*wt/del2) mating scheme, with resultant genotypes recovered in the expected mendelian ratios (1:2:1). Only male subjects were assessed on subsequent behavioral measures, 7 *Dcdc2* KO mice and 7 wild-type littermate controls. All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water ad lib, until the start of operant testing (when food restriction was implemented). Subjects were gradually food restricted to 80%-85% of their baseline body weight (3 weeks prior to operant testing). During the last week before training, subjects were given a sample of the food reward (Strawberry Ensure Plus, Abbott, IL, USA) in their home cage, to habituate them to its taste and eliminate hyponeophagia. All behavioral testing occurred during the light cycle.

Procedures were performed blind to genotype, and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

5.3.2 Touch-screen Operant Conditioning

Pairwise visual discrimination was tested in the automated Bussey-Saksida touchscreen apparatus for mice (Campden Instruments Ltd/Lafayette Instruments, Lafayette, IL, USA), using a procedure modified from methods described previously (Brigman and Rothblat, 2008; Brigman et al., 2013; Bussey et al., 2012; DePoy et al., 2013; Oomen et al., 2013; Silverman et al., 2013). The reinforcer was 20 µl of a palatable liquid nutritional supplement (Strawberry Ensure Plus). Each session was conducted under overhead lighting (~60 lux). A standard tone cue was used to signal the delivery of the reinforcer during pre-training and acquisition. Prior to pre-training, subject mice were weighed, and placed on a restricted diet of 2–4 g of rodent chow per mouse per day, to induce a 15% weight loss. Body weight was carefully monitored throughout the experiment, to ensure that a minimum of 80% of free feeding age-corrected body weight was maintained for each mouse.

5.3.3 Pairwise Discrimination (P100-340)

Before subjects advanced to Pairwise Discrimination testing they underwent a series of 'pretraining' sessions, in which they learned to make instrumental responses in the touchscreen apparatus. Pre-training consisted of Habituation, Initial Touch Training, Must Touch Stimuli, Must Initiate, Punish Incorrect (Horner et al., 2013). After completion of training, subjects progressed to Pairwise Discrimination Testing. At the start of the session a free delivery of food was made and the tray light was turned on. The mouse had to nose poke and exit the food-tray to begin the first trial. When a trial was initiated, two novel stimuli were presented on the screen -- one programmed as correct (S+), and the other incorrect (S-). S+ and S- were presented on the

left or right pseudo-randomly. Food delivery was accompanied by illumination of the tray light and a tone. Entry to collect the food turned off the tray light and started the intertrial interval (ITI). After the ITI, the tray light was illuminated and the mouse was required to nose poke and exit to initiate the next trial. If the mouse touched the incorrect stimulus, no reward was delivered and a timeout followed, which entailed illumination of the house light and an audible tone. Pairwise Discrimination was performed in 4 stages, using progressively more complex stimuli. The first stage of testing used stock black/white images, followed by static black/white Gabors, then moving Gabors, and finally moving dots (Fig. 5.1).

5.3.4 Histology

At P360 subjects were weighed, anesthetized (ketamine/xylazine (100/15 mg/kg)), and transcardially perfused with 0.9% saline followed by 10% formalin. Brains were extracted and post-fixed in 10% formalin. Brains were serially sectioned in the coronal plane (60 μm) using a vibratome (Leica VT1000 S). Every second section was mounted on gelatin-subbed slides and stained for Nissl bodies using cresyl violet (coverslipped with DPX mounting medium).

5.3.5 **Stereological Measures**

All prepared tissue was analyzed using *Stereo Investigator* (MBF Biosciences, Williston, VT, USA) integrated with a Zeiss Axio Imager A2 microscope (Carl Zeiss, Thornwood, NY). Experimenters were blind to subject genotype. Volumes were reconstructed from serial area scores using the *Cavalieri Estimator* probe in *Stereo Invesigator*. Neuronal cell populations were estimated using the *Optical Fractionator* probe, with cross sectional neuronal cell area estimated concurrently using the *Nucleator* probe. Measurements were made using a sampling frequency of every 2nd section, with the LGN appearing on 8-10 sections. Contours of the LGN for

volumetric measures were drawn at 2.5× magnification, and a standard stereotaxic atlas was used to determine borders (Paxinos & Franklin, 2004). All cell estimations were performed under 60× oil-immersion using the fractionator with a sampling grid of 250 × 250 μm and a counting box of 30 × 30.

5.3.6 Statistical Analysis

All subjects were used for analysis (WT, n = 7; *Dcdc2* KO, n = 7). Group differences on Pairwise Discrimination performance were analyzed using a 2 (Genotype; WT and *Dcdc2* KO) x # (Weeks) repeated measures analysis of variance (ANOVA), where number of weeks varied by stimulus. All analyses used performance on the standard visual Pairwise Discrimination task as a covariate, to account for baseline differences between subjects. Group differences in volume and cellular measurements of the LGN (estimated neuron population and size within defined boundaries) were assessed using univariate ANOVAs. Examination of cell size distribution was conducted using a cumulative percent distribution. To examine group differences in cell size distribution, non–parametric analyses (Kolmogorov Smirnov; K-S) were conducted on the cumulative percent distributions of each Genotype. All statistical analyses were conducted using SPSS 19 with an alpha criterion of 0.05, two-tailed.

5.4 Results

5.4.1 Pairwise Discrimination

A repeated measures ANOVA revealed no significant differences in Genotype on the standard Pairwise Discrimination task for percent correct across a 5 week period [$F_{1,12} = .688$, $p>.05$] (Fig. 5.2a). There was, however, a significant Week effect [$F_{4,48} = 22.640$, $p<.01$]. Both WT and *Dcdc2* KOs scored progressively higher percent correct scores, indicative of learning. For

the next stage of testing (static Gabors), a repeated measure ANOVA was also performed on percent correct. This also revealed no significant differences in Genotype for percent correct across a 3 week period [$F_{1,11} = 3.689$, $p>.05$] (Fig. 5.2b). There was again, however, a significant Week effect [$F_{2,22} = 6.683$, $p<.01$] and a marginally significant Week x Genotype interaction [$F_{2,22} = 3.356$, $p=.053$], with WTs showing gradual improvement on this task while the *Dcdc2* KOs did not (Fig. 5.2b). For the next stimulus (moving Gabors), repeated measures ANOVA revealed marginally significant differences in Genotype on the Pairwise Discrimination task (percent correct across a 6 week period, [$F_{1,11} = 4.223$, $p=.064$]; Fig. 5.2c). There was no significant effect of Week [$F_{5,55} = .591$, $p>.05$], with WTs showing only slight improvement over time (although they started at 84 percent correct). Lastly, a repeated measures ANOVA showed a significant Genotype effect [$F_{4,44} = 1.752$, $p<.05$] on the Pairwise Discrimination task when more difficult moving dots were used (percent correct across a 5 week period; Fig. 5.2d). WTs consistently scored higher compared to *Dcdc2* KOs throughout this session, although we saw no Week effect [$F_{4,44} = 1.752$, $p>.05$] or interaction [$F_{4,44} = .653$, $p>.05$] (Fig. 5.2d).

5.4.2 LGN Assessment

A univariate ANOVA comparing LGN volumes revealed no main effect of Genotype [left: $F_{1,12} = .537$, $p>.05$; right: $F_{1,12} = .3.73$, $p>.05$; total: $F_{1,12} = .510$, $p>.05$] (Fig 5.3a). Examination of LGN neuronal population also failed to show a significant effect of Genotype [left: $F_{1,12} = .894$, $p>.05$; right: $F_{1,12} = .363$, $p>.05$; total: $F_{1,12} = 2.781$, $p>.05$] (Fig. 5.3b), although results did trend for *Dcdc2* KOs to exhibit fewer neurons than WTs. Comparisons of cumulative percent distributions between *Dcdc2* KOs and WT controls using a Kolmogorov Smirnov test did reveal a significant K-S statistic ($p<.05$; Fig. 5.3c). Specifically, analysis of cell size distribution

revealed that *Dcdc2* KO brains contain more small and fewer large LGN neurons compared to controls.

5.5 Discussion

The purpose of this study was to behaviorally and neuroanatomically evaluate *Dcdc2* KO mice on motion perception, as well as visual thalamic (LGN) cellular composition. *Dcdc2* KOs did in fact exhibit significant deficits on motion perception, especially as stimuli became more complex (i.e., moving dot patterns). We did not observe any significant learning impairments on the standard Pairwise Discrimination task, although learning and memory deficits have previously been observed in this mouse model on other more cognitive demanding tasks (Gabel et al., 2011; Truong et al., 2014). We did, however, observe deficits on the Pairwise Discrimination task specifically as motion was added to visual stimuli. Interestingly, both WTs and *Dcdc2* KOs displayed better performance when motion was added to the stimuli, however, *Dcdc2* KOs consistently scored lower on percent correct compared to WTs. After weeks of moving Gabor testing, subjects advanced to the most difficult task, moving dots. Here, *Dcdc2* KOs showed percent correct means that hovered at chance levels while WTs consistently scored above chance after the first week of testing, and these scores showed a significant Genotype effect.

Our findings are generally consistent with a "magnocellular" theory of dyslexia (Galaburda et al., 1994; Livingstone et al., 1991; Stein, 2001). This theory posits that both visual and auditory processing dysfunction associated with reading disability can be attributed to low-level processing deficits resulting from specific disruption to the magnocellular sub-systems of thalamic nuclei, and potentially elsewhere in sub-cortical sensory structures (Galaburda et al., 1994; Livingstone et al., 1991; see Stein, 2001, for review). It is well established that the magnocellular component of the visual system is specialized for processing rapidly-changing

temporal information (e.g., motion). Although the literature surrounding this theory remains controversial, early studies conducted by Livingstone et al. (2001) and Galaburda et al. (2004), examining post mortem human brain tissue from dyslexic individuals revealed a reduction in the distribution of large cells ("magnocells") versus small cells ("parvocells") in both the lateral geniculate (visual) and medial geniculate (auditory) nuclei of the thalamus of these subjects (noting that a distinct magnocellular sub-system has not been identified in the human MGN). It is thought that a resulting disruption in temporal processing in both visual and auditory domains might make it difficult for individuals with dyslexia to process rapidly changing sensory input (Stein & Walsh, 1997).

In the current study, all subjects underwent histological preparation and stereological evaluation and analysis of the cumulative distribution revealed that *Dcdc2* KOs exhibited more small neurons and fewer larger neurons in the LGN. This is similar to what has been reported in dyslexic post mortem brains. Although, the mouse LGN does not contain the 6 layers of neurons as seen in cats and primates (Connolly and Van Essen, 1984; Dreher et al. 1976; Kaas et al., 1972; Malpeli and Baker, 1975; Sherman et al., 1976), there has been evidence suggesting magno and parvo-like cells are scattered in "salt and pepper" fashion throughout the LGN in mouse (Piscopo et al., 2013). The findings of more small and fewer large LGN neurons observed in *Dcdc2* KOs parallel clinical findings, and do support a magnocellular theory of dyslexia.

It should be noted that the low sample size of 7 per genotype was a major limitation of this study. Additionally, the development and adaptation of the pairwise discrimination task to moving stimuli was another limitation. Future experiments should incorporate a larger n and may adjust the parameters of the stimuli (e.g., speed and contrast). The level of difficulty when transitioning

from moving Gabors to moving dots was greater than anticipated, and it would be ideal to insert an intermediate version of the task in future studies.

Overall, our results compliment findings that *DCDC2* genetic alteration results in anomalies in visual motion pathways in a subpopulation of dyslexic patients (Cicchini et al., 2015; Gori et al., 2015). *DCDC2* appears to play a role in the development neural processes governing typical motion perception, and our results are the first to directly evaluate the association between *DCDC2* and motion perception using transgenic mice. Through ongoing phenotyping of mouse models, we can continue to gain insight on the role of individual genes critical to the polygenic developmental cascade subserving language abilities, including reading. Continued research may support more advanced early screening, as well as genetically-driven individualized treatment techniques for neurodevelopmental disorders that include language and communicative impairments.

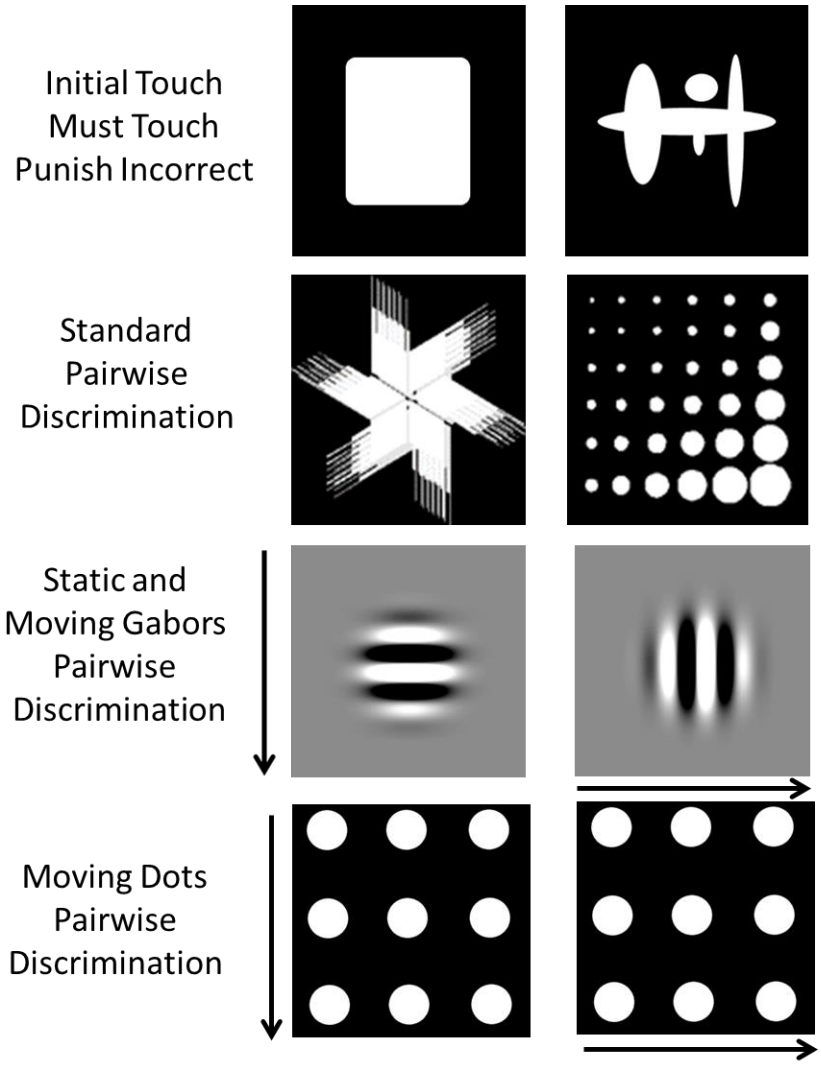

Figure 5.1. Visual stimuli presented throughout the different stages of Pairwise Discrimination.

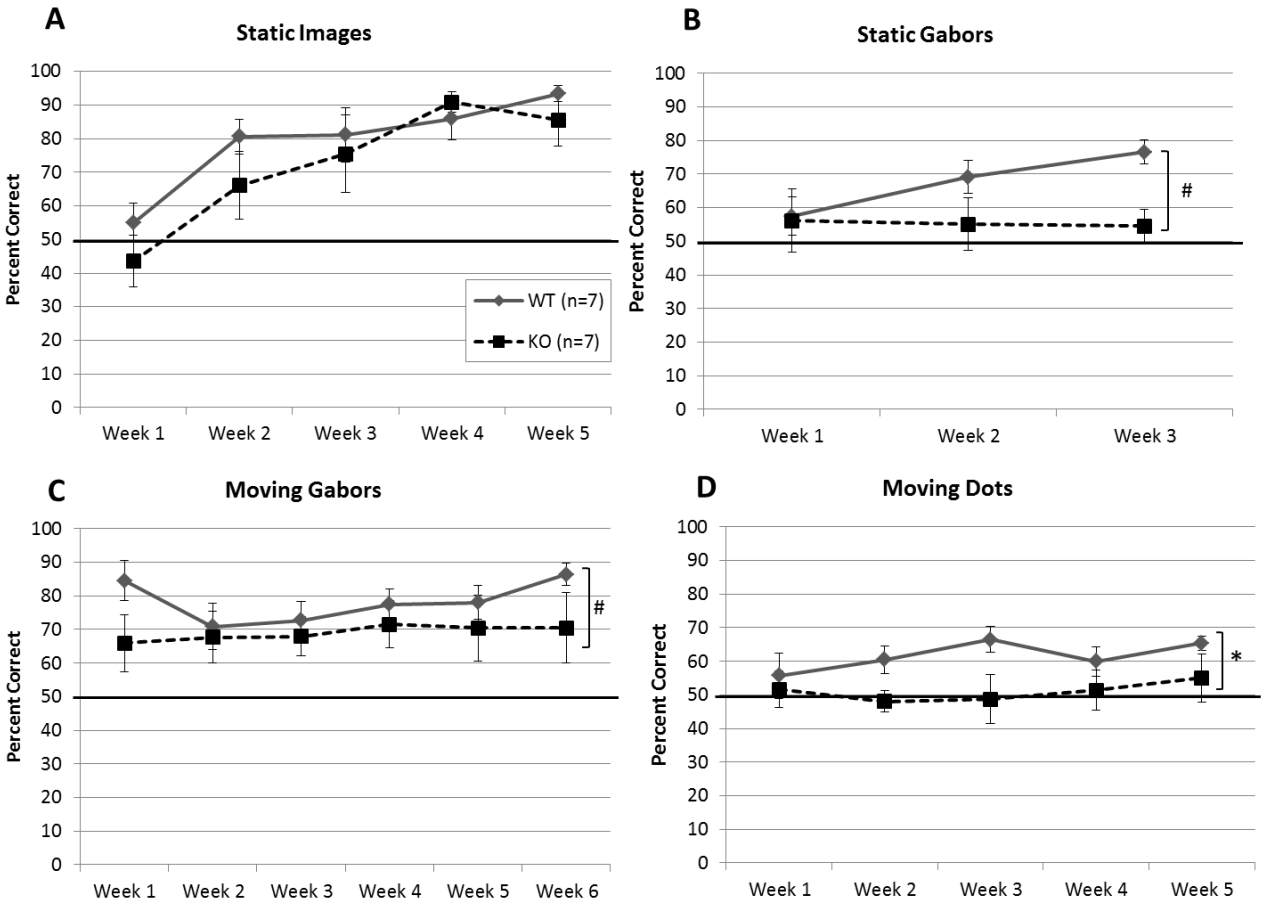

Figure 5.2. Pairwise discrimination performance of *Dcdc2* KO mice across various stimuli. Overall percent correct was analyzed across weeks. (a) Static Images. (b) Static Gabors. (c) Moving Gabors. (d) Moving Dots. Data shown are mean ±SEM for each group, n = 7 for each genotype. * $p < .05$; # $p < .10$

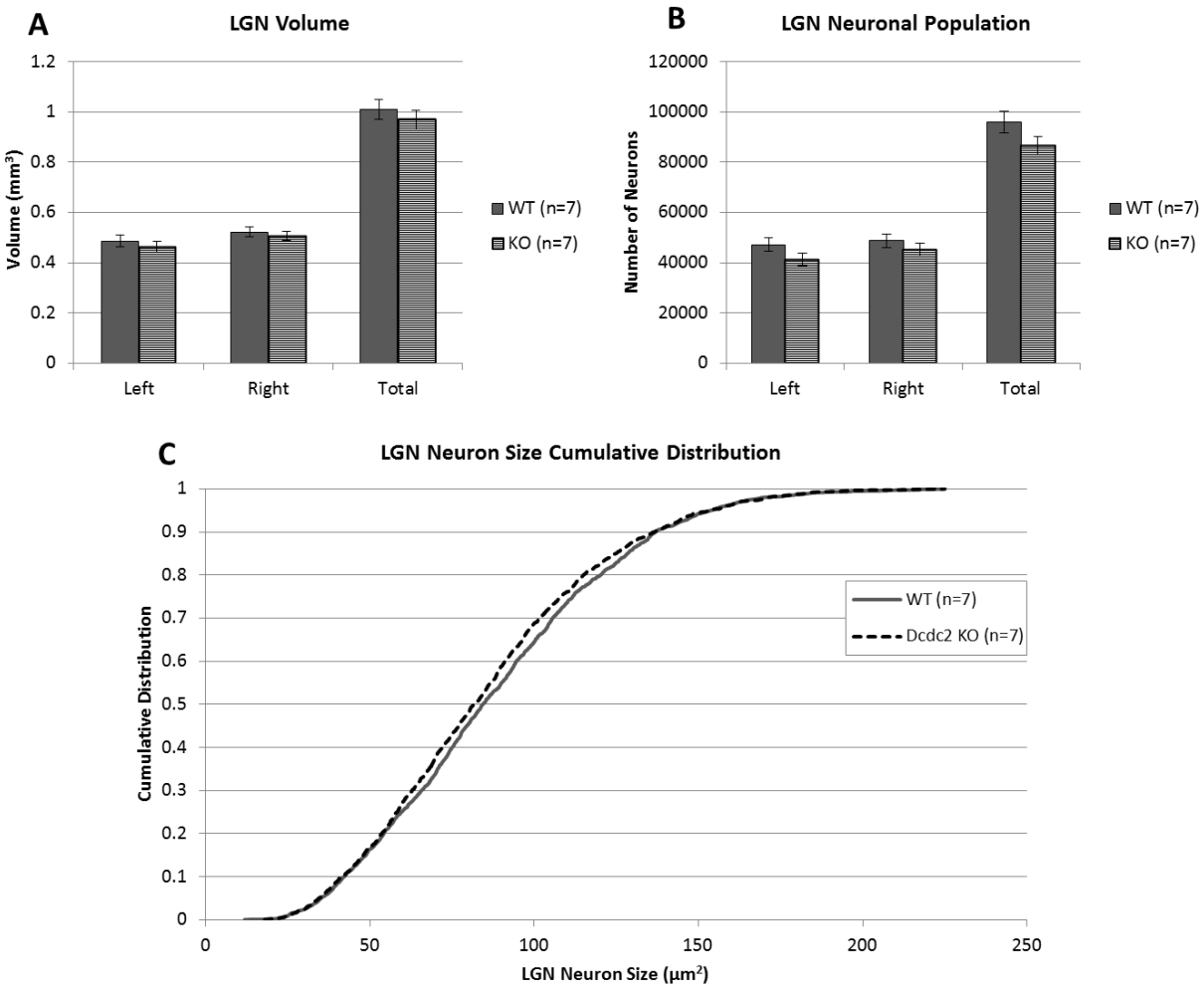

Figure 5.3. Lateral geniculate nucleus assessment of *Dcdc2* KO mice. (a) LGN Volume. (b) LGN Neuronal Population.(c) LGN Neuron Size Cumulative Distribution.

Chapter 6

Behavioral characterization of *Shank3B* knockout mice

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In preparation.

6.1 Abstract

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by a core set of atypical behaviors in social-communicative and repetitive-motor domains. Individual profiles are highly heterogeneous, and language abilities in ASD range from nonverbal to hyperlexic. To date, causal mechanisms underlying ASD remain poorly understood, but appear to include a complex combination of polygenic and environmental risk factors. Heritability rates vary from 70-90% and up to 1,000 risk genes have been identified, pointing to a complex genetic architecture. *SHANK3* (SH3 and multiple ankyrin repeat domains 3) is among a handful of highly-replicated ASD-risk genes, with haploinsufficiency of *SHANK3* following deletion or *de novo* mutation seen in about 1% of non-syndromic ASD. *SHANK3* is a synaptic scaffolding protein enriched in the postsynaptic density of excitatory synapses. In order to more closely evaluate the contribution of *SHANK3* to neurodevelopmental expression of ASD, a knockout mouse model with a mutation in the PDZ domain was developed. Initial research showed compulsive/repetitive behaviors and impaired social interactions in these mice, replicating two core ASD features. The current study was designed to further examine *Shank3*B heterozygous and homozygous knockout mice for behaviors that might map onto atypical language in ASD (e.g., auditory processing, and learning/memory). We report findings of atypical sensorimotor and social behaviors (replicating prior reports), as well as new evidence that *Shank3* KO mice have typical auditory processing abilities with specific low-level enhancements (mapping onto heightened pitch discrimination seen in ASD), along with robust learning impairments.

6.2 Introduction

Autism Spectrum Disorders (ASDs) are a set of neurodevelopmental disorders characterized by a complex behavioral phenotype, encompassing deficits in both social and cognitive domains. The core symptoms range from atypical social interactions and language impairments to repetitive behaviors (American Psychiatric Association, 2013). Accordingly, individual cases range substantially in severity and presentation of symptoms. Currently, the estimated prevalence in the United States identifies 1 in 68 children as having ASD, with a strong male bias among those diagnosed (1 in 42 boys versus 1 in 189 girls) (Elsabbagh et al., 2012; Newschaffer et al., 2007). To date, causal mechanisms underlying ASD remain poorly understood, but likely include a complex combination of polygenic and environmental risk factors (Moreno-De-Luca, 2013).

Ongoing ASD research has been focused on investigating the genetic and neurobiological mechanisms of ASD, based on the notion that characterization of the varied neurogenetic features of ASD could provide insight to the diverse behavioral symptoms and variability observed. Multiple lines of evidence suggest the genetic contribution in ASD appears to be strong. For example, monozygotic twin studies estimate concordance rates as high as 70% - 90%, which is greater than that of any other known cognitive and/or behavioral disorders (Bailey et al., 1995; Rosenberg et al., 2009; Steffenburg et al., 1989). Furthermore, the recurrence estimates of infants with at least 1 older sibling with ASD are more than tenfold higher (Constantino et al., 2010; Ozonoff et al., 2011). Additionally, there are documented familial patterns of inheritance for qualitatively similar phenotypes (albeit with less severe behavioral and cognitive deficits, but falling under the broader autism phenotype) in first-degree relatives of identified probands, further supporting heritability of ASD (Bolton et al., 1994;

Bishop et al., 2004). Besides the strong monogenic risk factors that underlie syndromes such as Fragile X and Rett, the relative proportion of ASD that can be accounted for by either rare or common genetic variation remains to be determined and no single gene has been identified as a major cause. In fact, it is suggested that over 1,000 genes are involved in ASD, reflecting a complex genetic architecture (De Rubeis & Buxbaum, 2015). Additionally, no one of these known genetic contributors accounts for more than 1-2% of the phenotypic variance seen in ASD, despite the strong inheritance pattern (Abrahams & Geschwind, 2008).

One highly replicated ASD-risk gene is *SHANK3* (SH3 and multiple ankyrin repeat domains 3), which is a synaptic scaffolding protein enriched in the postsynaptic density of excitatory synapses, and that plays a crucial role in synaptic plasticity Grabrucker et al., 2011; Naisbitt et al., 1999). Heterozygous deletions or point mutations of *SHANK3* are thought to be the main cause of Phelan–McDermid Syndrome (PMS, also referred to as 22q13 Deletion Syndrome) -- a genetic disorder characterized by global developmental delays, delayed or absent speech, moderate to severe intellectual disability, dysmorphic features, neonatal hypotonia, seizures, and a strong co-morbidity with ASD (Bonaglia et al., 2001; Harony-Nicolas et al., 2015; Phelan, 2008; Phelan and McDermid, 2012). Haploinsufficiency of *SHANK3* due to deletion or *de novo* mutations is seen in approximately 1% of autism spectrum disorder (ASD) cases, making *SHANK3* abnormalities one of the most common genetic causes of autism (Durand et al., 2007; Moessner et al., 2007; Buxbaum, 2009; Betancur and Buxbaum, 2013; Boccuto et al., 2013). Additionally, *SHANK3* variants have been linked to other non-syndromic ASDs, as well as schizophrenia and intellectual disability (Gauthier et al., 2010; Gong et al., 2012).

Abnormal dosage of *SHANK3* shows particularly robust effects on cognitive and language development (Bonaglia et al., 2001; Durand et al., 2006). Language is the most

significantly affected developmental domain, although deficits are variable and unpredictable (Durand et al., 2006; Zwanenburg et al., 2016). Impairments are also more prevalent in expressive than receptive language, consistent with severely delayed or absent speech in individuals with PMS. For instance, in a study evaluating 32 participants with PMS, none used phrase speech on a daily basis, and 19% used only single words to communicate (Soorya et al., 2013). Individuals with PMS also show deficits in using and understanding gestures and other forms of nonverbal communication, such as eye contact and facial expression. Therefore, it is thought that *SHANK3* participates in the assembly of specialized postsynaptic structures and neural circuitry necessary for the development of language and social communication (Durand et al., 2007).

There has been accumulating evidence suggesting that language impairments observed in ASD could be a consequence of atypical auditory processing. Whereas many studies have linked deficits in rapid acoustic processing with emergent language and reading disorders (e.g., SLI and dyslexia; Benasich ref here), there are also robust findings within ASD populations of specific *enhancements* in low-level acoustic tasks (e.g., pitch discrimination) (Bonnel et al. 2010, 2003; Eigsti & Fein, 2013; Heaton 2003, 2005; Jones et al. 2009). Increased sensitivity to pitch may be particularly associated with difficulties generalizing across simple acoustic information to form broader phonemic categories, which would negatively impact language development (Eigsti & Fein, 2013). These aberrant perceptual processing may also relate to the core features of ASD, for example social and communication deficits. However, little animal or genetic research has focused on the low-level perceptual superiorities seen in ASD, regardless of their implications for higher-level cognition and language. Therefore, it is difficult to associate particular

underlying genetic and neurobiological mechanisms to the auditory enhancements seen in individuals with ASD.

In order to more closely evaluate the contribution of *SHANK3* in neurodevelopment and behavior, a knockout mouse model with a mutation within the PDZ domain was created by the Feng lab (Peca et al., 2011). This *Shank3B* line includes a neo-cassette which replaces exons 13–16 of the *Shank3* gene, resulting in a deficiency of isoforms *Shank3*α and *Shank3*β, and a reduction in expression of the *Shank3*γ isoform. Initial research found that genetic disruption of *Shank3* in mice leads to compulsive/repetitive behavior and impaired social interactions, thus modeling two of the core features of ASD. Biochemical and electrophysiological studies further revealed synaptic dysfunction at cortico-striatal synapses -- part of the atypical neural circuitry strongly implicated in ASD (Peca et al.,2011). More recent research revealed that *Shank3B* heterozygous mice were slower to reach criterion in a pairwise visual discrimination task, indicating a deficit in discrimination learning in the *Shank3B* model of PMS and ASD (Copping et al., 2016).

The current study was designed to further examine the behavioral profile of *Shank3B* heterozygous and homozygous knockout mice, specifically with regard to features that might map onto atypical language. Measures included acoustic processing of rapid and complex stimuli, sensorimotor functions, and social/communicative interactions, as well as learning and working memory. Our findings provide additional insight on how disruption of *SHANK3* may alter fundamental processes required to develop typical language abilities. Ongoing research may promote enhanced early screening of infants, as well as individualized early-intervention treatments for children with ASD risk factors that indicate high probability of subsequent language and communicative impairments.

6.3 Methods and Materials

6.3.1 Subjects

Heterozygous breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, Maine, B6.129-*Shank3*^{tm2Gfng}; stock #01768). Subjects were generated from Het × Het breedings. Genotypes were determined by PCR of mouse ear punch DNA using (GAGACTGATCAG CGCAGT TG) Common, (TGACATAATCGCTGGCAAAG) Wild type Reverse and (GCTATACGAAGTTATGTCGACTAGG) Mutant Reverse. Only male subjects were assessed on subsequent behavioral measures. All subjects were single-housed in standard mouse tubs (12 h/12 h light/dark cycle), with food and water *ad libitum.* Procedures were performed blind to genotype, and in compliance with the National Institutes of Health and University of Connecticut's Institutional Animal Care and Use Committee (IACUC).

6.3.2 Rotarod P60

Subjects were assessed at age P60 for sensorimotor ability and motor learning using a rotarod task. All mice were habituated to the rotarod a day prior to testing, where they were placed on a rotating cylindrical drum that was held at a constant speed of 4 rotations per minute. Subjects underwent 4 trials that maxed out at 2 minutes. Testing began the following day with subjects placed on a rotating cylindrical drum that accelerated from 4 to 40 rotations per minute across 2 minutes. Four trials were administered per day, across five consecutive days. Latency to fall from the rotating drum was averaged across the four trials for each day.

6.3.3 Marble Burying P65

Subjects were placed in a standard polycarbonate cage (26 cm × 48 cm × 20 cm) filled with fresh mouse bedding (5cm deep) for the marble burying test. Standard glass toy marbles (assorted

styles and colors, 15 mm diameter, 5.2 g in weight) were placed on the surface of the bedding in 3 rows of 7 marbles. A marble was considered buried if at the end of the 45 minute session the marble was more than half-way covered by bedding. Subjects were given 45 minutes to explore the area; number of marbles buried and time spent grooming were reported and used for analysis.

6.3.4 Social Dominance – Tube Task P67

The Tube Test was administered to evaluate social dominance/aggression. The tube used for this task was a clear plexiglas tube (length 30.5cm; outer diameter 4.5cm; inner diameter 3.5cm). This narrow space is just sufficient for a mouse to walk through without being able to reverse direction. Mice were trained to walk through the tube before testing. A WT and a mutant mouse were randomly paired on different sides of the tube (balanced), and released at the same time into the tube. The mouse that forced the other mouse to back out of the tube was considered the "winner" of the trial (recorded for analysis). Each mouse underwent 4 trials paired with a different randomly assigned subject and the percentage of wins was calculated and analyzed. Mice were not paired within Genotype since by definition this would yield a score of 50%.

6.3.5 Three Chamber Social Preference and Recognition P72

The Three-Chamber test was used to assess general sociability as well as social recognition. This test derives from observations that healthy wild-type mice typically prefer to spend time with a conspecific (social stimulus) rather than an object (non-social stimulus). After a 5 min habituation period, the subjects were allowed to freely explore three chambers, with one containing another "stranger" mouse, and another chamber on the opposite side containing a novel object. The subject was placed into the middle (empty) chamber and was able to freely explore all the chambers for 10 min. Next we placed an unfamiliar conspecific mouse ("stranger

2") where the novel object was previously located, and subjects were given another 10 min to explore the chambers. The percent time spent interacting with the mouse during the social preference phase, and percent time spent interacting with the novel mouse during the social recognition phase, were recorded and analyzed.

6.3.6 Auditory Processing P78 – P114.

Subjects then advanced to auditory processing testing, which utilizes a modified pre-pulse inhibition paradigm (see Fitch et al., 2008 for review). Subjects were placed on individual load-cell platforms (Med Associates, St. Albans, VT) and presented with auditory stimuli generated using RPvdsEx on a Dell Pentium D PC and RZ6 multifunction processor (Tucker Davis Technologies, Alachua, FL). Sounds were amplified using a Niles SI-1260 Integration Amplifier (Niles Audio Corp., Carlsbad, CA) and delivered through powered Yamaha YHT-M100 speakers (Buena Park, CA). The acoustic startle reflex (ASR; a reflexive response elicited by an unexpected, intense stimulus) was recorded by an iMac 7.1 running Acknowledge 4.1, and obtained via the voltage output from each load cell platform through a linear amplifier (PHM-250U; Med Associates, St. Albans, VT) connected to a Biopac MP150 acquisition system (Biopac Systems, Goleta, CA). The modified pre-pulse inhibition paradigm measured differences in ASR to a loud startle-eliciting stimulus (SES; 105dB, 50 ms, broadband white noise burst (1kHz-10kHz)) when presented with or without a preceding acoustic cue. The ASR difference on cued versus uncued trials provided a measure of cue detection and/or discrimination. If the auditory cue was detected, a reduction (attenuation) in the ASR was expected relative to the ASR elicited when the auditory cue was not present (or not detected). This phenomenon was quantified using an "attenuation score" (ATT) that compared the average amplitude of the ASR

from the cued trial to the average ASR of the uncued trial ([average cued ASR/average uncued ASR]*100).

6.3.6.1 Normal Single Tone P78

Animals were initially tested on Normal Single Tone (NST) to measure baseline pre-pulse inhibition and auditory ability (P78). This auditory PPI control task was used to establish whether subjects exhibited hearing deficits and/or impaired gross motor reflexes that could confound other auditory PPI tests, and provided an index of baseline auditory pre-pulse inhibition ability across test groups. Testing sessions consisted of 104 pseudorandomly presented cued and uncued trials at inter-trial intervals (ITI) of varying duration (16–24 s). The task comprised a silent background and a simple single tone cue (50 ms, 75 dB, 8,000 Hz tone) presented 50 ms prior to the 50 ms, 105 dB SES. All subjects were able to perform this task, and therefore were used for further auditory processing evaluation.

6.3.6.2 Embedded Tone P82-P93

The variable duration Embedded Tone (EBT) task (300 sequential pseudorandom trials) assessed ability to detect a change in tone frequency relative to a standard background tone (cue was a variable duration 5.6 kHz pure tone embedded in a 10.5 kHz background pure tone). On cued trials, the cue was presented 100 ms before the SES, while uncued trials used a "cue" of 0 ms. Two EBT tasks were used in this study – a long-duration EBT (0 ms to 100 ms), and a short-duration EBT (0 ms to 10 ms). A range of cue durations were used to evaluate specific thresholds for performance differences between the genotypes, since groups might perform comparably on longer durations yet differ on shorter durations (which are more difficult to detect). Using a range of cue durations enables ascertainment of stimulus features that all animals can

discriminate (ceiling), that no animals can discriminate (basement), as well as any group differences in the mid-range. Both EBT tasks were administered for five consecutive days (P82-P93).

6.3.6.3 Silent Gap P96-107

A Silent Gap (SG) task was used to assess ability to detect silent breaks in continuous white noise (P96 to P107). A session included 300 trials with a continuous 75 dB broadband white noise background, with pseudorandomized cued and uncued trials. On cued trials, a silent gap of variable duration (0-100 ms or 0-10 ms) was presented 100 ms before the SES, while a "0 ms" trial served as the uncued condition. Subjects were tested on the Silent Gap task for five consecutive days using both versions of the task.

6.3.6.4 Pitch Discrimination P110-P114

Pitch discrimination testing also took place across five consecutive days of testing (300 trials/day). This task assessed ability to detect very small changes in pitch embedded in a background tone. A variable ITI (16–24s) was used, and the cue was a 300 ms, 75 dB tone of variable frequency embedded in a standard 75 dB, 10500 Hz background pure tone (2 ms up/down linear frequency ramp) prior to the SES. The experimental frequencies used for the pitch discrimination task deviated from the standard background frequency by as much as 75 Hz, to as little as 5 Hz. Uncued trials did not include a frequency deviant prior to the SES.

6.3.5 Water Maze Testing P120-135

Subjects were initially tested on a water escape to assess any underlying impairments that might confound further maze testing (i.e., deficits in motivation, swimming, or visual acuity). Subjects were placed in the far end of an oval tub (103 cm × 55.5 cm) filled with room temperature water,

and given 45 seconds to swim to a visible escape platform (8.5 cm in diameter; 1 cm above water surface) at the opposite end of the tub. Latencies to reach the visible platform were recorded. None of the subjects displayed any impairment on this task, and thus all subjects then progressed to a water version of the 4/8 radial arm maze (adapted from Hyde, Hoplight & Denenberg, 1998).

This task was used to assess spatial reference and working memory ability simultaneously, using a standard 8 arm radial maze with 4 arms baited (i.e., containing a submerged goal platform), and 4 arms open but never baited. Configuration of goal arms were counterbalanced between subjects but remained fixed per subject across all sessions. Additionally, high contrast extra-maze cues were present, and the locations of these remained static for the experiment.

The day prior to testing (Day 1), subjects were given a training session where all arms that would not contain a platform were blocked, forcing the animals to only enter arms containing a platform. Subjects were placed in the start arm and given 120 seconds to locate a platform. Every subject completed 4 training trials, and each time they found a platform, that platform was removed and the entrance to that arm was blocked. This ensured that the subject could no longer enter this arm for the remainder of the training session. If the subject failed to find a platform in this window, they were guided to the nearest available goal. Once on the platform, subjects remained for 20 seconds and then were removed from the maze to their home cage (30 second inter-trial interval; ITI).

Testing began on Day 2 and continued for an additional 14 consecutive days. Here, instead of blocking the goal arm of the most recently located platform, the platform was removed during the 30 second ITI. However, the arm remained open and unbaited for the remainder of the

test session. Animals were required to locate all 4 platforms, and thus received 4 test trials per day. Test sessions were recorded using a Sony camera integrated with the SMART video-tracking program (Panlab, Barcelona, Spain). Total number of errors were recorded and used for analysis.

6.4 Results

6.4.1 Rotarod

A repeated measures ANOVA examining average rotarod latency across 5 days of testing showed no main effect of Genotype on rotarod [$F_{2,25} = .148$, $p>.05$], indicating no overall difference in performance on the rotarod (and hence sensorimotor function) between the wildtype and mutant groups. There was, however, a significant Genotype × Day interaction [$F_{8,100} = 2.130$, $p<.05$], with WTs and HTs showing similar learning curves across days but *Shank3B* KOs exhibiting a delay in improvement (increasing latency) across days. This interaction indicates a motor learning impairment in the KO group. There was also a main effect of Day [$F_{4,100} = 20.307$, $p<.01$], with all Genotypes showing some increase in latency on the rotarod as testing progressed, indicative overall motor learning of varying degrees (Fig. 6.1a).

6.4.2 Marble Burying

A univariate ANOVA revealed a main effect of Genotype [$F_{2,25} = 9.636$, $p<.01$] on marble burying, with WTs burying significantly more marbles (Fig. 6.1b). However, further analysis revealed *Shank3B* KOs spent significantly more time grooming during the marble burying task [$F_{2,25} = 9.827$, $p<.01$] (Fig 6.1c).

6.4.3 Social Dominance

Social dominance (tube task) was conducted to evaluate social dominance. Number of wins was calculated as a percentage of the 4 trials. A univariate ANOVA revealed a main effect of Genotype [$F_{2,25} = 3.373$, p=.05], with *Shank3B* KOs winning significantly more trials compared to HTs and WTs (Fig. 6.1d).

6.4.4 Three Chamber - Social Preference and Recognition

On the three-chamber social interaction tasks we did not find a main effect of Genotype, either in the social preference phase [$F_{2,25} = .151$, p>.05] (Fig. 6.1e), or the social recognition phase [$F_{2,25} = .352$, p>.05] (Fig. 6.1f). On all tasks, WTs, HTs and KOs mice spent a comparable percentage of time exploring the social stimuli (social preference - mouse; social recognition - novel mouse).

6.4.5 Auditory Processing

All subjects were initially tested on a normal single tone (NST) task, to establish baseline hearing and PPI ability. None of the subjects showed impairments on NST, nor was there a main effect of Genotype [$F_{2,25} = 1.305$, p>.05]. We did however control for individual variations in baseline auditory prepulse inhibition (PPI) performance by using NST attenuation scores as a covariate for analysis of additional tasks. On the embedded tone (EBT) 0-100 ms task, we found no main effect of Genotype [$F_{2,25} = .171$, p>.05], with all subjects able to discriminate the stimuli based on cued/uncued amplitude comparisons (Fig. 6.2a). On the more difficult embedded tone task (cue durations ranging from 0 to 10 ms, where 0 ms is the uncued condition) we also failed to find a main effect of Genotype [$F_{2,25} = .379$, p>.05] (Fig. 6.2b). For silent gap detection, we assessed performance on the 0-300 ms task and again saw no main effect of Genotype [$F_{2,25} =$

.041, p>.05] (Fig. 6.2c). On the silent gap 0-100 ms task we saw overall worse performance (attenuation scores hovering around 100 or chance levels), and again no main effect of Genotype [$F_{2,25}$ = 1.667, p>.05] (Fig. 6.2d). On the pitch discrimination task, we also failed to find a main effect of Genotype when analyzing across all Days [$F_{2,25}$ = .331, p>.05] (Fig. 6.2e). However, we did see a Day [$F_{4,100}$ = 2.774, p<.05], and a Day x Genotype interaction [$F_{8,216}$ =2.113, p<.05] reflecting the fact that *Shank3b* KOs exhibited significantly better discrimination of subtle differences in pitch during the initial testing sessions [Days 1-2; $F_{1,13}$=5.132, p<.05] (Fig. 6.2f).

6.4.6 Water Maze Testing

Prior to spatial water maze testing, a visible platform control task was conducted to assess any underlying impairments that could confound subsequent water maze performance (e.g., impairments in swimming ability, visual acuity, or motivation). A univariate ANOVA on latencies found no main effect of Genotype [$F_{2,25}$ = 1.060, p>.05] indicating that genetically modified groups had no impairments on underlying aspects of the water task (e.g., swimming).

The 4/8 radial arm water maze was used to simultaneously measure spatial working and reference memory performance. A repeated-measures analysis of the average number of total errors (working memory, initial reference, and repeated reference memory errors) revealed significant effect of Genotype [$F_{2,25}$ = 4.031, p<.05] (Fig. 6.3a). A main effect of Day [$F_{13,325}$=5.611, p<.01] was observed, confirming that all groups reduced errors across days (i.e., showed learning). We also examined group differences in error types including total reference memory, initial reference memory, repeated reference memory, and working memory. Again repeated measures ANOVA revealed a significant main effect of Genotype on total reference memory [Genotype: $F_{2,25}$ = 5.600, p<.05], initial reference memory [Genotype: $F_{2,25}$ = 6.488, p<.05] and repeated reference memory errors [Genotype: $F_{2,25}$ = 4.491 p<.05]) but not working

memory errors working memory [$F_{2,25}$ =1.461, p>.05] (Fig. 6.3c). There were no significant differences in Genotype on latency to platform [$F_{2,25}$ = .067, p>.05] (Fig. 6.3d).

6.5 Discussion

The current study was designed to further examine the behavioral profile of *Shank3B* heterozygous and homozygous mutant mice, specifically with regard to features that might map onto atypical language in ASD (e.g., auditory processing skills, learning and memory). Subjects' sensorimotor ability and social behavior were also examined to confirm consistency with prior reports. Results showed that *Shank3B* KOs display particular enhancements in pitch discrimination, together with repetitive behaviors and learning impairments.

6.5.1 Shank3B KOs exhibit ASD-like behaviors

Previous studies behavioral characterizing *Shank3B* KO mice found that genetic disruption of *Shank3* leads to compulsive/repetitive behavior and impaired social interaction, which are two core symptoms of ASD (Peça et al., 2011). Here, we assessed *Shank3B* mutants on marble burying -- a task typically used to evaluate repetitive behaviors in transgenic mouse models of ASD. *Shank3B* KO mice did not show an increase in marble burying, however, when examining video recordings we found that *Shank3B* KOs spent significantly more time grooming compared to HTs and WTs, consistent with prior reports (Peça et al., 2011). Thus KOs did in fact exhibit enhanced repetitive behaviors, just not the behaviors we expected in that particular task. Related studies have shown that *Shank3B* KOs display early cortical hyperactivity, which triggers increased striatal spiny projection neurons excitatory synapse and corticostriatal hyperconnectivty. This tight functional coupling between cortex and striatum during early

postnatal development may explain the repetitive/perseverative behaviors seen in this mouse model (Peixoto et al., 2016).

Our results also expanded the atypical social phenotype observed in *Shank3B* mutants with *Shank3B* KOs, specifically by showing that they display more dominant behaviors on the social dominance tube task. Although generally interpreted to reflect enhanced aggression, these results could also indicate a failure to perceive and process social cues when confronted with another mouse. Interestingly, we saw no significant differences on the three chamber task for both social preference and social recognition. This may reflect discrepancies in protocol across studies. Specifically, our subjects were given a choice to explore a mouse or a novel object, whereas other investigators have offered a choice between a caged cohort and an empty wired cage (Peça et al., 2011). Overall, our findings do replicate prior reports for the *Shank3B* mouse model, and affirm that this mouse model exhibits core ASD-like traits such as repetitive behaviors and altered social behavior.

6.5.2 Shank3B KOs exhibit enhancements on pitch discrimination.

Based on the language deficits observed in clinical patients with disruptions of *SHANK3,* and in ASD more generally, we explored auditory processing abilities in the *Shank3B* mutant mice. Enhanced low-level auditory processing has previously been associated with language deficits seen in ASD (Mottron et al., 2006). We found that *Shank3*B KO subjects performed equivalently to wild-types on all of the acoustic discrimination tasks, with the exception of a significant KO superiority observed on the pitch discrimination task. This is the most difficult of the tasks, since it requires detection of very small differences in frequency. Here, the *Shank3B* KOs were initially (Days 1 & 2) significantly better than wild types at the four conditions where any discrimination was seen: 10425 vs 10500 (∆75 Hz), 10450 vs 10500 (∆50 Hz), 10525 versus

10500 (Δ25 Hz) and 10550 vs 10500 (Δ50 Hz; $F_{1,13}$=5.132, p<.05 for KO vs WT, with NST covariate). [The 5 more difficult PD conditions (from Δ5) failed to show discrimination by any group (ATT >95)]. This specific KO enhancement suggests an initial (intrinsic rather than learned) superiority among KOs on a fine-grained acoustic frequency discrimination (with HTs showing intermediate performance). This is highly consistent with related studies showing robust local acoustic superiorities in other ASD mouse models. Specifically, *Cntnap2* KOs showed superiority over matched wild-types on a fine-grained embedded frequency (EBT) and pitch discrimination (PD) task, while TS2-Neos showed acoustic superiority over matched wild-types on virtually all]acoustic discrimination tasks employed (Rendall et al., 2017; Truong et al., 2015). Combined findings could reflect an interaction between intrinsic local acoustic superiorities in ASD models, and simultaneous learning deficits that degrade performance. In support of this view, TS2-Neo mice showed no learning deficits on a radial arm maze, coupled with significant superiority over wild-types on virtually all acoustic tasks employed (Rendall et al., 2017). *Cntnap2*s showed some mild (acquisition only) learning/memory deficits on a radial arm maze, coupled with significant acoustic superiorities on several (but not all) fine-grained acoustic tasks (EBT and PD; Truong et al., 2015). Here, *Shank3B*s showed very robust (acquisition and retention) learning/memory deficits on a radial arm maze, along with more limited initial superiority on a PD task only. These results are important, since other investigators have shown that low-level acoustic enhancement in ASD may correlate with impaired language development, reflecting an initial failure to form distinct phonemic categories containing broadly generalized (e.g., co-articulated) variants of the core phoneme (Fein & Eigsti, xxxxx). Although, *Shank3B* KOs superiorities in auditory processing were not as prominent compared to the *Cntnap2* and TS2-neo mouse model, our findings do parallel clinical evidence on sensory

sensitivity in children with PMS compared to children with idiopathic ASD (Mieses et al., 2016). Together this evidence suggests that language impairments seen in individuals with *SHANK3* mutations may be correlated to robust cognitive deficits as well as specific enhancements in pitch discrimination.

6.5.3 Shank3B KOs demonstrated learning and memory impairments

Investigators have previously assessed *Shank3B* on a Morris water maze task, and found KOs to performed comparably to WT controls in both learning and probe trials. There were also no differences found on reversal learning (Peça et al., 2011). However, another study showed *Shank3*B HTs were slower to reach criterion on a more difficult pairwise visual discrimination task (Copping et al., 2017). Here, we examined *Shank3B* HTs and KOs performance on a complex 4/8 radial arm water maze, and report that *Shank3B* KOs displayed robust deficits across different error types. Findings may relate to evidence that *Shank3*, KOs also exhibit a decrease in NMDA/AMPA excitatory postsynaptic current ratio in area CA1 of the hippocampus, as well as reduced long-term potentiation in area CA1, and deficits in hippocampus-dependent spatial learning and memory (Kouser et al., 2013). Both spatial learning deficits and decreased LTP in area CA1 of the hippocampus have also been reported in the *Shank3*$^{e4–9}$ homozygous mutant mice (Bozdagi et al., 2010; Wang et al., 2011). Overall findings are consistent with *SHANK3*'s role in assembling postsynaptic structures and maturation of dendritic spines, which is altered in individuals with learning disabilities (Boeckers et al., 2002; Carlisle & Kennedy, 2005; Naisbitt et al., 1999).

6.5.4 Conclusion

The goal of the current study was to assess fundamental processes necessary to the development of typical language abilities in the *Shank3B* KO mouse model. We found that

Shank3B KOs exhibited normal sensorimotor learning, along with deficits in spatial learning. Interestingly, *Shank3B* KOs expressed some specifically enhanced pitch discrimination abilities compared to WT controls. These findings suggest that abnormal auditory processing as well as learning may contributing to language impairments seen in individuals with *SHANK3* mutations. Both clinical studies and transgenic animal models provide us with indispensible tools in elucidating the underlying causes of neuromorphological, neurophysiological, and behavioral differences associated with language-related developmental disorders. Animal models can in particular be utilized to parse apart specific genetic contributions to the etiology/pathogenesis of core functional anomalies that underlie language-related disorders -- including some genetically mediated features that may be shared across clinically distinct disorders. Future studies of language-relevant processing skills in mouse ASD models will help to improve early screening, as well as the development of mechanism-based behavioral and possibly targeted pharmacological interventions for language-based developmental disorders in general.

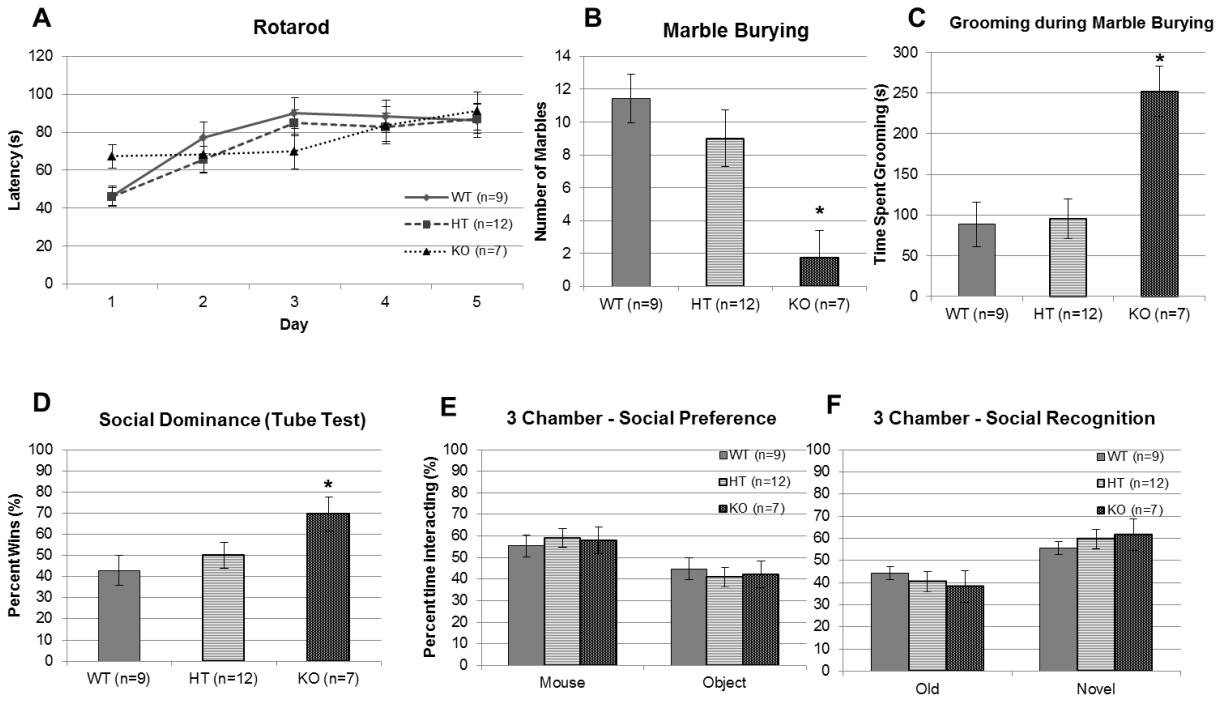

Figure 6.1 – *Shank3B* KOs exhibit ASD-like behaviors. (A) Rotarod performance across 5 days. (B) Number of marbles buried. (C) Grooming during Marble Burying. (D) Percent wins during Social Dominance Tube Test. (E) Social preference in 3 Chamber task. (D) Social Recognition in 3 Chamber task. *p<.05

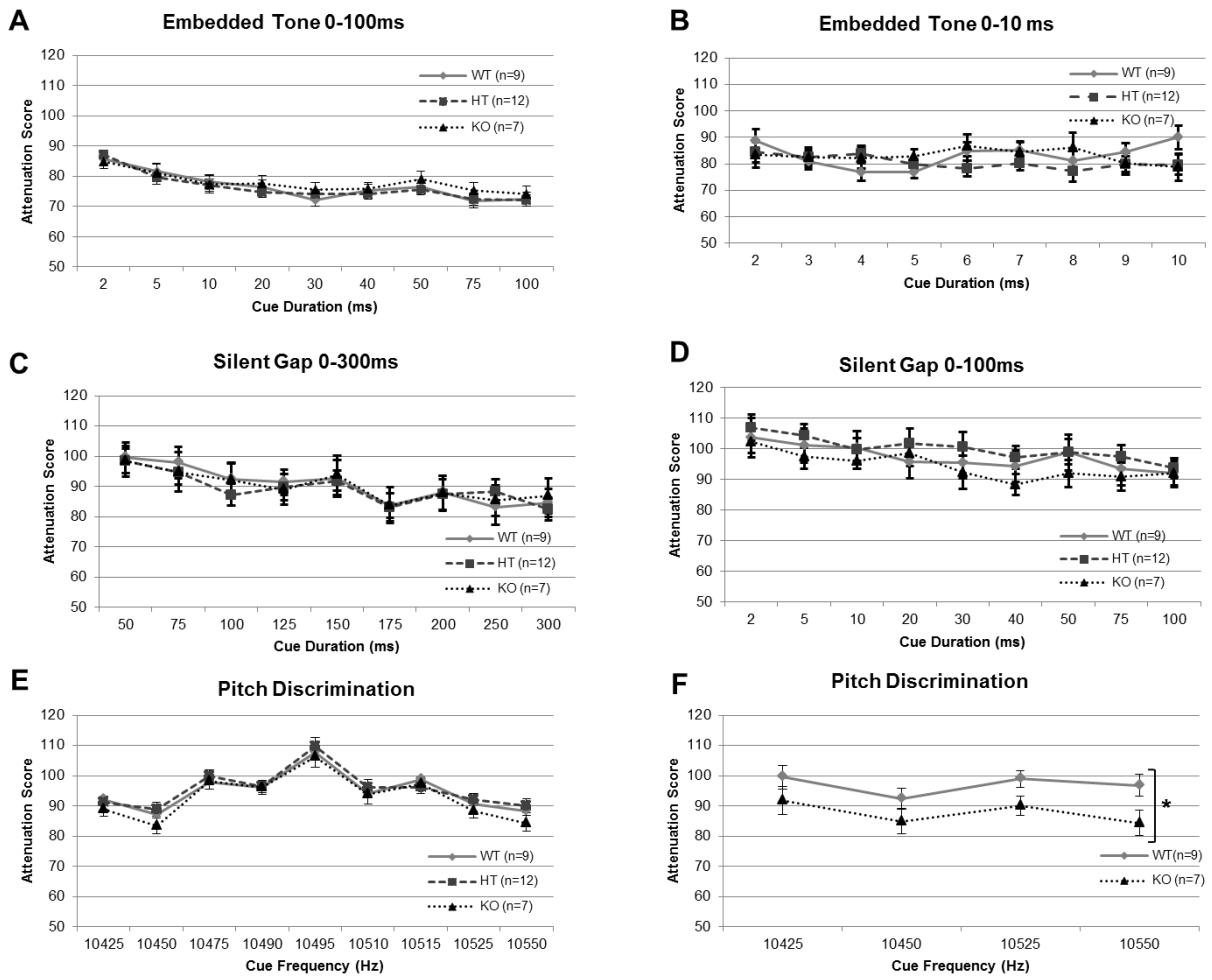

Figure 6.2 - *Shank3B* KOs exhibit enhancements on pitch discrimination. (A) Embedded Tone 0-100 ms. (B) Embedded Tone 0-10 ms. (C) Silent Gap 0-300 ms. (D) Silent Gap 0-100 ms. (E) Pitch Discrimination. (F) Pitch Discrimination during Day 1 -2. *p<.05

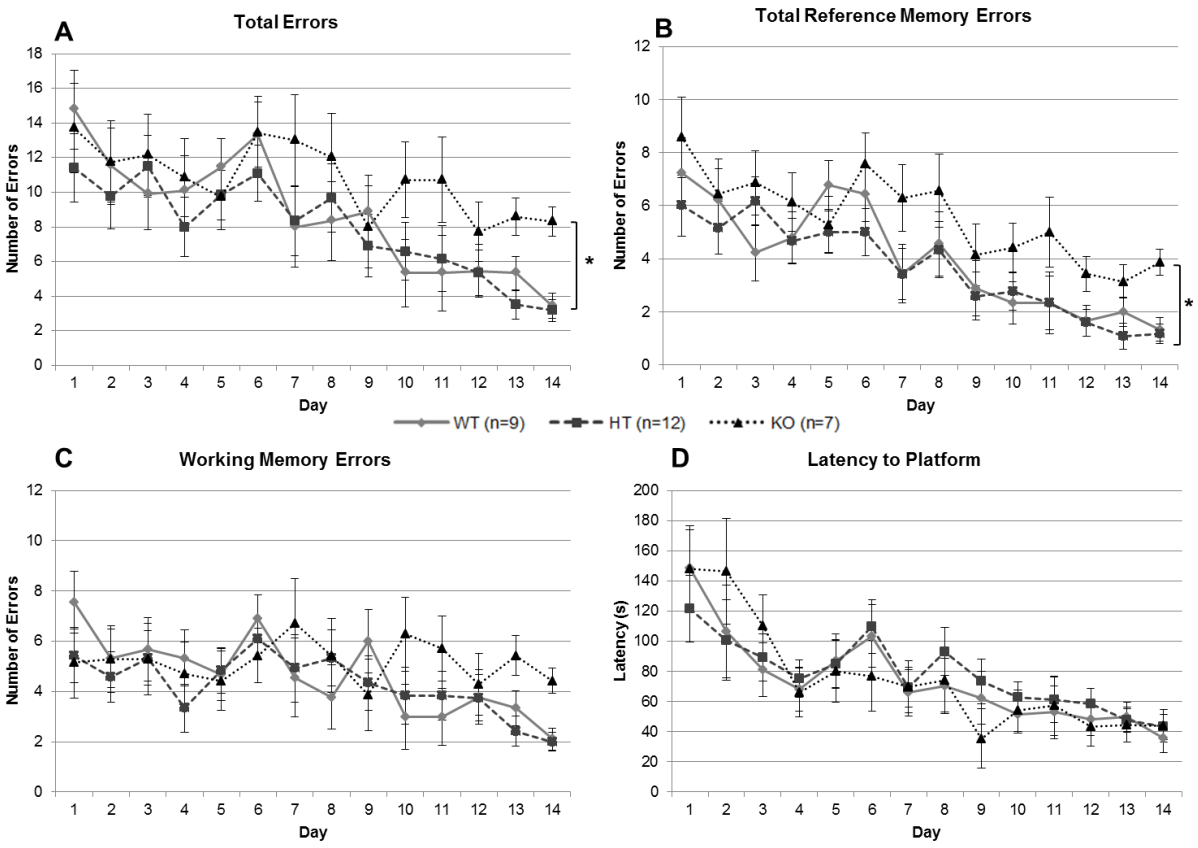

Figure 6.3 - *Shank3B* KOs demonstrated learning and memory impairments. (A) Number of total errors. (B) Number of total reference memory errors. (C) Number of working memory errors. (D) Latency to platform. *p<.05

Table 7.1 – Summary of behavioral data (Chapters 2 - 6)

Mouse Model	*ASD - like behaviors*		*Language intermediate phenotypes*				
	Social Behavior	**Repetitive Behavior**	**Motor Coordination/ Learning**	**Auditory Processing (Pitch)**	**Auditory Processing (Temporal)**	**Learning Memory**	**Motion Perception**
***Cntnap2* KO**	-	+	=	+	-	-	N/A
TS2-neo	-	+	-	=	+	=	N/A
***Shank3b* KO**	-	+	=	+	=	-	N/A
***Dyx1c1* co KO**	N/A	N/A	=	=	=	-	N/A
***Dcdc2* KO**	N/A	N/A	=	=	-	-	-

Chapter 7

Discussion

The collection of studies presented here provides important insight into the intricate relationship between genetic expression, neuroanatomical development, and behavioral outcomes in the context of language and reading related endophenotypes. To achieve our goal, we performed five experiments using transgenic mouse models. Each study was designed to examine loss or alteration of function of key genes highly associated with impaired language/communication and/or reading ability in clinical populations. Our findings provide valuable knowledge that may assist future development of personalized treatments targeted to underlying atypical neurobiology associated with language-related neurodevelopmental disorders (NDDs) (e.g., dyslexia and autism), including early behavioral interventions tailored to individual genetic risk factors.

7.1 Summary of studies

Acoustic processing ability, visual motion perception impairments, and working memory deficits all appear to contribute to emergent language problems. Here, we evaluated these behavioral disruptions across 5 models to understand how genetic and cellular mechanisms could influence behavioral impairments. A summary of all behavioral findings from the five models studied is displayed in Table 7.1. Overall, our data suggest that underlying disruptions in genetic factors crucial to neurodevelopment lead to subsequent deficits in fundamental processes, as well as anomalies of anatomic structure that underlie language related disorders. The present studies allow us to assess how these aberrant processes differ and overlap across varying targeted genetic and molecular mechanisms. This provides us with a foundational understanding of

behavioral and neuroanatomical vectors that could be involved in language disabilities, even though the biological etiology and neural substrates associated with language and reading related dysfunction in neurodevelopmental disorders such as dyslexia and autism remains unclear. Nonetheless, some major biological themes have emerged that pertain to our current results. These include genetic modulation of enhanced/diminished (maladaptive) synaptic plasticity; global disconnection disorders (i.e., hypo/hyper-connectivity, as observed in ASD); and/or developmental sub-cortical magnocellular defects as well as excitation/inhibition imbalance implicated in dyslexia.

The enhanced acoustic processing observed in *Cntnap2* KOs as well as Ts2-neo mice may be explained by enhanced synaptic plasticity, which is thought to be responsible for the superior perceptual processing observed in ASD (Mottron et al., 2014). A majority of the genes associated with ASD play a crucial role in the construction and maintenance of synapses, thus altering synaptic plasticity (Kelleher et al., 2008 and Baudouin et al., 2012; Bourgeron, 2015 for review). Furthermore, brain imaging studies have shown large-scale cortical reorganization in the autistic brain (Gillis and Rouleau, 2011 and Ronemus et al., 2014). Interestingly, the enhanced cross-modal plasticity observed in the ASD population is strikingly similar to that observed among individuals who are sensorially deprived (congenitally blind, congenitally deaf), wherein regional reorganization of brain functions also contributes to some modality-specific enhanced perceptual processing (Mottron, 2014). This superior perceptual processing could cause an inadvertent challenge for speech development (Heaton et al., 2008), since neural processes may shift to focus overly on irrelevant or low-level dimensions of language that are typically "ignored." This theory of ASD is consistent with the "functional hypo-connectivity" and/or "weak central coherence" accounts (Happé and Frith, 2006). Weak central coherence

purports that individuals with ASD are biased towards local processing, at the expense of global processing crucial to language skills. It has been also been suggested that working memory may be specifically disrupted in ASD, also in association with a connectivity deficiency of corticocortical regions (Di Martino et al., 2014). Moreover, observed impairments in working memory in individuals with ASD seem be the result of a global disconnection rather than a focused deficit in the prefrontal cortex, as revealed in neuroimaging research (Barendse et al., 2013). This disconnection may result in problems with sensory integration, and therefore disrupt learning, which could explain why *Cntnap2* KOs exhibit delayed learning on a complex maze task. The magnocellular theory of dyslexia may further support the findings observed in the *Dcdc2* KOs performance on a motion perception discrimination task (Galaburda et al., 1994; Livingstone et al., 1991; Stein, 2001). The magnocellular theory of dyslexia posits that both visual and auditory processing dysfunction associated with reading disability can be attributed to lower level processing deficits resulting from specific disruption to the magnocellular sub-systems of thalamic nuclei, and potentially elsewhere in sub-cortical sensory structures (Galaburda et al., 1994; Livingstone et al., 1991; see Stein, 2001, for review). It is well established that the magnocellular component of the visual system is specialized for processing rapidly-changing temporal information (e.g., motion). Although the literature surrounding this theory remains controversial, early studies conducted by Livingstone et al. (2001) and Galaburda et al. (2004), examining post mortem human brain tissue from dyslexic individuals, revealed a reduction in the distribution of large cells ("magnocells") versus small cells ("parvocells") in both the lateral geniculate (visual) and medial geniculate (auditory) nuclei of the thalamus of these subjects (with disclaimer that a distinct magnocellular sub-system has not been identified in the MGN). It is thought that temporal imprecision in both visual and auditory domains might

lead to deficits for individuals with dyslexia in processing rapidly changing sensory input (Stein & Walsh, 1997).

Future research should further investigate the above biological theories to aid in the understanding of the casual mechanisms underlying these disorders. Additionally, studies should incorporate female subjects into studies to gain a better understanding of the sex differences observed in neurodevelopmental disorders. Sexually dimorphic disease prevalence is well recognized but poorly understood. For instance, males are diagnosed with dyslexia much more frequently than females (Finucci &Childs, 1981; Vogel, 1990; Hawke et al., 2007; Olson, 2002), and autism also shows a strong male bias with approximately 4 affected males for every 1 affected female. These sex differences currently remain unexplained.

7.2 The Research Domain (RDoC) approach.

It is essential to gain better understanding of the causes of NDDs because this will allow for earlier detection and more refined innervation that is mechanistically driven. By studying transgenic mouse models we are able to assess the role of individual genes in modulating biological and behaviorally phenotypes. Due to the heterogeneity and complexity of higher-order functions implicated in disorders of language and social communication, it is crucial to deconstruct diagnostic outcomes into simpler behavioral "intermediate phenotypes" to examine what fundamental processes are contributing to deficits. Thus behavioral phenotypes can more easily be associated with individual genetic mechanisms, and also can identify shared features across disorders. As we know, genes implicated in NDDs are overlapping for numerous complex disorders including autism, dyslexia, specific language impairment, intellectual disability, schizophrenia, and bipolar disorder. The use of more fine-grained functional measures will allow for a highly focused analysis of how genes are specifically contributing to behavioral and/or

functional components within a broader domain. This approach is parallel to the National Institute of Mental Health's (NIMH) Research Domain Criteria (RDoC) project that aims to create a framework for research on pathophysiology, especially for genomics and neuroscience, which will ultimately influence future classification techniques of neuropsychiatric disorders, including neurodevelopmental disorders (Insel et al., 2010).

Current versions of the Diagnostic and Statistical Manual of Mental Disorders (DSM) and International Classification of Diseases (ICD) have historically facilitated reliable clinical diagnosis and research. However, recent evidence emerging from clinical neuroscience and genetics fail to support diagnostic categories based on these established clinical criteria. This suggests that the boundaries of these categories are not capturing fundamental underlying neurobiological and genetic mechanisms that are contributing to the disorder as a whole. A major consequence of these limitations is the slow development of novel mechanism-based treatments that appropriately target the pathophysiology of the disorder (Insel et al., 2010). Therefore, it is essential for research as well as application to move towards developing a classification system based upon dimensions of neuroscience, genetics, and observable behavior.

7.3 Treatment of neurodevelopmental disorders

Neurodevelopmental disorders first appear during early development and are indexed by atypical milestones that emerge from infancy to school age. NNDs are caused by a variety of genetic and environmental conditions (Ehninger et al., 2008). The molecular and neuronal mechanisms underlying the clinical phenotype of non-syndromic NNDs remain largely unknown. However, NDDs are relatively common, affecting ~1-2% of the population, yet clinicians possess limited options for intervention and treatment. Yet, there is a growing and

constant demand for effective services and treatments for NDDs due to the fact that symptoms persistent across an individual's life span.

Abnormal brain development in NDDs comprises a sequence of critical periods and abnormalities occurring during early development. Alterations in development, (i.e., disruptions in neurogenesis, cell migration, and neuronal connectivity) are all potential vectors responsible for the irreversible behavioral deficits that emerge in childhood and persist in adulthood. However, recent animal model studies of neurodevelopmental disorders show that targeting the underlying molecular and cellular dysfunction could substantially reduce symptoms and improve cognitive function, possibly even via treatments initiated in adulthood. For example, it is possible that biochemical amelioration of the underlying genetic deficits may promote plasticity in the adult brain to compensate for, or even correct, specific developmental pathologies (Ehninger et al., 2008). These findings suggest that pharmacological treatments in combination with rehabilitation might alleviate and possibly reverse the symptoms of NDDs even after the end of critical developmental periods. Although this field of research is in its infancy, cumulative findings provide a rational and promising basis for treatment of NDDs.

7.3.1 Dyslexia

Currently, treatment of dyslexia consists of defining the disorder and advising parents. Subsequent treatment depends on the severity of dyslexia and psychological symptoms or concurrent disorders. There are no current pharmacological treatments available for dyslexia since it is thought that drug treatments are not beneficial; however, this avenue has not been fully explored. Only if the individual has ADHD or another comorbid disorder they will receive drug treatment along with psychotherapy.

Early studies focused on Piracetam, a nootropic compound known for its learning and memory enhancing properties. Specifically, Piracetam modulates neurotransmission of cholinergic and glutamatergic systems and exhibits neuroprotective and anticonvulsant properties while also improving neuroplasticity. Although findings have been inconsistent, most studies reported a significant effect of Piracetam relative to placebo for outcomes that include verbal learning, reading speed and/or accuracy (Di Ianni et al., 1985; Levi & Sechi, 1987; Tallal et al., 1986; Van Hout & Giurgea, 1990; Wilsher, Atkins &Manfield, 1978; Wilsher et al., 1987). Overall, the improvement of cognitive symptoms with Piracetam have been shown to be modest, however, outcomes took months to manifest and were optimized when combined with educational programs (Winbald, 2005). More recent studies also point to targeting the glutamergic system, which may have significant potential for treating dyslexia.

Although there are no current pharmacological agents available to treat dyslexia, there are some potential avenues that can be explored. For instance, there was inconsistent spike timing observed in *Dcdc2* KO mice that may be due to an up-regulation in *Grin2B* expression (a gene that encodes the 2B subunit of NMDA receptors) observed in these animals (Che et al., 2013). The NMDA receptor is an ionotropic glutamate receptor that plays a role in synaptic plasticity, as well as memory function. Further electrophysiological experiments found that treating affected mice with an NMDA receptor antagonist led to increased temporal precision, thus rescuing the noisy spike timing phenotype in *Dcdc2* KOs. This is consistent with evidence that low-level sensory processing deficits that require precise timing mechanisms underlie phonological impairments seen in dyslexia (Hancock, Pugh & Hoeft, 2017). Neural excitability and neural noise are crucial to these temporal processes and therefore, targeting these mechanisms may be beneficial to individuals with dyslexia. Studies of endogenous

neurochemistry in humans also point towards increased excitability. Specifically, an overall negative association between reading skill and levels of glutamate was observed in the visual cortex of children with dyslexia (Pugh et al., 2014). Moreover, this excitation-inhibition imbalance is also suggested to be an outcome of abnormal pyramidal cell migration, as well as abnormal GABergic interneuron localization that has been associated with dyslexia risk genes *Kiaa0319* and *Dyx1c1* (Currier et al., 2011; Peschansky et al., 2010). Fortunately, Memantine is a FDA-approved drug on the market, which is an uncompetitive NMDA-receptor antagonist used to treat cognitive symptoms of Alzheimer's disease and vascular dementia. Future research (both pre-clinical and clinical) should study new pharmacological and behavioral approaches aimed at potentiating glutamatergic neurotransmission, particularly at NMDAR-type glutamate receptors.

7.3.2 ASD

There is a growing and constant demand for effective services and treatments for ASD, due to the fact that symptoms persistent across the life span. Currently, there are no FDA-approved drugs available for the specific treatment of core symptoms of ASD (social/communication deficits and repetitive behaviors). Despite this lack of targeted treatment, it is believed that 75% of patients with ASD receive some kind of pharmacological treatment, usually directed to treat a non-core symptom such as hyperactivity, irritability or aggression (Esbensen et al., 2009). Moreover, other patients may receive off-label prescriptions on an experimental basis; in efforts to treat components of ASD. Since there are no specific biological targets, drug prescription has been limited to drugs that are approved for other disorders (e.g. ADHD). To date, the only drugs that are approved by the United States Food and Drug Administration (FDA) to treat symptoms of ASD are atypical antipsychotics, Risperidone (approved in 2006) and Aripiprazole (approved in 2009), which treat irritability, hyperactivity

and aggression (Penagarikano, 2015). These drugs usually have a secondary effect of improving overall social behavior by alleviating hyperactivity/aggression. However, atypical antipsychotics are prescribed on a limited basis, due to undesirable side effects (Miral et al., 2008). Current research is focused on understanding the neurobiological etiology to develop more specific and effective medicines.

Although, the identification of the underlying mechanisms of ASD has been challenging, common neurobiological themes have emerged. The prevalent neurobiological pathways identified in ASD are thought to be an avenue for developing pharmacological therapies aiming to restore or compensate neurochemical imbalances. Potential targets include synaptic transmission, and serotonin and oxytocin signaling (Penagarikano, 2015). There have been a number of pharmacological studies, both human and animal, investigating drug manipulations of these systems that have been successful. Moreover, animal studies have shown that certain behavioral and molecular defects can be reversed in mature mouse brain, making the outlook for potential pharmacological treatments in human patients positive. (Penagarikano, 2015, for review).

A reoccurring synaptic defect seen in ASD is alteration in dendritic spines, including spine density, morphology and or dynamics; this has been observed in both postmortem studies of ASD patients as well as animal models (De Rubeis et al., 2014). Therefore, drugs that could enhance spine maturation may serve as a therapeutic option. For instance, insulin-like growth factor 1 (IGF-1) may be a potential treatment since it regulates and promotes synapse formation. IGF-1 has been shown to promote the formation of mature excitatory synapses in neurons generated from induced pluriplotent stem cells from patients with Phelan-McDermid syndrome (PMS), a complex disorder that is considered to be a monogenic form of ASD due to disruptions

in the *SHANK3* gene (Shcheglovitov et al., 2013). A mouse knockout of *Shank3* was treated with daily IGF-1 injections over a 2-week period, resulting in the rescue of motor and long term potentiation deficits (Tavassoli & Buxbaum, 2013). In a pilot study, children with PMS received a 3-month treatment of IGF-1 and showed significant improvements in both social and restrictive behaviors without showing any adverse side effects (Kolevzon et al, 2014). To date, there have been no studies examining the differences in efficacy of IGF-1 between children and adults. IGF-1 seems like a viable potential drug candidate, specifically for individuals who have a mutation in the *SHANK3*, which is responsible for at least 0.5% of ASD cases (Durand et al, 2007). These findings inform us that an imbalance between excitatory and inhibitory at the cellular level due to the failure to form the correct number of excitatory synapses and the reduction of glutamate receptors may be responsible for social deficits as well as repetitive behaviors seen in ASD. Disruption of synapses and signal transmission is thought to be a major cause of ASD and various types of drugs may be able to target these aberrations at different levels. Recent preclinical trials have been utilizing glutamergic and GABAergic agents with some success.

Memantine, a NMDA receptor antagonist of glutamate, has been used in several ASD studies, and has resulted in improvements in language and social behaviors (Chez et al., 2007; Erickson et al., 2007). Memantine has also been administered in adjunction of Risperidone and reported reduction in repetitive behaviors, hyperactivity and irritability in addition (Ghaleiha, 2013). Memantine's efficacy has been evaluated in open-label trials in adults and has resulted in improvement in social/communication skills and without any serious adverse events (Joshi et al., 2016). However, when Memantine's safety, tolerability, and efficacy was evaluated in children with ASD, the trial did not demonstrate clinical efficacy, but results indicated that short and long term treatments of Memantine was well tolerated and safe (Aman et al., 2016). Therefore, further

research is indicated for Memantine use in children, since it may represent a potential drug candidate for the treatment of core symptoms seen in ASD.

Dysfunction in the GABAergic system also fits into this prominent excitatory-inhibitory imbalance hypothesis of ASD. An imbalance of excitatory/inhibitory neurotransmission is further consistent with comorbid anxiety and seizures observed in ASD (Bourgeron, 2009; Geschwind and Levitt, 2007; Gogolla et al, 2009; LeBlanc and Fagiolini, 2011; Rubenstein and Merzenich, 2003). Furthermore, multiple transgenic mouse models associated with ASD (*Fmr1*, *Cntnap2*, *Cadps2* and *En2*) have shown a reduction in the number of cortical GABAergic interneurons, as well as reduced GABAergic neurotransmission. Therefore, elevating GABAergic signaling might be a promising target for pharmacological treatments. In clinical trials for Fragile X syndrome, Fragile X with an autism diagnosis, and ASD, the selective GABA(B) enantiomer Arbaclofen was used as a therapeutic strategy. A phase 2 clinical trial detected improvements on Aberrant Behavior Checklist (ABC)-Social Avoidance scores (Berry-Kravis et al., 2012) in Fragile X patients. Another open-label trial of Arbaclofen in patients with ASD *not* associated with Fragile X showed beneficial effects on ABC-irritability social withdrawal scale and on the social responsiveness scale (Erickson et al, 2014). When Arbaclofen and Racemic baclofen were administered to *Fmr1* knockout mice, protein synthesis was restored, their increased dendritic spine density was reduced, and audiogenic seizures were reduced (Henderson et al, 2012). Additionally, R-baclofen was used to treat two other mouse models of ASD (BTBR and C58 mice), with low doses leading to a significant reduction in repetitive behaviors, as well as reversed social deficits, without any deleterious effects (Silverman et al., 2015). In clinical trials, Abraclofen has been effective in both children and adults. The efficacy of R-baclofen could be the result of its ability to dampen hyperexcitability via both pre- and

postsynaptic mechanisms. These data support the hypothesis that enhancing inhibitory transmission improves ASD relevant deficits through targeting GABAergic mechanisms.

Another potential target for treatment is the serotonin system, which plays an important role in neurodevelopmental processes such as cell proliferation, migration and differentiation. Elevated whole blood serotonin, or hyperserotonemia, was the first biochemical change observed in individuals with ASD and 45% of patients see increased levels of serotonin (Schain & Freedman, 1961; Cook and Leventhal, 1996). Abnormal 5-HT synthesis and signaling, platelet hyperserotonemia, and amelioration of repetitive behaviors by selective serotonin reuptake inhibitors (SSRIs) are some of the key findings that contribute to the notion that the serotoninergic system may be involved in ASD. Furthermore, it has been hypothesized that serotonergic genes might act as ASD susceptible genes. Variants in genes involved in the serotonin system such as the serotonin transporter (SLC6A4) and the monoamine oxidase A gene (MAOA) involvement in the deamination of serotonin has been linked to ASD (Conroy et al, 2004; Cohen et al, 2003). Animal studies looking at transgenic mice of these genes displayed altered serotonergic transmission along with social deficits (Lira et al., 2003; Bortolato et al., 2013). Currently the only drugs used to target this system are SSRIs, which have not been consistent with treating the symptoms of ASD. SSRIs such as Fluoxetine and Citalopram have been used to treat repetitive behavior and impulsivity but tend to have adverse side effects. Adult studies suggested that SSRIs relieve symptoms of irritability and rigid-compulsive behavior in autism (Gordon et al., 1993, Hollander et al., 2005 and Hollander et al., 2012). However, studies in children have been less supportive, perhaps because of greater adverse events in children or methodological shortcomings (King et al., 2009 and King et al., 2013). More consistent data support the use of Risperidone and Aripiprazole (the two approved treatments for ASD)

(McCracken et al., 2002 and McDougle et al., 2005), which are antagonists at multiple monoamine receptors, including the serotonin receptor 5-HT2A. Drugs targeting serotonin receptors, particularly 5-HT1A and 5-HT2A, have shown promise for increasing social interaction or decreasing cognitive rigidity (File et al., 1996, Edwards et al., 2006, Boulougouris and Robbins, 2010, Gould et al., 2011 and Amodeo et al., 2014). Most of this work has been conducted in animal models with limited research in humans. There is potential for developing drugs that target different levels of the serotonin system (i.e., receptor, transport, and processing) that may target the core symptoms of ASD unlike the current SSRIs available (Muller, Anacker & Veenstra-VanderWeele, 2015). Future research should investigate ways on using serotonin as a potential biomarker for ASD to help aid treatment.

Oxytocin (OXT), a naturally occurring neuropeptide has received substantial attention for its potential therapeutic value for treating social deficits in ASD. OXT is produced in the hypothalamus and is involved in the modulation of a broad range of prosocial behaviors including maternal behavior, mother-infant bonding, pair bonding and social memory and recognition (Ross & Young, 2009). According to clinicaltrials.gov there is currently 21 total studies investigating the effects of oxytocin in individuals with ASD, 11 of which are pediatric. The results of the effectiveness of OXT have been promising, with 85% of controlled trials finding improvements in social behaviors such as eye gaze, facial emotion recognition (Preti et al., 2014). Recently studies have demonstrated neurobiological and behavioral changes to intranasal OXT including enhanced resting state functional connectivity between anterior cingulate cortex and dorso-medial prefrontal cortex and improvements in social reciprocity and interaction (Aoki et al., 2014; Tachibana et al, 2013; Watanbe et al.,). However, there have also been numerous studies with negative findings --to date, the results have been inconclusive. For

instance, two pediatric placebo-controlled oxytocin trials did not observe improvements in social function when compared to placebo (Dadds et al., 2014). As of now, OXT appears to be more effective as a treatment for adults compared to children. Animal models can be utilized to gain understanding on how OXT is acting on the system and influencing social behavior by targeting oxytocin at multiple levels including, gene, receptor, and release (Modi & Young, 2012). Future research is required to understand the best administration route, dosage, developmental time point, as well as duration of treatment for particular ASD subgroups.

In many neurodevelopmental disorders the exact underlying pathology of the disorder is unknown making it difficult to develop treatments based on biological mechanisms. However, there has been substantial progress in the development of targeted pharmacological treatments for the core symptoms of ASD. The main neurochemical correlates of ASD identified are a part of the following transmitter systems: glutamate, GABA, serotonin, and oxytocin. This means that there might be several different pathophysiological CNS differences that ultimately yield one generalized symptom or a group of symptoms of ASD. These widespread alternations in cell to cell communication appear to be contributing to deficits in social, as well as repetitive behavior and cognitive function including language. There is great potential for drugs to act on these systems to alleviate the core deficits of ASD.

Overall, it appears adults respond better to treatment compared to children with NDDs. This may be a result to the fact that most pediatric drug studies include broad ranges of age, which is important to note since significant changes occur during development, meaning a year or two can make a substantial difference in response to treatment. These changes in maturation can be influencing the clinical trials effects. Further work is necessary to establish biomarkers and development effective pharmacological interventions to treat NDDs. Early discovery of

biomarkers in pediatric psychiatric and NDDs has been supported by NIH/foundation funding, although early studies focused on candidate genes; more recent studies are investigating CNS imaging biomarkers. Hopefully, with the push to establish early biomarkers this will accelerate the development of effective targeted pharmacological therapies.

7.4 Final concluding remarks

In summary, both clinical studies and transgenic animal models provide us with indispensible tools in elucidating the underlying causes of neuromorphological, neurophysiological, and behavioral differences associated with language-related developmental disorders. Animal models in particular are well suited to parse apart specific genetic contributions to the etiology/pathogenesis of core functional anomalies that underlie language-related disorders, including some genetically mediated features that may be shared across clinically distinct disorders. Ongoing research will help to improve early screening, as well as promoting the development of mechanism-based behavioral therapies and targeted pharmacological interventions for language-based developmental disorders.

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