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Functional Loss of Cntnap2 in the Rat Leads to Autism-related Alterations in Behaviour and Auditory Processing

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience

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Abstract

The contactin-associated protein-like 2 gene, *CNTNAP2*, is a highly penetrant gene thought to play a role in the genetic etiology of neurodevelopmental disorders such as autism spectrum disorder (ASD). Despite its link to ASD, the field lacks a complete understanding of the role *CNTNAP2* plays in the hallmarks of ASD: repetitive behaviours and abnormalities in social interaction, language, and sensory processing. Therefore, this thesis first examines if a loss-of-function mutation in the *CNTNAP2* gene in the rat (*SD-Cntnap2^{tm1Sage}*) is sufficient to cause alterations in social interactions, stereotypic behaviour, and sensory processing. *Cntnap2* knockout rats showed deficits in sociability and social novelty, displayed repetitive circling and hyper-locomotion, and demonstrated exaggerated acoustic startle responses, an increased avoidance of sounds of moderate intensity, and a lack of rapid audiovisual temporal recalibration; indicating changes in sensory processing at both the pre-attentive and perceptual levels. Therefore, this study established the *Cntnap2* knockout rat as an effective model to study the neural mechanisms underlying behavioural differences in ASD. Next, the role of *Cntnap2* in acoustic stimulus processing was determined by examining the development of brainstem temporal processing, sensitivity, and sensory filtering using the auditory brainstem response (ABR) and acoustic startle response (ASR). Delayed maturation of the ABR and persistent differences in the ASR across age were identified in knockout rats. Since the sound-induced neural activity was found to be transmitted slower through the brainstem in juvenile *Cntnap2^{-/-}* rats compared to wildtypes, the consequences of this altered development on cortical processing in adulthood were explored. Despite mature ABRs in adulthood, cortical auditory function remains altered. Specifically, immature cortical evoked potentials, delayed multi-unit response latencies, impaired temporal processing, and a pattern of hyper-excitability in both multi-unit and single-cell recordings were found. All these observations show striking parallels to disruptions reported in ASD. Overall, this work demonstrates that developmental disruptions in the *Cntnap2* gene are associated with persistent changes in autism-associated behaviours, auditory evoked behaviour, and the neural circuitries responsible for processing acoustic information.

Keywords

CNTNAP2, Autism Spectrum Disorder, Animal Behaviour, Acoustic Startle Response, Sensory Filtering, Auditory Evoked Potential, Extracellular Electrophysiology, Rat

Summary for Lay Audience

Neurodevelopmental disorders, such as autism spectrum disorder (ASD), are caused by an interaction between a person's genome and their environment during development. To study how different genes are related to autism, researchers study the behaviour and the brain of animal models with mutations in those genes. In this thesis, the contactin-associated protein-like 2 gene, *Cntnap2*, was studied using a rat model lacking a working version of this gene. Behaviours related to the diagnostic criteria of autism, namely social behaviour, restrictive and repetitive behaviour, and auditory sensory behaviours, were examined in these rats. We found the rats showed differences in these behaviours similar to what we see in autism. For example, they exhibited fewer social interactions, more repetitive behaviours, a greater response to startling sounds, and found moderately loud sounds more aversive. Because we know that the way the brain processes sounds is different in autism, in this thesis we also explored how the brain of the rats lacking a working *Cntnap2* gene responds to sound stimuli. Similar to autistic individuals, we found that the *Cntnap2* knockout rats' brainstem auditory response was slower to mature. We also know that in ASD, and other language-related disorders, the cortical auditory response to sound appears immature and that the cortex cannot process rapidly presented sounds. We examined these characteristics in the rat's auditory cortex and found a similar profile of immaturity. Since the *Cntnap2* knockout rat model reflects ASD so well, it can now be used to explore the underlying cellular and molecular mechanisms through which *Cntnap2* works to influence ASD-related behaviours and sound processing.

Co-Authorship Statement

Chapter 2 of this thesis was published in *Autism Research* and was coauthored by K. Kazazian, R.S. Mann, D. Möhrle, A.L. Schormans, S. Schmid, and B.L. Allman. I designed, oversaw, and conducted all experimental procedures, performed all data collection and analyses, and wrote/edited the manuscript. K. Kazazian assisted in the design and collection of the social behaviour, cognitive flexibility, and spatial learning and memory data sets. R.S. Mann assisted in the design and collection of the pre-attentive sound processing behaviour data set. D. Möhrle designed, collected, and assisted in the analysis of the stereotypic and exploratory behaviour data set. A.L. Schormans designed and assisted in the collection and analysis of the audiovisual temporal order judgement behaviour data set. S. Schmid and B.L. Allman were involved in experimental design and edited the manuscript.

Chapter 3 of this thesis was published in *The Journal of Neuroscience* and was coauthored by A.L. Schormans, K. Pacoli, C. De Oliveira, B.L. Allman, and S. Schmid. I designed, oversaw, and conducted all electrophysiological recordings, behavioural testing, and imaging, performed all data analysis, and wrote/edited the manuscript. K. Pacoli assisted with behavioural testing. C. De Oliveira performed immunohistochemistry. B.L. Allman and S. Schmid were involved in experimental design and edited the manuscript.

Chapter 4 of this thesis is being prepared for submission to *eLife* and was coauthored by R.S. Mann, A.L. Schormans, S. Schmid, and B.L. Allman. I designed, oversaw, and conducted all *in-vivo* electrophysiological recordings, behavioural testing, and immunohistochemistry, performed the associated data analysis, and wrote/edited the manuscript. R.S. Mann designed, oversaw, and conducted all *in-vitro* electrophysiological recordings, performed the associated data analysis, and assisted in writing the corresponding sections of the manuscript. A.L. Schormans assisted in the design and analysis of *in-vivo* electrophysiological recordings. S. Schmid and B.L. Allman were involved in experimental design and edited the manuscript.

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Chapter 1

1 General Introduction

1.1 Genetic Etiology of Neurodevelopmental Disorders

The development of the brain is regulated by numerous genes that, when mutated, can lead to maladaptive processing, altered development, and ultimately neurodevelopmental disorder. Genetic differences arise in several ways; for example, mutations can be chromosomal rearrangements, copy number variation, small indels, or nucleotide substitutions (Cardoso et al., 2019; Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; de la Torre-Ubieta et al., 2016; Pinto et al., 2014). Moreover, genetic variants give rise to complex genotype-phenotype associations (Cardoso et al., 2019). Several hundreds of genes are thought to partially play an overlapping role as risk-factors for a group of heterogeneous neuropsychiatric disorders all showing symptomologies associated with altered brain development, including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), epilepsy, intellectual disability (ID), and developmental language disorder (DLD; Cardoso et al., 2019; Deriziotis & Fisher, 2017; Masi, DeMayo, Glozier, & Guastella, 2017; Sahin & Sur, 2015). Although the genetic landscape is heterogeneous, the function of the genetic products converge onto common molecular and cellular pathways, including protein translation, synapse development and function, and epigenetic regulation (Chen et al., 2015; Cheroni, Caporale, & Testa, 2020; de la Torre-Ubieta et al., 2016; Sahin & Sur, 2015). By manipulating risk-genes in preclinical research settings, scientists can begin to narrow the developmental events that lead to neurodevelopmental phenotypes and identify mechanistic targets for intervention.

1.1.1 The *CNTNAP2* Gene

One example of a highly penetrant risk gene that leads to a neurodevelopmental phenotype is the contactin-associated protein-like 2 gene, *CNTNAP2*. *CNTNAP2* has enriched expression in the frontal and anterior temporal lobes, striatum, and dorsal thalamus (Alarcón et al., 2008). Both common and rare inherited variants of *CNTNAP2* are linked to

neurodevelopmental alterations (Cardoso et al., 2019; Chen et al., 2015; Strauss et al., 2006). For example, the single nucleotide polymorphism (SNP), rs7794745, is a risk variant localized on the *CNTNAP2* gene and is associated with ASD across a variety of human populations (Cardoso et al., 2019). Conversely, a single base pair deletion in exon 22 of the *CNTNAP2* gene leads to a frameshift mutation resulting in a non-functional protein and is present only in a small Amish community (Strauss et al., 2006). In this population, a homozygous loss of *CNTNAP2* function results in cortical-dysplasia and focal epilepsy (CDFE) which is characterized by seizures, language regression, altered social interactions, and a restricted behavioural repertoire (Strauss et al., 2006).

Large-scale association studies have identified several different variations in *CNTNAP2*, with most cases presenting with four central features: intellectual disability (ID), seizures, autistic features, and language problems (reviewed in Rodenas-Cuadrado, Ho, & Vernes, 2014). As such, the *CNTNAP2* gene has been linked to ASD, DLD, ID, and other language-related disorders (Arking et al., 2008; Newbury et al., 2011; Poot, 2017; Rodenas-Cuadrado et al., 2014; Vernes et al., 2008; SFARI). A homozygous loss-of-function in *CNTNAP2* causes the most severe phenotype, including cortical dysplasia, seizures, intellectual disability, language impairment, and certain features of ASD (Rodenas-Cuadrado et al., 2016; Strauss et al., 2006). However, most *CNTNAP2* mutations leading to neurodevelopmental disorder are heterozygous, with the mutation type and genetic background factoring into the displayed phenotype (Poot, 2015; Rodenas-Cuadrado et al., 2014, 2016). Moreover, genetic variation in *CNTNAP2* is also present in neurotypicals, seemingly responsible for differences in language development and brain activation during language tasks (Whalley et al., 2011; Whitehouse, Bishop, Ang, Pennell, & Fisher, 2011; Worthey et al., 2013). The association between *CNTNAP2* variation and ASD is particularly strong. Studies have found: (1) significant variation in SNPs in *CNTNAP2* in ASD subjects (Alarcón et al., 2008), (2) *CNTNAP2* polymorphisms that are significantly associated with inherited autism susceptibility (Arking et al., 2008), and (3) altered *CNTNAP2* gene expression in brains of autistic patients compared to age-matched control subjects (Sampath et al., 2013).

1.2 Autism Spectrum Disorder (ASD)

Autism spectrum disorder is a life-long neurodevelopmental condition, affecting males at four times the rate as females, and has a global prevalence of 1/161 (Elsabbagh et al., 2012; Maenner et al., 2020). In 2016, the Autism and Developmental Disabilities Monitoring Network estimated a prevalence of 1/54 for children aged 8 years in the United States (Maenner et al., 2020). In 2018, the Public Health Agency of Canada's National Autism Spectrum Disorder Surveillance System (NASS) estimated a prevalence of 1/66 for youth aged 5 - 17 years across Canada (Public Health Agency of Canada., 2018).

1.2.1 Genetic Basis of ASD

CNTNAP2 is only one of many genes that have been strongly linked to ASD. The etiology of autism is very complex and thought of as a genetic predisposition for the disorder combined with an environmental impact (Lord, Elsabbagh, Baird, & Veenstra-Vanderweele, 2020; Masi et al., 2017). Environmental risk factors include prenatal exposure to heavy metals, air pollution, and chronic exposure to certain medications or alcohol (Cheroni et al., 2020; Guinchat et al., 2012; Lyall et al., 2017), as well as advanced parental age and maternal infection during pregnancy (Guinchat et al., 2012; Lyall et al., 2017; Mandy & Lai, 2016). For a detailed review of the gene × environment perspective on ASD see Cheroni et al., 2020, Kim & Leventhal, 2015, and/or Lyall et al., 2017.

A consequence of the frequent occurrence of a genetic predisposition for ASD is a high heritability; i.e., the proportion of variation in a condition that is attributable to variation in genetics. ASD heritability is estimated to range from 0.5 to 0.8 (Bai et al., 2019; De Rubeis & Buxbaum, 2015; Sandin et al., 2017), with 0 meaning no genetic predisposition, and 1 meaning certain genetic predisposition. Although the heritability of ASD is high, there is heterogeneity in the genetic architecture of autism, primarily arising from three factors: genetic variation, comorbidity, and sex (Masi et al., 2017). As described in section 1.1, genetic variation in ASD exists due to the type of mutation, the gene that is affected, and the molecular mechanisms that are altered as a result (Cheroni et al., 2020). The sex of an individual affects the likelihood of ASD diagnosis. Given its increased prevalence in males,

differential sex-related genetic mechanisms and hormonal factors are suggested to play a *female protective* role (Masi et al., 2017). However, there are arguments for differences in the presentation of ASD in females, and that ASD is masked by female gender-related social differences, leading to the perspective that ASD may be underdiagnosed in females (Lord et al., 2020; Masi et al., 2017).

1.2.2 Behavioural Diagnosis of ASD

As of 2013, ASD diagnosis by the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; American Psychiatric Association, 2013) requires the presence of (1) social interaction and communication impairments and (2) restrictive, repetitive patterns of behaviour, interests, or activities. Moreover, these symptoms must be present in early development, cause clinically significant impairment, and not be better explained by intellectual disability (ID) or global developmental delay (DSM-5; Lord et al., 2020). The behavioural alterations in ASD are exemplified by abnormal social approach or failure to initiate social interactions, stereotyped motor movements, rigid thinking patterns, and altered reactivity to sensory input (DSM-5).

Importantly, diagnosis ranges from mild to severe, determined on a three-tiered scale by the degree of support required to enable daily functioning within the two core diagnostic criteria (DSM-5). Not only does heterogeneity exist in clinical symptomology, but also in phenotype presentation, etiology, comorbid diagnoses, and outcome, reflecting the spectrum that is ASD (Lord et al., 2020; Masi et al., 2017). For example, associated symptoms that are commonly, but not always, present include intellectual impairment, ADHD, anxiety (DSM-5; Lord et al., 2020), and altered processing of sensory stimuli (Baum, Stevenson, & Wallace, 2015; Gomes, Pedroso, & Wagner, 2008; Lord et al., 2020; Marco, Baret, Hinkley, Hill, & Nagarajan, 2011; Masi et al., 2017; O'Connor, 2012; Robertson & Baron-Cohen, 2017; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016). Moreover, the sensory symptoms have a huge variation in their presentation (Baum et al., 2015). Previous behavioural studies in autistic individuals have identified differences in social interaction, behavioural flexibility, and sensory processing, which will be explored in following sections.

1.2.2.1 Social Behaviour in ASD

Social behaviour is a complex behaviour based on the ability to interact and communicate with others, and in ASD is characterized by hypo-sociability, or the lack of social connections (Barak & Feng, 2016). Social differences arise in early development in ASD, with newborns showing a lack of preference for the human voice over other sounds, and poor eye-gaze abilities (Barak & Feng, 2016; Frazier et al., 2017). As a child ages, the social phenotype expands to include differences in social motivation, social orienting, and social cognition, exemplified by a lack of interest in and avoidance of social interactions (e.g. preference for solitary play), reduced orientation to social cues (e.g. human voices), and a tendency to incorrectly interpret social information (e.g. sarcasm; Barak & Feng, 2016; Vivanti, Hamner, & Lee, 2018). Social alterations can be seen in nonverbal social interactions and in communication deficits involving impaired speech development (Barak & Feng, 2016).

1.2.2.2 Restrictive and Repetitive Behaviour in ASD

Reduced *behavioural flexibility* associated with ASD often manifests as repetitive sensory-motor behaviours and insistence on sameness, with 81% of autistic individuals displaying stereotypy (Chebli, Martin, & Lanovaz, 2016; Jiujiias, Kelley, & Hall, 2017). Repetitive sensory motor behaviours include stereotyped movements (e.g. rocking) and repetitive use of objects (e.g. spinning objects). Insistence on sameness is exemplified by adherence to strict routines and restricted interests or preoccupations (Jiujiias et al., 2017). Executive functioning deficits are thought to underlie restrictive and repetitive behaviours, and a common measure of executive functioning is cognitive flexibility. For example, autistic individuals perform worse in tasks that require switching behaviours, the ability to sort based on a given rule, and the ability to maintain a new behaviour over a preferred/learned behaviour (D’Cruz et al., 2013; Jiujiias et al., 2017; Van Eylen et al., 2011).

1.2.2.3 Sensory Phenotypes in ASD

The study of sensory processing in ASD has rapidly gained traction in the last 20 years. Altered sensory processing is thought to be related to difficulty with adaptive behaviour

and/or contribute to maladaptive behaviour, making it important to study in ASD (DuBois, Lymer, Gibson, Desarkar, & Nalder, 2017). Since the inclusion of sensory symptoms in the DSM-5, research has taken two approaches to studying sensory processing: the first focuses on identifying sensory symptoms in daily interactions, and the second on basic sensory detection and/or discrimination in research settings (Burns, Dixon, Novack, & Granpeesheh, 2017; DuBois et al., 2017; Schauder & Bennetto, 2016). The first approach commonly uses assessment measures such as behavioural questionnaires (e.g. The Sensory Profile) and behavioural observation (e.g. Sensory Processing Assessment; Burns et al., 2017). Research using these metrics have identified an estimated 75% - 90% of children with ASD show sensory processing differences (Crane, Goddard, & Pring, 2009; Robertson & Baron-Cohen, 2017; Schoen, Miller, Brett-Green, & Nielsen, 2009; Talay-Ongan & Wood, 2000; Tomchek & Dunn, 2007). The second approach to studying sensory processing in ASD addresses basic sensory abilities that are thought to underlie complex behaviours (Baum et al., 2015; Schauder & Bennetto, 2016). Psychophysical behavioural assessments are used to objectively evaluate sensory responses and the neural mechanisms underlying sensory processing (Baum et al., 2015; Robertson & Baron-Cohen, 2017; Schauder & Bennetto, 2016). Importantly, these latter methods find overlapping similarities with symptoms identified via questionnaires (DuBois et al., 2017; Schauder & Bennetto, 2016). Sensory issues identified in ASD include both hypo- and hypersensitivity, sensory-seeking, and sensory-avoidance behaviours across sensory domains (Crane et al., 2009; DuBois et al., 2017; Robertson & Baron-Cohen, 2017; Schauder & Bennetto, 2016; Talay-Ongan & Wood, 2000; Tomchek & Dunn, 2007). Experimentally, behavioural assessments identify differences in the reflexive response, gating, perception, and integration of stimuli (Baum et al., 2015; O'Connor, 2012; Robertson & Baron-Cohen, 2017; Sinclair et al., 2016).

1.2.2.4 Auditory Sensory Differences in ASD

A variety of differences in acoustic stimulus processing have been found in ASD (Baum et al., 2015; O'Connor, 2012; Sinclair et al., 2016). Differences in automatic-response (i.e., pre-attentive) behaviours mediated by brainstem and midbrain structures have been

identified. Autistic individuals show differences in their acoustic startle response (ASR), that is the motoric reaction to an acoustic stimulus. Dependent on the symptom severity, they exhibit increased startle amplitudes and latencies (Kohl et al., 2014; Perry, Minassian, Lopez, Maron, & Lincoln, 2007; Sinclair et al., 2016; Takahashi, Komatsu, Nakahachi, Ogino, & Kamio, 2016; Takahashi et al., 2014; Takahashi, Nakahachi, Stickley, Ishitobi, & Kamio, 2017), and changes in the ASR response are correlated with differences identified in sensory behavioural questionnaires (Takahashi, Nakahachi, Stickley, Ishitobi, & Kamio, 2018). Sensorimotor gating, a process that limits some sensory information from reaching higher cognitive centers, can be operationally measured using prepulse inhibition (PPI) of startle. Typically, when a non-startling acoustic stimulus (i.e. the prepulse) precedes a startling stimulus, there is a reduction in the ASR; however, this process is disrupted in ASD and other neurodevelopmental disorders (Madsen, Bilenberg, Cantio, & Oranje, 2014; Perry et al., 2007; Sinclair et al., 2016; Takahashi et al., 2014 cf. Kohl et al., 2014). Sensory filtering, a process whereby the repeated exposure to a behaviour-inducing stimulus progressively decreases the behavioural response following the stimulus presentation, can be operationally measured using habituation. Normally, the repeated presentation of a startle-eliciting acoustic stimulus leads to a reduction in the ASR. Habituation differences are not consistently reported in ASD, and it has been suggested differences may only exist in sub-populations (Kohl et al., 2014; Kuiper, Verhoeven, & Geurts, 2019; Perry et al., 2007; Sinclair et al., 2016; Takahashi et al., 2016).

Perceptually, autistic individuals do not have inherent differences in their ability to register the presence of an acoustic stimulus, termed their auditory detection threshold (Khalifa et al., 2004; Kuiper et al., 2019). That is, the level at which they can detect a sound in the absence of any external sound is typical. However, autistic individuals find lower intensity sounds to be louder and more uncomfortable (i.e., lower loudness discomfort levels; Khalifa et al., 2004), although their ability to discriminate between sound intensities remains intact (Bonnell et al., 2010). Auditory differences are also seen in temporal processing ability. To date, this has been measured in two ways. First, temporal ability has been assessed by determining the extent that a subject can detect a brief silent gap in an otherwise continuous background noise. This gap detection ability is poorer in autistic individuals, meaning a

longer gap is required for it to be detected (Foss-Feig, Schauder, Key, Wallace, & Stone, 2017). Second, auditory and audiovisual temporal discrimination tasks in which participants must report either the order of presentation of two stimuli (i.e., temporal order judgement), or if the stimuli occur simultaneously (i.e., simultaneity judgment), have found that autistic individuals have an altered temporal binding ability, i.e. they have a reduced sensitivity to the temporal order of stimuli compared to neurotypical individuals (de Boer-Schellekens, Eussen, & Vroomen, 2013; Kwakye, Foss-Feig, Cascio, Stone, & Wallace, 2011; Stevenson et al., 2014).

1.2.3 The Brain and ASD

In addition to behavioural symptomologies, autistic people also have differences in brain structure and function. Altered structural connectivity in excitatory and inhibitory neural networks is proposed to contribute to neural dysregulation in ASD (reviewed in Zikopoulos & Barbas, 2013). Many areas of the brain, including the frontal and temporal cortices, amygdala, and cerebellum show atypical structural characteristics in ASD, which can affect attention, social interactions, emotions, sensory responses, and executive control. In autism, structural connectivity is altered in the form of changes in axonal structure (e.g. reduced diameter and myelination), axonal growth, dendritic arborization (e.g. dendrite branching and density), dendritic spines (e.g. spine number and size), neuronal morphology (number, location, density), and/or inhibitory neurotransmission (e.g. GABAergic innervation or receptor density; Zikopoulos & Barbas, 2013). Short- and long-range connectivity is also disrupted, with local overconnectivity and/or long-distance underconnectivity, depending on the brain region (Zikopoulos & Barbas, 2013).

Autistic individuals also have altered patterns of neural activity. Functional magnetic resonance imaging (fMRI) has supported the concept of altered connectivity in ASD (Kana, Uddin, Kenet, Chugani, & Müller, 2014). Findings suggest the activity in different brain regions is not as strongly correlated in autistic individuals as in neurotypical individuals, with network nodes unable to effectively transfer and integrate information (reviewed in Baum et al., 2015). Electroencephalography (EEG) techniques have been used to investigate the rhythmic neural activity generated by groups of neurons firing

synchronously, called oscillations. Oscillations reflect changes in the local field potential; that is changes in the extracellular voltage recorded by an electrode. At the circuit level, oscillations regulate network organization (reviewed in Simon & Wallace, 2016). In autism, alterations in brain oscillations, namely in the gamma and alpha bandwidths, are proposed to contribute to alterations in sensory and cognitive processing because of a reduced ability to integrate information across different neural networks (i.e. reduced synchrony; Simon & Wallace, 2016; Takarae & Sweeney, 2017). EEG coherence patterns provide information about how well-connected different brain regions are since they reflect the consistency and magnitude of a relationship between simultaneously recorded brain areas. In autistic individuals, EEG coherence patterns are different dependent on the compared brain regions and frequency band of interest, although there remains considerable debate about the details and their significance (Schwartz, Kessler, Gaughan, & Buckley, 2017). Apart from oscillatory activity, differences in the latency and amplitude of evoked potentials have been found in a variety of subcortical and cortical regions in response to various sensory stimuli (Baum et al., 2015, Sinclair et al., 2016). This reduced temporal control of neural activity could affect the perception of stimuli because perception depends on the duration of cortical sensory responses (Takarae & Sweeney, 2017).

1.2.4 Auditory Processing Differences in ASD

Abnormal auditory processing is well documented in ASD (reviewed in Baum et al., 2015; Hitoglou, Ververi, Antoniadis, & Zafeiriou, 2010; Marco et al., 2011; O'Connor, 2012; Sinclair et al., 2016). In the brainstem, delayed neurotransmission through the successive relay nuclei exists (Fujikawa-Brooks, Isenberg, Osann, Spence, & Gage, 2010; Gonçalves, Wertzner, Samelli, & Matas, 2011; Kwon, Kim, Choe, Ko, & Park, 2007; Magliaro, Scheuer, Assumpcao Junior, & Matas, 2010; Miron et al., 2016; Rosenhall, Nordin, Brantberg, & Gillberg, 2003; Roth, Muchnik, Shabtai, Hildesheimer, & Henkin, 2012; Tas et al., 2007; Wong & Wong, 1991). In addition to the brainstem, the auditory cortex also responds differentially to sound in ASD (Baum et al., 2015; O'Connor, 2012; Sinclair et al., 2016). For example, the cortical response to acoustic stimuli is commonly found to be delayed, reflected by slower response latencies as measured by the M50 or M100 using

magnetoencephalography (MEG; Port et al., 2016; Roberts et al., 2010, 2019) and by cortical auditory evoked potentials using EEG (Bruneau, Roux, Adrien, & Barthélémy, 1999; Gage, Siegel, & Roberts, 2003). Specifically, the N1 potential which is associated with activity from the primary auditory cortex, has prolonged latencies (Brandwein et al., 2015; Bruneau et al., 1999; Høyland et al., 2019; Stroganova et al., 2013). Several studies suggest that slower response latencies might reflect immature auditory processing ability in autistic individuals (Berman et al., 2016; Brandwein et al., 2015; Bruneau et al., 1999; Edgar et al., 2015; Høyland et al., 2019; Matsuzaki et al., 2014; Port et al., 2016; Roberts et al., 2019; Stroganova et al., 2013).

1.3 Modelling Neurodevelopmental Disorders in Animals

Animal models are an essential component of preclinical research focused on uncovering the underlying mechanisms that lead to altered brain structure and function, and ultimately neurodevelopmental disorder.

1.3.1 Establishing Validity of Animal Models

The validity of an animal model must be assessed for it to effectively serve as an ASD model. There are three levels of validity to be considered to establish an animal model: construct (i.e., etiology), face (i.e., symptomology), and predictive (i.e., response to treatment; Servadio, Vanderschuren, & Trezza, 2015). The first is construct validity, or to what degree does the model mimic a known cause of ASD or the similarity in the underlying neurobiological mechanisms. In rodent models for ASD, this is achieved by an environmental insult to the rodent in early pre- or postnatal development, or by a targeted genetic mutation in a gene known to be associated with ASD (Möhrle et al., 2020; Servadio et al., 2015; Varghese et al., 2017). The second level is face validity, which addresses how well the model replicates the human phenotype of the disorder (Servadio et al., 2015). Given that an ASD diagnosis relies on evaluating behavioural traits, it is important that preclinical models be assessed for their face validity using behavioural assays that seek to mimic those in humans (Atanasova, 2015; Chen et al., 2015; Kazdoba, Leach, & Crawley, 2016; Möhrle et al., 2020; Servadio, Vanderschuren, & Trezza, 2015). A large number of

behavioural assays have been developed for rodents that are used to determine if behavioural alterations present in rodent models are similar to those observed in autistic individuals (Kas et al., 2014; Möhrle et al., 2020). In addition, brain architecture and neural responses are assessed for their similarity to ASD (Chen et al., 2015). The third level for establishing an animal model is predictive validity, which is how well the model responds to treatments for the disorder that are known to be effective in the human population (Chen et al., 2015; Servadio et al., 2015). Predictive validity is often the last to be established. In addition to the three core levels of validity, a model should also be reliable, and a consistent phenotype should be present in the model across studies (Servadio et al., 2015).

As a consequence of the heterogeneity in the causes and phenotypes of ASD, and the lack of an effective treatment, no one animal model is able to recapitulate all of the complex structural, electrophysiological, and behavioural features of autism. As such, models often focus on replicating the behavioural diagnostic symptoms of ASD: impaired social communication and repetitive behaviours (reviewed in Servadio et al., 2015). Beyond these criteria, models are also tested for associated symptom domains such as altered memory and sensory function.

1.3.2 Assessing ASD-related Behaviour in Rodents

Over the last 30 years, there has been a significant increase in the number of tasks which have shown face validity to assess rodent behavioural phenotypes relevant for autism. Below is a summary; for a comprehensive review see Möhrle et al. (2020).

1.3.2.1 Social behaviours

Social interaction is quantitatively measured in rodents using the 3-chamber apparatus. Sociability is measured by examining the preference of the test animal to spend time with a conspecific in one chamber or a novel object in the opposing chamber, with the center chamber left empty. Social novelty is examined using the same apparatus, but preference for a novel conspecific over a familiar conspecific is assessed (Crawley, 2007; Kas et al., 2014; Moy et al., 2004). Juvenile play is also used to measure social behaviour in rodents. By assessing the interaction of two freely moving rodents, behaviours extending beyond

social approach can be measured, including social reciprocity, aggression, play behaviour, parental behaviour, and sexual approach (Crawley, 2007; Kas et al., 2014). Furthermore, rodents emit ultrasonic vocalizations, and both call number and type can be assessed to provide an indication of social communication. Distress calls emitted by pups separated from the nest, and play vocalizations emitted during free interaction can provide information about differences in communicative behaviours in different models (Crawley, 2007).

1.3.2.2 Repetitive behaviours and behavioural inflexibility

Commonly measured repetitive behaviours in rodents include grooming, jumping, circling, rearing, and digging (Crawley, 2012; Kas et al., 2014). Behavioural inflexibility and increased frequency of perseveration can be measured using a variety of reversal paradigms that assess resistance to change. These paradigms are based on an animal's ability to change its behavioural response strategy based on environmental cues (Kas et al., 2014). Example tasks include a change in location of a food reward in a T-maze, relocating a hidden platform in the Morris water maze, and tasks in operant boxes that require rodents to learn a new rule in order to receive a food reward (i.e., set-shifting and reversal learning paradigms; Crawley, 2007; Gilmour et al., 2013; Kas et al., 2014).

1.3.2.3 Learning and Memory

Cognition is assessed using learning and memory tests such as spatial navigation tasks including the Morris water maze, Barnes maze, radial maze and T-maze, fear conditioned freezing, 5-choice serial reaction time task, and operant reinforcement schedules (Crawley, 2007, 2012). The time to learn the task, accuracy, and the memory of the task after a given period of time are common measurements.

1.3.2.4 Auditory behaviours

Pre-attentive. Behavioural paradigms relying on the acoustic startle response (ASR), i.e. the motor reaction to an acoustic stimulus, have gained traction in the last 20 years for assessing pre-attentive acoustic responsivity in ASD (Sinclair et al., 2016). Importantly, the acoustic startle pathway is a relatively simple neural circuit, well defined, and

conserved across species (Koch, 1999), making the study of the ASR a translational and accessible behavioural readout of pre-attentive auditory function. The acoustic startle response is elicited by presenting a sudden, loud acoustic stimulus and assessed in rodents by measuring the whole-body contraction using a movement-transducing platform (Valsamis & Schmid, 2011). The ASR can be modulated by both internal and external stimuli, and has a non-zero baseline, meaning it can show both reduction (e.g., habituation and prepulse inhibition) and enhancement (e.g., sensitization, fear potentiation, or prepulse facilitation). Sensory filtering, a process that limits some sensory information from reaching higher cognitive centers, can operationally be measured using habituation and/or prepulse inhibition (PPI). Habituation of the ASR is the progressive decrease in the startle response to the repeated presentation of a loud acoustic stimulus. PPI is the modification of the ASR by a sensory event preceding the startling stimulus by 30 – 500 msec, called a prepulse stimulus (Hoffman & Ison, 1980). Typically, when a non-startling acoustic prepulse precedes a startling stimulus, there is a reduction in the ASR.

Perceptual. There are several tasks that have been developed to assess sound perception in rodents in order to discern sensory processing ability at a higher-cognitive level. A light/dark box test apparatus is normally used to assess anxiety and exploratory behaviour based on rodents' natural preference for dark spaces over light spaces (Bourin & Hascoët, 2003; Crawley, 2007). Recently, this natural behaviour was harnessed to assess the averseness of sound stimuli by measuring the sound level at which rats preferred a quiet + brightly lit area over a loud + dark area as the sound level gradually increased in the dark area (Manohar, Spoth, Radziwon, Auerbach, & Salvi, 2017). Given reports of increased loudness discomfort levels and hyper-responsiveness to auditory stimuli in ASD (Khalifa et al., 2004; Schauder & Bennetto, 2016), this task offers a unique way to study subjective perception and averseness of sound in rodents. Second, various operant tasks have been developed that allow for psychophysical testing of acoustic stimulus perception. In these tasks a single stimulus characteristic is manipulated. For example, rodents are trained using a two-alternative forced choice task to categorize acoustic stimuli using template sounds on the extreme ends of a continuum varied in a single stimulus characteristic (e.g., sound level), and, once trained, their perception of ambiguous stimuli are tested. Sound quality

perception has been studied with amplitude or frequency detection paradigms and the temporal processing of auditory and visual stimuli has been studied using temporal order judgement paradigms (Sarro & Sanes, 2011; Schormans et al., 2017). In addition, a sound level detection task was created by pairing conditioned avoidance behaviour with silence to determine at what sound intensity a rat can perceive a sound (i.e., acoustic threshold; Möhrle et al., 2019; Rüttiger, Ciuffani, Zenner, & Knipper, 2003).

1.3.3 Creating Animal Models

In preclinical ASD research, animal models fall into two categories: environmental or genetic, with models simulating risk factors or causative agents that lead to ASD. Given the heterogeneity in the cause of ASD, understanding the role of environmental and genetic risk-factors will greatly improve our understanding of the convergent and underlying processes leading to the disorder (Chen et al., 2015; Cheroni et al., 2020; de la Torre-Ubieta et al., 2016). Researchers have pursued this endeavour over the past decades by creating rodent models through exposure to environmental insults, or through targeted gene deletions or mutations that mimic the genetic alterations present in ASD (Chen et al., 2015; Möhrle et al., 2020; Patterson, 2011; Pinto et al., 2014; Varghese et al., 2017).

1.3.3.1 Environmental Rodent Models for ASD

Rodent models for ASD based on environmental risk factors are created by exposing the pup *in utero* to the risk factor. This is accomplished by altering the dam's drinking water, food, air, or by injection. The most common models are based on maternal infection or valproic acid (VPA) exposure. Epidemiological evidence has found associations between maternal infection in the first trimester and autism (Patterson, 2011; Servadio et al., 2015). In rodents, the dam's innate immune system can be activated using polyinosinic:polycytidilic acid [poly(I:C)], a synthetic, double-stranded RNA analog that evokes an antiviral-like immune reaction, or lipopolysaccharide (LPS), which evokes an antibacterial-like immune reaction (Patterson, 2011; Servadio et al., 2015). VPA is a common medication for epilepsy and mood disorders and has been identified as a risk factor for ASD in children whose mother had taken VPA during pregnancy. In rodents, a

single injection of VPA in pregnant dams can lead to consistent autism-like alterations in social, stereotypic, and sensory behaviours in the offspring (Patterson, 2011; Servadio et al., 2015; Varghese et al., 2017). Other models include ethanol exposure or thalidomide exposure (Patterson, 2011).

1.3.3.2 Genetic Rodent Models for ASD

Over the last 15 years, there has been a large increase in the number and type of genetic models for ASD (for review see Chen et al., 2015; Möhrle et al., 2020). Alterations in genes that encode cell-adhesion molecules are one avenue researchers have taken in the creation of genetic models, since several of these genes have been linked to ASD and autistic-like behaviours (Chen et al., 2015). These include presynaptic neurexins, postsynaptic neuroligins, the SHANK postsynaptic scaffolding proteins, and contactin and associated proteins for axonal and dendritic organization (Chen et al., 2015). Indeed, several genes in this group cause rare monogenic ASD. For example, deletion of *SHANK3* in human causes Phelan–McDermid syndrome, mutations in the *FMRI* gene leads to fragile X syndrome, variants of *NLGN-3* are associated with ASD, and homozygous mutations in *CNTNAP2* cause CDFE. All of these disorders present with the behavioural symptoms of ASD (Chen et al., 2015; Patterson, 2011; Varghese et al., 2017). To date, both mice and rats with mutations in *Shank3*, *Fmr1*, *Nlgn-3*, and *Cntnap2* have been created and display behavioural alterations that mimic those of autistic individuals (Chen et al., 2015; Möhrle et al., 2020; Patterson, 2011; Varghese et al., 2017).

1.4 *Cntnap2* Rodent Models

The *CNTNAP2* gene has been implicated in autism and neurodevelopmental disorders starting in 2006, with a report by Strauss et al. (2006), in which a homozygous gene mutation was discovered in a population of Amish children who presented with language regression, hyperactivity, impulsive and aggressive behaviours, seizures, and cognitive delays, of which ~ 70% were diagnosed with ASD (see section 1.1.1 for more information). Since this initial report, a variety of mutation types in the gene has been linked to other neurodevelopmental disorders including intellectual disability, developmental disability,

and language impairment (SFARI; Arking et al., 2008; Newbury et al., 2011; Poot, 2017; Rodenas-Cuadrado et al., 2014; Vernes et al., 2008). As such, the SFARI gene-scoring database has ranked it as a strong candidate risk gene (SFARI). This led to the development of *Cntnap2* rodent models to uncover the role of the gene in autism-like behaviours, language, and the neural mechanisms through which it alters function. Importantly, the *Cntnap2* gene is highly (87%) conserved between rats and humans, indicative of a preserved functional role (Abrahams et al., 2007; Rodenas-Cuadrado et al., 2014).

To date, both mouse and rat models have been created with alterations in *Cntnap2*. There are 35 different *Cntnap2* mouse models (SFARI, mouse model database), the majority generated and donated by Dr. Elior Peles from the Weizmann Institute of Science. The mouse models have differing mutations in the *Cntnap2* gene and exhibit a range of autistic-like behaviours (SFARI, mouse model database). More recently, rat models with mutations in the *Cntnap2* gene have been created. The first rat model was originally created at SAGE Laboratories, Inc. in conjunction with Autism Speaks and the line is now maintained by Envigo (SD-*Cntnap2*^{tm1Sage}). It was produced using zinc finger nuclease targeted gene editing on a Sprague Dawley background to create a five base pair deletion in exon 6 of the *Cntnap2* gene (Envigo). A second rat model was created by Simons Foundation Autism Research Initiative (SFARI; LE-*Cntnap2*^{em1Mcwi}). This rat strain was produced by injecting CRISPR/Cas9 targeting rat *Cntnap2* into Long-Evans rat embryos, resulting in a one base pair deletion in exon 6 of the gene (Rat Genome Database). Given the recent creation of these models (since 2014), little literature exists on their face validity. This thesis focuses on establishing face validity and uncovering the way *Cntnap2* alters auditory processing in the SD-*Cntnap2*^{tm1Sage} rat model.

1.4.1 Classical Role of *Cntnap2*: K⁺ Clustering in the Axon

The function of the *Cntnap2* gene was first discovered by Dr. Elior Peles and colleagues in 1999, where its protein product, Caspr2, was identified as a neurexin-like protein located in the juxtaparanodal region of the nodes of Ranvier in myelinated axons. Here, Caspr2 colocalized with Shaker-like K⁺ channels, suggesting a role in the differentiation of the axon into distinct functional subdomains (Poliak et al., 1999; Traka et al., 2003). To further

probe the role of Caspr2, mice with mutations in the *Cntnap2* gene that led to a non-functional protein product were created (Poliak & Peles, 2003; Poliak et al., 2003). Caspr2 interacts with TAG-1, an immunoglobulin-like cell adhesion molecule, to form a scaffold needed to cluster the K⁺ channels in the juxtaparanodal region (Poliak et al., 2003). Through this association, Caspr2 is also involved in maintaining axon-glia interactions mediated by the homophilic binding of TAG-1 present on myelinating glia cells (Fig. 1-1; Poliak & Peles, 2003; Poliak et al., 2003; Traka et al., 2003). In mice, the loss of *Cntnap2* led to the loss of accumulation of Kv1.1, Kv1.2, and Kvβ2 in the juxtaparanodal region and their spread into the internodes. Despite this rearrangement, nerve conduction appeared to remain intact in the CNS (optic nerve; Poliak et al., 2003). In addition to clustering K⁺ channels in the juxtaparanodal region, Caspr2 also localizes Kv1.2 channels to the distal region of the axon initial segment of human neocortical pyramidal cells (Inda, DeFelipe, & Muñoz, 2006). This function contributes to the regional segregation of the axon initial segment, and therefore could influence pyramidal cell activity. More recent findings suggest that altered K⁺ clustering does in fact affect the action potential waveform of long-range myelinated axons, with wider action potential leading to increases in neurotransmitter release and postsynaptic excitatory responses in *Cntnap2* knockout mice layer 2/3 cortical neurons (Scott et al., 2019). Altered K⁺ clustering due to loss of *Cntnap2* could therefore have a postnatal influence on the temporal aspects of action potentials, neurotransmitter release, and excitability.

1.4.2 Role of *CNTNAP2* in Neurodevelopment

Since *CNTNAP2* is expressed embryonically before neuronal myelination, additional roles in prenatal development have been explored as alternative mechanisms through which mutations in *CNTNAP2* could affect early brain development and lead to disorder (Alarcón et al., 2008; Poliak et al., 1999; Rodenas-Cuadrado et al., 2014). Neuronal circuit assembly depends on proper neuron migration, and the regulation and maintenance of synaptic contacts. Research suggests that *CNTNAP2* may play a role in circuit assembly in the CNS, since its protein product is a cell adhesion molecule. In people with CDFE (homozygous

mutations in *CNTNAP2* gene) and in *Cntnap2* knockout mice, abnormal cortical neuron migration patterns have been observed, such that there are fewer upper layer projection neurons as they appear to be redistributed to lower cortical layers (Penagarikano et al., 2011; Strauss et al., 2006). Caspr2 is present in the dendritic spines, axon, and soma of neurons and interacts with other proteins outside of the juxtapanodal region to influence the development of spiny synapses (Fernandes et al., 2019; Horresh et al., 2008; Varea et al., 2015). Altered dendritic arborization and spine development have also been observed

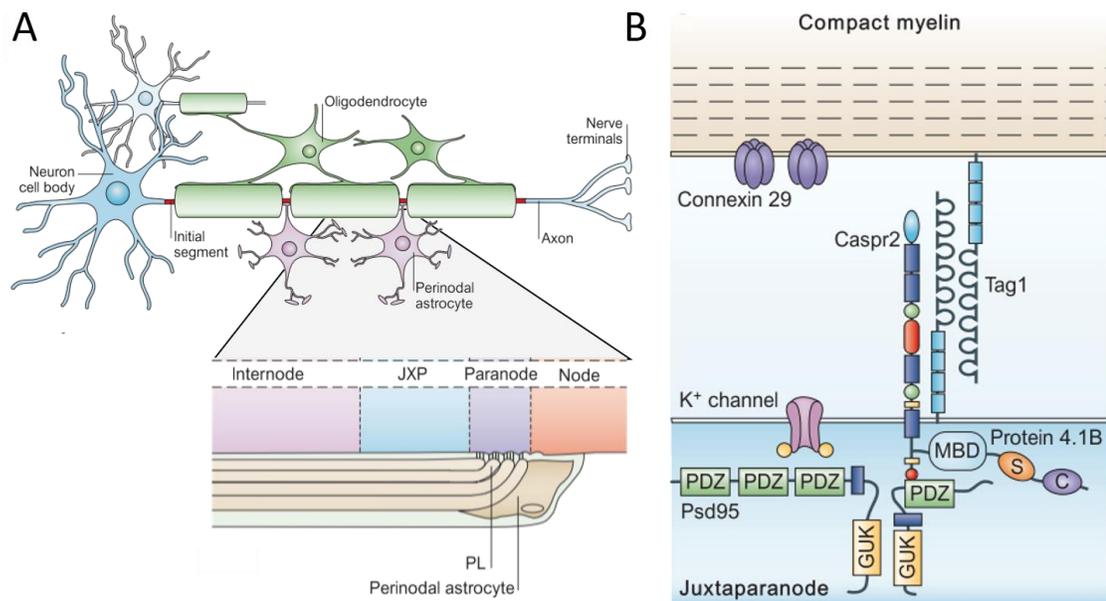


Figure 1-1: Caspr2 location and function.

(A) Depiction of myelinating glial cells and oligodendrocytes in the central nervous system (CNS) forming the myelin sheath. Myelin covers the axon at intervals, leaving bare gaps termed nodes of Ranvier. Nodal region is expanded below, depicting the compartmentalized regions. (B) Juxtapanode (JXP) where the Caspr2 protein is located. Caspr2 interacts with TAG-1 to cluster K⁺ channels and anchor the myelin sheath. Adapted with permission from RightsLink: Springer Nature, Nature Reviews Neuroscience (The local differentiation of myelinated axons at nodes of Ranvier, Poliak & Peles, 2003), Copyright © Nature Publishing Group (2003).

in *Cntnap2* mutants. RNAi-mediated knockdown of Caspr2 in cultured cortical neurons from newborn mice have revealed decreases in dendritic arborization, which leads to decreases in synapse numbers and transmission (Anderson et al., 2012). Other studies have shown that Caspr2 is involved in maintaining spine density and morphology (Gdalyahu,

Lazaro, Penagarikano, & Golshani, 2015; Varea et al., 2015). In mice lacking *Cntnap2*, dendritic spines were less dense because of an increase in spine elimination due to an impairment in the stabilization of new spines (Gdalyahu et al., 2015). The role of *Cntnap2* in spine stabilization may arise through its interaction with CASK (calcium/calmodulin-dependent serine protein kinase 3), a scaffolding protein involved in anchoring and trafficking proteins, and previously associated with spine development (Gdalyahu et al., 2015; Horresh et al., 2008). Alternatively, *Cntnap2* may be involved in spine stabilization through its role in trafficking GluA1 AMPA receptor subunits to the spine head of pyramidal neurons, with a knockout of *Cntnap2* in neurons leading to somatic aggregates of GluA1 (Fernandes et al., 2019; Varea et al., 2015). The reduction of GluA1-AMPA receptors trafficking to the cell surface and into synapses was found to impair AMPAR-mediated synaptic transmission in the mouse visual cortex (Fernandes et al., 2019). Ultimately, reduced spine stabilization and altered AMPA receptor function affect synaptic signaling and may be a mechanism of *Cntnap2* that leads to altered neurodevelopment.

The expression pattern of *CNTNAP2* points to roles in sensory and cognitive development and function. In humans, *CNTNAP2* is enriched in cortico-striatal-thalamic circuitry (temporal lobes, striatum, and dorsal thalamus), in the frontal and prefrontal cortex, and in language-related association cortices (Abrahams et al., 2007; Alarcón et al., 2008). In both humans and mice, the gene is expressed throughout regions involved in sensory processing in both the brainstem and cortex (Alarcón et al., 2008; Gordon et al., 2016).

1.4.3 *Cntnap2* Models and Autistic-like Behaviours

Social behaviour and communication. Generally, *Cntnap2* knockout mice show ASD relevant impairments in social ability. *Cntnap2* knockout mice performing the 3-chamber interaction test have impaired sociability, as they do not prefer to interact with a conspecific over an inanimate object (Penagarikano et al., 2011; Xing et al., 2019 cf. Brunner et al., 2015; R. Scott et al., 2019), and they show poor reciprocal social interaction (Xing et al., 2019 cf. Brunner et al., 2015; R. Scott et al., 2019). *Cntnap2* knockout mice have also been shown to emit significantly fewer ultrasonic vocalizations than wildtype littermates (Brunner et al., 2015; Penagarikano et al., 2011).

Repetitive behaviours and behavioural flexibility. In the classical metrics of repetitive behaviours for mice, *Cntnap2* knockout mice consistently show increased grooming and digging behaviours (Penagarikano et al., 2011; Scott et al., 2019; Thomas, Schwartz, Saxe, & Kilduff, 2016; Xing et al., 2019), and both rats and mice exhibit hyper-locomotion (Brunner et al., 2015; Scott et al., 2019; Thomas et al., 2016; Xing et al., 2019). Moreover, knockout mice exhibit more preservative behaviours in Morris water maze reversal learning and T-maze spontaneous alternation tasks (Penagarikano et al., 2011), indicating they may have difficulty in changing to newly learned behaviours.

Cognitive behaviours. Learning and memory appear intact in *Cntnap2* mutants. Visual spatial learning and reference memory tasks have shown intact, though moderately delayed, learning and memory using the Morris water maze (Penagarikano et al., 2011) and radial-arm maze (Rendall, Truong, & Fitch, 2016).

Sensory behaviours. The most commonly assessed sensory behaviour is the acoustic startle response and associated prepulse inhibition. Behaviourally, no ASR differences are reported in knockout *Cntnap2* mice and prepulse inhibition differences vary with experimental protocol (Brunner et al., 2015; Penagarikano et al., 2011; Truong, Rendall, Castelluccio, Eigsti, & Fitch, 2015). However, increased reactivity to other sensory stimuli have been reported, specifically in response to mechanical, heat, or chemical stimuli, and increased sensitivity to olfactory cues in a buried food assay (Dawes et al., 2018; Penagarikano et al., 2011). In addition, impaired cortically mediated temporal processing has been identified using silent gap detection (Truong et al., 2015).

As mentioned above and in reviews of several studies using *Cntnap2* mouse models, no single model species is able to capture the full complexity of ASD (Möhrle et al., 2020; Vecchia et al., 2019). Several reviews compare the utility of rats and mice on various behavioural tasks such as social behaviour, communication, learning, and cognition (Ellenbroek & Youn, 2016; Homberg, Wöhr, & Alenina, 2017; Wöhr & Scattoni, 2013). For example, rats may be preferred when assessing social behaviour as they have a more complex social repertoire and exhibit less aggression, stress, and variability compared to mice (Homberg et al., 2017; Kondrakiewicz, Kostecki, Szadzińska, & Knapska, 2019).

Rats may also be preferred in tasks involving repeated stimuli exposure, learning, and cognition, such as the probabilistic reversal learning, sound intensity categorization, and audiovisual temporal order judgement tasks, because rats typically require shorter habituation periods and fewer training sessions to perform the task (Ellenbroek & Youn, 2016).

1.5 Uncovering the Role of *CNTNAP2* in Auditory Processing

1.5.1 *CNTNAP2* and Language

The *CNTNAP2* gene is closely linked to language and auditory processing. In the neurodevelopmental disorders to which *CNTNAP2* has been linked, differences in language development are prevalent (Deriziotis & Fisher, 2017; Kang & Drayna, 2011; Poot, 2015; Rodenas-Cuadrado et al., 2014). Moreover, heterozygous *CNTNAP2* mutations (e.g. SNPs) lead to impairments in speech and language such as dysarthric language, language delay or absent speech/language and genetic variation in *CNTNAP2* in neurotypicals is responsible for differences in linguistic processing and development (Fisher & Scharff, 2009; Poot, 2015; Rodenas-Cuadrado et al., 2014; Whalley et al., 2011; Whitehouse et al., 2011; Worthey et al., 2013).

Supporting the involvement of *CNTNAP2* in language development is the fact that it is a downstream target of the *FOXP2* gene, one of the most well studied genes linked to language disorders (Fisher & Scharff, 2009; Lai, Fisher, Hurst, Vargha-Khadem, & Monaco, 2001; Vernes et al., 2008; White, 2010). *FOXP2* downregulates *CNTNAP2* expression with a binding site in intron 1, and they show complimentary expression throughout the cerebral cortex (Fisher & Scharff, 2009; Vernes et al., 2008). While language is uniquely human, *FOXP2* has conserved expression in the cortex, thalamus, basal ganglia, and cerebellum in vertebrates, and mutations in mice lead to altered USVs and neural plasticity (Deriziotis & Fisher, 2017). Moreover, in songbirds, the *Cntnap2* gene is differentially expressed in females and males (Carmen Panaitof, Abrahams, Dong, Geschwind, & White, 2010). *Cntnap2* expression is enriched in key song control nuclei of

the zebra finch, but only in males, since vocal learning and song are not acquired by females of this species (Carmen Panaitof et al., 2010; Condro & White, 2014).

1.5.2 Studying *Cntnap2* and Sound Processing

Sensory-evoked potentials (i.e., event-related potentials) are the most accessible cross-species platform for studying the neural activity related to stimulus processing. In rodents, the dynamics of the brain's response to an acoustic stimulus can be determined at the level of the brainstem, midbrain, and cortex by using subdermal electrodes, bone screws, or *in-vivo* extracellular recordings. This approach has helped to uncover the neural mechanisms of stimulus processing, with a consistent finding of delayed brainstem and cortical processing of sound in a variety neurodevelopmental disorders, including ASD, and respective animal models (Ankmal-Veeranna et al., 2019; Kwok et al., 2018; O'Connor, 2012; Scott et al., 2018; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016).

1.5.2.1 Brainstem Auditory Processing

The electrical response of the auditory brainstem to the presentation of acoustic stimuli is called the brainstem auditory evoked potential (BAEP), also known as the auditory brainstem response (ABR). It occurs within the first 10 – 15 milliseconds of stimulus presentation (Petrova, 2009). In humans, the ABR consists of five to seven main positive waves (I – VII), representing the auditory nerve (proximal - I, distal - II), cochlear nucleus (CN - III), superior olivary complex (SOC - IV), lateral lemniscus (LL - V), and inferior colliculus (IC - VI-VII; Petrova, 2009). In rats, four or five waves are detectable, depending on stimulus frequency (Alvarado, Fuentes-Santamaría, Jareño-Flores, Blanco, & Juiz, 2012), thought to represent the auditory nerve (I), CN (II), SOC (III), and LL terminating at the IC (IV-V). Therefore, latency metrics often taken in human subjects focusing on waves I, III, and V, correspond to waves I, II, and IV in the rat.

Auditory brainstem responses help to understand auditory processing relevant for pre-attentive behaviours. The acoustic startle response is a pre-attentive behavioural response mediated by a short and conserved pathway, with information passing through the auditory nerve (and cochlear root nucleus in rodents), to the caudal pontine reticular nucleus (PnC),

before reaching the spinal cord (Koch, 1999). Sensorimotor gating, specifically the pathway for acoustic prepulse inhibition, is more complex, involving midbrain and cortical structures (Fendt, Li, & Yeomans, 2001; Semba & Fibiger, 1992). That said, the basic pathway depends on the auditory nerve through to the inferior colliculus, before information is processed in the caudal pedunculopontine tegmental nucleus (PPT) which projects to the PnC (Koch, 1999).

1.5.2.2 Cortical Auditory Processing

The electrical response of the cortex to the presentation of acoustic stimuli is called the cortical auditory evoked potential (CAEP), and can be measured with electroencephalography (EEG) in humans, and a variety of electrode types in rodents (e.g. surface, intracranial, or indwelling). The primary potentials of interest are the N1 and P2 potentials which are consistently elicited by simple acoustic stimuli (e.g. click, noise burst, or tone), with additional components present if the stimulus contains information significant to the subject (e.g. as with speech stimuli in humans; Näätänen, Simpson, & Loveless, 1982). The N1 and P2 potentials represent activity of the primary auditory cortex and mesencephalic reticular activating system (RAS; Ponton, Eggermont, Kwong, & Don, 2000).

Cortical activity can also be studied at the microcircuit level in rodents by conducting invasive *in vivo* neural recordings. Extracellular recordings allow for the spiking activity of surrounding neurons to be recorded in response to a stimulus.

1.6 Thesis Overview

1.6.1 Chapter 2: Loss of *Cntnap2* in the Rat Causes Autism-Related Alterations in Social Interactions, Stereotypic Behaviour, and Sensory Processing

Rational & Objective. The *CNTNAP2* gene is a highly penetrant risk gene thought to play a role in the genetic etiology of neurodevelopmental disorders such as ASD. Despite its link to ASD, the field lacks a complete understanding of the role *CNTNAP2* plays in the hallmarks of ASD: social interactions, repetitive behaviours, language, and sensory

processing. Therefore, in Chapter 2, I examine if a loss-of-function mutation in the *Cntnap2* gene in rats is sufficient to cause autism-related alterations in social interactions, stereotypic behaviour, and sensory processing.

Experimental Approach. Using behavioural assays that seek to mimic those behaviours observed in humans, I investigated the consequences of a functional loss of *Cntnap2* on ASD-related behaviours by assessing social interactions, behavioural flexibility, spatial learning/memory, and sensory processing at the pre-attentive and perceptual levels. Specifically, I compared the performance of adult male and female rats with a homozygous or heterozygous knockout of *Cntnap2* to their wildtype littermates across a comprehensive test battery.

Predicted Results & Significance. Given the relationship between *CNTNAP2* and ASD, and the presentation of autistic-like behaviours in mouse models with *Cntnap2* mutation, I predicted a loss of *Cntnap2* function in the rat would be sufficient to cause alterations in social, restricted and repetitive, and sensory behaviours. Ultimately, this study may establish the *Cntnap2* knockout rat model as an effective preclinical platform to investigate (1) the neural mechanisms underlying sensory differences in ASD and (2) the complex relationship between altered sensory processing and the diagnostic behaviours for ASD.

1.6.2 Chapter 3: Altered Auditory Processing, Filtering, and Reactivity in the *Cntnap2* Knockout Rat Model for Neurodevelopmental Disorders

Rational & Objective. Autistic people have altered responses to sensory stimuli, and my previous work identified that a loss of *Cntnap2* in rats leads to a behavioural profile reflective of differential sensory processing, including an increase in the acoustic startle response magnitude and reduced sensorimotor gating (i.e., Chapter 2 findings). As such, it was hypothesized that altered processing of sound in the brainstem could explain these pre-attentive differences in behaviour.

Experimental Approach. I investigated the developmental trajectory of *Cntnap2*-related deficits in electrophysiological and behavioural measures of brainstem function in male

and female juvenile, adolescent, and adult male and female rats with a homozygous or heterozygous knockout of *Cntnap2* compared with wildtype controls. Consistent with electrophysiological testing in humans, the four characteristic waves of the rat auditory brainstem response (ABR) to acoustic stimuli were used to assess hearing sensitivity (i.e., hearing threshold), neural responsiveness (i.e., ABR wave amplitude), and speed of neurotransmission (i.e., ABR wave latency) across development, which provided an index of the reliability of auditory information processing. Behaviourally, pre-attentive responses to startle-eliciting sounds were used to determine how *Cntnap2* dysfunction affected the maturation of the acoustic startle response, sensory filtering (i.e., habituation), and sensorimotor gating (i.e., prepulse inhibition).

Predicted Results & Significance. Given that *Cntnap2* is expressed in the cochlear nucleus, superior olive, and inferior colliculus in rodents (Gordon et al., 2016), I predicted the loss of *Cntnap2* would alter the maturation of brainstem auditory processing and brainstem-mediated behaviours. Overall, this study provides the first comprehensive investigation of the direct contribution of the autism-linked gene *CNTNAP2* to the development of brainstem-mediated auditory processing and behaviour.

1.6.3 Chapter 4: Immature and Hyperexcitable Auditory Cortex in Rats with a Functional Loss of the Language-linked Gene *Cntnap2*

Rational & Objective. The *CNTNAP2* gene has been linked to language related disorders such as ASD and DLD, and individuals with these disorders show altered cortical response profiles to sound. Several studies suggest the cortical response of individuals with language-related disorders appears delayed, reflecting immaturity in the auditory cortex, and that there are impairments in rapid temporal processing (Berman et al., 2016; Brandwein et al., 2015; Oram Cardy, Flagg, Roberts, & Roberts, 2005; Port et al., 2016; Samson et al., 2011). Despite its candidacy for influencing language development, few preclinical studies have examined the role of *CNTNAP2* in auditory processing. I endeavoured to determine if a functional loss of *Cntnap2* in rats would lead to altered cortical auditory processing in adulthood similar to what is seen in ASD and DLD.

Experimental Approach. I examined differences in auditory processing in adulthood in wildtype and *Cntnap2* knockout rats by (1) measuring brainstem and cortical responses to simple noise bursts using auditory evoked potentials (AEP) and cortical multi-unit response dynamics, (2) determining the temporal processing ability of the cortex, (3) examining the single-cell response properties of pyramidal cells, and (4) assessing the expression of markers of excitation and inhibition in the auditory cortex.

Predicted Results & Significance. Given that atypical sensory input during early development can influence adult function, and my previous work identified delayed development of the auditory brainstem response in *Cntnap2* knockout rats (i.e., Chapter 3 finding), I predicted that cortical auditory processing in *Cntnap2* rats would reflect an immature profile, and that they would have a reduced ability to process rapidly presented sounds. This study provides the first evidence that a loss of *Cntnap2* function is sufficient to cause changes in adult cortical auditory function similar to ASD and DLD, which is characterized by immature cortical evoked potentials, delayed multi-unit response latencies, impaired temporal processing, and a pattern of hyper-excitability in both multi-unit and single cell recordings.

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Chapter 2

2 Loss of *Cntnap2* in the Rat Causes Autism-Related Alterations in Social Interactions, Stereotypic Behaviour, and Sensory Processing ¹

Autism spectrum disorder (ASD) is characterized by social interaction and communication impairments, as well as restrictive/repetitive patterns of behaviour, interests or activities, which can coexist with intellectual disability and altered sensory processing. To study the mechanisms underlying these core features of ASD, preclinical research has developed animal models with manipulations in ASD-linked genes, such as *CNTNAP2*. In order to fully interpret the findings from mechanistic studies, the extent to which these models display behaviours consistent with ASD must be determined. Toward that goal, we conducted an investigation of the consequences of a functional loss of *Cntnap2* on ASD-related behaviours by comparing the performance of rats with a homozygous or heterozygous knockout of *Cntnap2* to their wildtype littermates across a comprehensive test battery. *Cntnap2*^{-/-} rats showed deficits in sociability and social novelty, and they displayed repetitive circling and hyper-locomotion. Moreover, *Cntnap2*^{-/-} rats demonstrated exaggerated acoustic startle responses, increased avoidance to sounds of moderate intensity, and a lack of rapid audiovisual temporal recalibration; indicating changes in sensory processing at both the pre-attentive and perceptual levels. Notably, sensory behaviours requiring learned associations did not reveal genotypic differences, whereas tasks relying on automatic/implicit behaviours did. Ultimately, because these collective alterations in social, stereotypic and sensory behaviours are phenotypically

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similar to those reported in individuals with ASD, our results establish the *Cntnap2* knockout rat model as an effective platform to study not only the molecular and cellular mechanisms associated with ASD, but also the complex relationship between altered sensory processing and other core ASD-related behaviours.

2.1 Introduction

ASD is a neurodevelopmental disorder, with diagnosis by the DSM-5 (American Psychiatric Association, 2013) requiring the presence of (1) social interaction and communication impairments and (2) restrictive, repetitive patterns of behaviour, interests or activities. These behavioural alterations are exemplified by abnormal social approach or failure to initiate social interactions, stereotyped motor movements, rigid thinking patterns, and altered reactivity to sensory input (DSM-5). Additional associated symptoms include intellectual impairment (DSM-5), and altered processing of sensory stimuli (reviewed in Baum, Stevenson, & Wallace, 2015; Gomes, Pedroso, & Wagner, 2008; Marco, Baret, Hinkley, Hill, & Nagarajan, 2011; O'Connor, 2012; O'Connor & Kirk, 2008; Robertson & Baron-Cohen, 2017; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016). Previous behavioural studies on individuals with ASD have identified differences in social interaction, behavioural flexibility, intellectual ability, and sensory processing. Research on the social interaction phenotype in ASD has generally reported that these individuals orient less to social cues, prefer solitary activities, and tend to incorrectly interpret social information (Vivanti, Hamner, & Lee, 2018). Reduced behavioural flexibility associated with ASD often manifests as repetitive sensory motor behaviours and stereotypic behaviours (Chebli, Martin, & Lanovaz, 2016; Jiujiu, Kelley, & Hall, 2017), as well as cognitive inflexibility, in which individuals with ASD can show difficulty transitioning away from a preferred/learned behaviour to a new one (D'Cruz et al., 2013). Intellectual ability, which can be studied using spatial learning/memory tasks, and viewed in the context of spatial navigation, is thought to be altered in ASD (Edgin & Pennington, 2005; Lind, Williams, Raber, Peel, & Bowler, 2013; Smith, 2015). Lastly, a variety of differences in sensory processing have been found (Baum et al., 2015; Bonnel et al., 2010; O'Connor, 2012; Sinclair et al., 2016; Zhou, Nagarajan, Mossop, & Merzenich, 2008), including

atypical processing of auditory information (Khalifa et al., 2004; Takahashi, Nakahachi, Stickley, Ishitobi, & Kamio, 2018), and altered audiovisual temporal processing (Noel, De Nier, Stevenson, Alais, & Wallace, 2016; Stevenson et al., 2014; Turi, Karaminis, Pellicano, & Burr, 2016).

Currently, there are ~800 known genes associated with autism susceptibility (SFARI). One example of an autism-risk gene is contactin-associated protein-like 2 (*CNTNAP2*), which was identified through linkage and association studies (Alarcón et al., 2008; Arking et al., 2008; Rodenas-Cuadrado et al., 2016; Sampath et al., 2013). The importance of *CNTNAP2* for neurodevelopment and its link to ASD was first documented by Strauss and colleagues (2006), who reported that 67% of the individuals with a homozygous mutation of the gene were diagnosed with ASD, and all presented with hyperactivity, aberrant social interaction, and language regression. Since this initial report, heterozygous mutations and other alterations in *CNTNAP2* have also been implicated in developmental disorders, including autism, intellectual disability, and schizophrenia, as well as in neurotypicals (reviewed in Rodenas-Cuadrado, Ho, & Vernes, 2014; Poot, 2015), highlighting its complex role as a genetic risk factor in neurological disorders. Given the breadth of effects caused by disruption in *CNTNAP2*, it is important to uncover the specific mechanisms by which this gene contributes to the core features of ASD.

Over the past decades, researchers have attempted to better understand the convergent molecular and cellular pathways by which particular risk genes, such as *CNTNAP2*, increase ASD susceptibility by creating rodent models with targeted gene deletions or mutations (Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; Möhrle et al., 2020; Patterson, 2011; Pinto et al., 2014; Varghese et al., 2017). Indeed, preclinical studies investigating the role of *Cntnap2* in mouse models have confirmed that its protein product, CASPR2, is important for axon myelination (Traka et al., 2003), neuronal migration (Penagarikano et al., 2011), stabilization of new dendritic spines (Gdalyahu, Lazaro, Penagarikano, & Golshani, 2015), and control of pyramidal cell activity (Inda, DeFelipe, & Muñoz, 2006; Varea et al., 2015). Recently, our lab identified the importance of *Cntnap2* in sensory filtering and sensorimotor gating using a knockout rat model (K. E.

Scott et al., 2018). Our findings suggest that this rat model may also be effective for studying the fuller range of sensory processing alterations common in ASD. At present, however, it remains unclear to what extent these *Cntnap2* knockout rats display core behavioural features of ASD (i.e., altered social interactions and behavioural flexibility), and if the differences observed in pre-attentive sound processing extend to altered auditory perception and audiovisual integration, consistent with individuals with ASD.

Given that an ASD diagnosis relies on evaluating behavioural traits, it is important that preclinical models be assessed for their face validity using behavioural assays that seek to mimic those in humans (Atanasova, 2015; Chen et al., 2015; Kazdoba, Leach, & Crawley, 2016; Möhrle et al., 2020; Servadio, Vanderschuren, & Trezza, 2015). In the present study, we have extended our recent work that characterized low-level sound processing in *Cntnap2* knockout rats to now fully investigate the consequences of a functional loss of *Cntnap2* on ASD-related behaviours by assessing social interactions, behavioural flexibility, spatial learning/memory, and sensory processing at the perceptual level. Ultimately, by comparing the performance of rats with a homozygous or heterozygous knockout of *Cntnap2* to their wildtype littermates across a comprehensive test battery, we were able to confirm that the *Cntnap2* knockout rat model represents an effective preclinical platform for studying the mechanisms underlying ASD, as well as for investigating the complex relationship between altered sensory processing and the core ASD-related behaviours.

2.2 Materials and Methods

Additional experimental details and analyses calculations are described in 2.6 - Supplemental Methods.

2.2.1 Animals

Male (M) and female (F) adult (>P70) Sprague-Dawley wildtype, heterozygous knockout (*Cntnap2*^{+/-}) and homozygous knockout (*Cntnap2*^{-/-}) rats were used to investigate their ASD-related behavioural profile in a variety of tasks. Heterozygous breeders (SD-*Cntnap2*tm1sage) were obtained from Horizon Discovery (originally created at SAGE

Laboratories, Inc. in conjunction with Autism Speaks; the line is now maintained by Envigo). The model contains a five base pair deletion in exon six of the *Cntnap2* gene, created using the zinc finger nuclease target site CAGCATTTCGCACC|aatgga|GAGTTTGACTACCTG. All experimental animals were obtained from heterozygous crossings. Both male and female rats were used in each experiment, ranging from five to fifteen rats per genotype tested (sex/genotype sample sizes range from three to eight). Exact group sizes are reported in the figure legends. All procedures were approved by the University of Western Ontario Animal Care Committee, and were in accordance with the guidelines established by the Canadian Council on Animal Care.

2.2.2 Social Behaviours

Each rat's preference for a conspecific over an inanimate object (sociability), and its preference for a stranger rat over a familiar rat (social novelty) were assessed using previously established 3-chamber assays (Crawley, 2007; Moy et al., 2004). Sociability was determined by placing a stranger rat (stranger 1) in one chamber in a tube that allowed nose contact, and video monitoring the test rat's exploration of the apparatus (10 min; Fig. 2-1A). Next, to test social novelty, the test rat was re-exposed to the initial stranger (now familiar) rat in one tube, as well as a novel stranger rat (stranger 2) in a second tube (10 min; Fig. 2-1D). The amount of time spent sniffing the tubes, and total distance travelled were recorded.

2.2.3 Stereotypic and Exploratory Behaviours

Testing of spontaneous locomotion took place in a 4-walled, plastic, open-top chamber (Fig. 2-2A), in which rats were able to freely explore for 20 min. Number of full body rotations and self-grooming occurrences were used as measures of stereotypic behaviour. The rearing frequency and total distance travelled (m) were used as measures of exploratory behaviour, whereas the proportion of time spent in the perimeter of the box was used as an index of anxiety.

2.2.4 Cognitive Flexibility

Cognitive flexibility was assessed with a lever-pressing task that required probabilistic reversal learning (Dalton, Phillips, & Floresco, 2014), in which an intermittent reinforcement pattern was used to test the rat's ability to disengage from a previously-learned strategy when it became no longer beneficial, and then maintain a newly-learned behaviour (D'Cruz, Mosconi, Ragozzino, Cook, & Sweeney, 2016; D'Cruz et al., 2013). In an operant conditioning chamber, food-restricted rats were first trained on a 100% reward rate to press a left or right lever for a sucrose pellet that was dispensed into a center receptacle. Next, one of the two levers were inserted into the chamber and rats learned to respond within 10 s, with failure to respond scored as an omission and the house light extinguished. Lastly, familiarization with a probabilistic reward scheme took place, with only 50% of trials rewarded. Rats then performed a probabilistic test paradigm on three consecutive days, in which a sucrose pellet was delivered for 80% of the correct lever presses and for 20% of the incorrect lever presses. Over 200 total trials in which both levers were inserted into the chamber, the rats had to first determine which of the two levers was deemed correct based on probabilistic learning, and once they had performed eight consecutive presses on this correct lever, the protocol reversed so that the opposite lever was now deemed correct (Fig. 2-3A). This pattern of reversal continued in accordance with the rat's performance during the 200 trials, which ultimately allowed for an assessment of probabilistic learning (i.e., first discrimination; win-stay ratio; lose-shift ratio) and reversal learning (i.e., first reversal; number of reversals).

2.2.5 Spatial Learning and Memory

Spatial learning and memory were assessed using protocols associated with the Morris water maze (MWM; Morris, 1984), and followed the methods established by Levit et al., 2019. Relying on visual cues available on the walls of the testing room, the rats learned to swim to a hidden platform submerged below the surface of the opaque water over six 90-s trials that began from a fixed start location (Fig. 2-4A). Twenty-four hours after the sixth learning trial, the platform was removed, and each rat underwent a probe trial (90 s) to assess their recall of the previous platform location (Fig. 2-4F). Finally, cued platform

location trials were then conducted to rule out possible performance confounds associated with visual acuity (Fig. 2-4K).

2.2.6 Sound Processing: Pre-attentive & Perceptual

Pre-attentive Sound Processing. To assess sound processing at the pre-attentive level, acoustic reactivity and sensorimotor gating were measured using paradigms involving the rats' acoustic startle response (i.e., its motoric reaction to a sudden, loud acoustic stimulus). Using a pressure-sensitive platform, the rat's acoustic reactivity was measured as the magnitude of its startle response to acoustic stimuli (pulse: 20 ms) at varying intensities (65, 74, 83, 89 and 105 dB sound pressure level [SPL]; Fig 5A). Sensorimotor gating (expressed as the percentage of prepulse inhibition, %PPI) was determined by the extent that the rat's startle response to the 105 dB SPL pulse was attenuated when a brief prepulse was presented 100 ms earlier (prepulse: 10 ms, 65, 74, 83, or 89 dB SPL; Fig. 2-5D).

Sound Intensity Categorization. The perceptual ability to categorize the intensity (subjectively, loudness) of sounds was determined by first training food-restricted rats on a two-alternative forced choice paradigm to discriminate a "loud" (89 dB SPL) versus "quiet" (71 dB SPL) stimulus by responding to a left or right feeder trough, respectively, for a sucrose pellet reward (Fig. 2-6A). Specifically, each trial began with the rat placing its nose in the center nose poke, which triggered the acoustic stimulus after a 1.5 – 3 s delay. Correct feeder trough choices were reinforced with a sucrose pellet, whereas incorrect responses resulted in the house light extinguishing for up to 15 s, during which time a new trial could not be initiated. During their training to discriminate the two stimuli, the rats performed ~150-250 daily trials, which lasted ~30 min. Once the rats had achieved greater than 85% accuracy (~1-2 months), they began to perform test sessions. Testing sessions included trials of novel sound intensities (74, 77, 80, 83, and 86 dB SPL), which rats proceeded to categorize as either "loud" or "quiet" by responding to a given feeder trough. 70% of the test trials were the two training stimuli (i.e., 71 and 89 dB SPL), and the remaining 30% of the trials were made up of the random presentation of the novel stimuli. Furthermore, the trained stimulus conditions continued to be positively reinforced for correct responses and punished for incorrect responses, whereas a sucrose pellet was

delivered following each novel stimulus regardless of which feeder trough was chosen. Overall, the rats' categorization of sound intensities was quantified by generating psychometric curves of the percentage of trials they reported each of the novel stimuli to be "loud" (i.e., left feeder selection), which were then fit with a cumulative Gaussian using the maximum likelihood procedure of the open-source package *psignifit* 4 for MATLAB (Schütt, Harmeling, Macke, & Wichmann, 2016), from which the subjective category boundary (i.e., the intensity at which 50% of the responses were reported "loud") was determined.

Active Sound Avoidance. The rats' natural avoidance of sounds perceived as aversive was assessed using an active sound avoidance paradigm (adapted from Manohar, Spoth, Radziwon, Auerbach, & Salvi, 2017). Taking advantage of rodents' preference for dark and enclosed spaces (Bourin & Hascoët, 2003), the paradigm measured their change in preference for a dark or light box as sounds of increasing intensity were presented within the dark box (Fig. 2-7A). Stimulus levels known to elicit the acoustic startle response (namely 83 dB SPL or greater; K. E. Scott et al., 2018) were predicted to be perceived as aversive and cause rats to prefer the light box. Rats were first acclimated to the apparatus across four 20-minute sessions during which they could freely explore the dark and light boxes. During testing on three consecutive days, rats were exposed to three sessions consisting of the following sound conditions presented in the dark box: (1) no added sound (i.e., ~60 dB SPL ambient noise); (2) one of three test stimuli (65, 74, or 83 dB SPL); (3) 89 dB SPL (Fig. 2-7A). In each of the three daily sound sessions, rats started in the dark box and sound played for 10 minutes, with sessions separated by a 1.5-h inter-trial rest interval. Actual and relative time spent in the dark box were used for analyses. Actual time spent in the dark box was averaged across the three test days for each rat for the 60 and 89 dB conditions. Relative time spent in the dark box for the various test stimuli was calculated in comparison to the 60 dB condition for that day.

2.2.7 Perceptual and Pre-attentive Audiovisual Processing

Audiovisual Temporal Order Judgement. Audiovisual processing abilities were measured perceptually using an established temporal order judgement (TOJ) task in which

the ability of rats to accurately perceive the relative timing of a pair of auditory and visual stimuli was assessed (Schormans & Allman, 2018, 2019; Schormans et al., 2017). Food-restricted rats were trained to discriminate a pair of auditory and visual stimuli presented with a stimulus onset asynchrony (SOA) of 400 ms (e.g., noise burst 400 ms before light flash, and vice-versa). Using sucrose pellets as positive reinforcement, the rats learned to associate a given feeder trough with a specific stimulus condition (left trough = auditory-first; right trough = visual-first; Fig. 2-8A) across ~200–300 daily trials lasting ~30 min over ~2-3 months. Once trained (> 85% accuracy), test sessions were conducted in which seven SOAs were randomly presented (i.e., 0, ± 100 , ± 200 and ± 400 ms), with 70% of the trials being the training stimuli (i.e., ± 400 ms SOA), and the remaining 30% of the trials made up of the random presentation of the novel (0, ± 100 , and ± 200) SOAs. Performance was measured as the proportion of trials in which the rat reported having perceived the stimulus order to be visual-first (i.e., right feeder trough selection), and a psychophysical profile was generated for each rat. Data were fitted and the just noticeable difference (JND: the difference between the SOAs at which 25% and 75% of the responses were considered “visual- first”, divided by two) (Vroomen & Stekelenburg, 2011) and point of subjective simultaneity (PSS: the SOA at which 50% of the responses were “visual first”) (Vatakis, Bayliss, Zampini, & Spence, 2007) were obtained. It is worth noting that the JND provides a measure of the smallest interval between the separately presented auditory and visual stimuli that can be detected reliably, whereas the PSS represents the actual timing of the audiovisual stimuli when the observer is most unsure of the temporal order. Lastly, we determined whether the rats demonstrated rapid temporal recalibration by carrying out a one-back analysis (analyzing trial t 's response as a conditional of trial $t-1$'s). Using this approach, we focused on whether the prior trial had a negative SOA (i.e., auditory-first) or a positive SOA (i.e., visual-first), and again fit the psychometric function with a cumulative Gaussian. This approach allowed us to obtain the PSS, and the amount of change in PSS as a function of the prior trial ($\Delta\text{PSS} = \text{PSS auditory-first} - \text{PSS visual-first}$), also known

as the adaptation effect (Noel, De Nier, Stevenson, Alais, & Wallace, 2016; Turi, Karaminis, Pellicano, & Burr, 2016; Van der Burg, Alais, & Cass, 2013).

Pre-attentive Audiovisual Processing. Pre-attentive audiovisual processing was tested using the acoustic startle response. Specifically, audiovisual sensorimotor gating (Ison, Zuckerman, & Russo, 1975) was used to assess pre-attentive multisensory processing by measuring the extent that each rat's acoustic startle response was attenuated when a brief, non-startling stimulus (prepulse) preceded the startle stimulus (pulse: 20 ms at 105 dB SPL) by 100 ms (Fig. 2-8G). Three pre-pulse stimuli were used: an auditory (A; 10 ms, 68 dB SPL), a visual (V; 10 ms, 70 lux), or an audiovisual (AV; simultaneous presentation of both A and V).

2.2.8 Statistics

The overall objective of the present study was to assess the effect of the complete or partial functional loss of *Cntnap2* on a variety of behavioural assays. Based on the experimental design for each of the behavioural tasks, various statistical analyses were performed, including univariate analysis of variance (x-way ANOVA, repeated measures [RM] ANOVA), multivariate analysis of variance (MANOVA), and t-tests, to ultimately assess the effect of genotype, sex, and other independent variables (e.g. sound level) on the dependent variable of interest. In all experiments, interactions involving genotype or a main effect of genotype were of interest. If there was no genotype x sex interaction, data were plotted to include both sexes, with a main effect of sex reported if present. Unless otherwise stated, *Cntnap2*^{+/-} rats did not differ from wildtypes. All statistical analyses are presented in the figure legends.

2.3 Results

2.3.1 Social Behaviours

Each rat's sociability (Fig. 2-1A) and preference for social novelty (Fig. 2-1D) was assessed by measuring the time it spent sniffing the tubes in the two chambers in the different experimental conditions. The wildtype ($p = 0.031$) and *Cntnap2*^{+/-} ($p = 0.003$)

rats preferred to spend time with a stranger rat versus exploring an empty tube, whereas *Cntnap2*^{-/-} rats did not show a preference ($p = 0.114$), indicative of their lack of sociability (Fig. 2-1B). With respect to preference for social novelty, only the wildtype rats preferred to sniff a novel stranger compared to a familiar rat ($p = 0.003$), whereas there was no significant preference in sniff time for the *Cntnap2* knockout rats (*Cntnap2*^{+/-}: $p = 0.132$; *Cntnap2*^{-/-}: $p = 0.403$; Fig. 2-1E). These genotypic differences in social behaviours were not likely due to differences in the extent that the rats explored the testing apparatus (Fig. 2-1C and 2-1F); however, the *Cntnap2*^{-/-} females did travel more than wildtype females ($p = 0.028$) in the sociability phase of testing.

2.3.2 Stereotypic and Exploratory Behaviours

Stereotypic and exploratory behaviours were tested in a locomotor box; data was binned in 5 minute segments for analysis (Fig. 2-2A). Perimeter preference, a surrogate measure of anxiety-like behaviour, was compared between genotypes, with all animals preferring the perimeter to a similar degree (Fig. 2-2B). With respect to stereotypic behaviours, *Cntnap2*^{-/-} rats exhibited a greater number of circling behaviours than wildtype rats ($p = 0.002$; Fig. 2-2C), though no differences in self-grooming were observed (Fig. 2-2D). Genotype influenced exploratory behaviours and locomotion, with both *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats displaying a greater rearing frequency than wildtype rats (*Cntnap2*^{+/-}: $p = 0.050$; *Cntnap2*^{-/-}: $p = 0.011$; Fig. 2-2E) and travelling a greater distance (in comparison to wildtype rats *Cntnap2*^{+/-}: $p = 0.039$, *Cntnap2*^{-/-}: $p < 0.001$; *Cntnap2*^{+/-} vs. *Cntnap2*^{-/-}: $p < 0.001$; Fig. 2-2F).

2.3.3 Cognitive Flexibility

To assess whether cognitive flexibility differed amongst the genotypes, we trained groups of wildtype and *Cntnap2* knockout rats to perform a probabilistic reversal learning task (Dalton et al., 2014; Fig. 2-3A). Overall, there was no main effect of genotype or its interactions in the number of trials to discrimination (i.e., acquisition; Fig. 2-3B), win-stay ratio (Fig. 2-3C) or lose-shift ratio (Fig. 2-3D); collective findings indicative of typical probabilistic learning. Furthermore, there were no genotypic effects in the number of trials

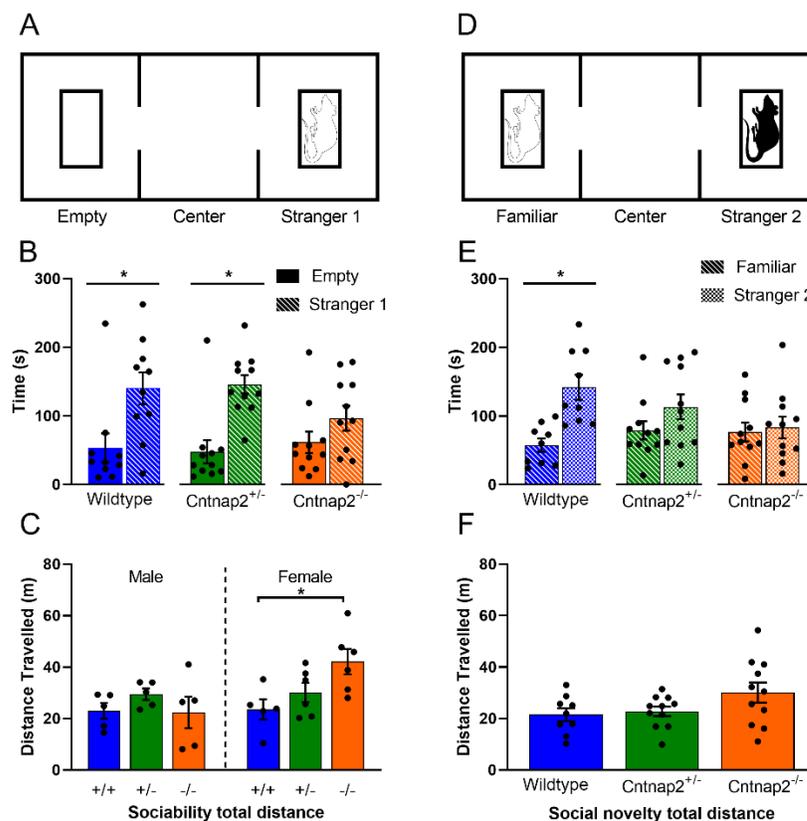


Figure 2-1: *Cntnap2*^{-/-} rats do not exhibit sociability nor a preference for social novelty.

Left column describes the sociability of wildtype, *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats (A-C). Sociability was tested by examining the preference for a stranger rat over an empty tube (A). Unlike the wildtype and *Cntnap2*^{+/-} rats which showed evidence of sociability, the *Cntnap2*^{-/-} rats did not prefer to spend time sniffing a stranger rat over an empty tube (B; 1-tailed paired t-test, WT: $t(9) = -2.141$, $p = 0.031$, HET: $t(10) = -3.528$, $p = 0.003$, KO: $t(10) = -1.122$, $p = 0.114$). Right column describes the social novelty behaviour of these rats (D-F), tested by examining a rat's preference for sniffing a novel stranger over a familiar rat (D). Wildtype rats showed the typical preference for social novelty; however, neither the *Cntnap2*^{+/-} nor *Cntnap2*^{-/-} rats prefer to spend time sniffing a novel stranger versus a familiar rat (E; 1-tailed paired t-test, WT: $t(8) = -3.862$, $p = 0.003$, HET: $t(10) = -1.187$, $p = 0.132$, KO: $t(10) = -0.253$, $p = 0.403$). The potential confound of differences in activity level was assessed by measuring distance travelled during testing stages (C; 2-way ANOVA, Genotype vs. Sex: $F(2, 26) = 3.410$, $p = 0.048$, post-hoc simple main effect 1-way ANOVA for genotype, Males: $F(2, 12) = 0.882$, $p = 0.439$, Females: $F(2, 14) = 4.774$, $p = 0.026$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.028$, WT vs. HET: $p = 0.912$, HET vs. KO: $p = 0.182$. F; 2-way ANOVA, Genotype vs. Sex: $F(2, 25) = 1.108$, $p = 0.346$, Genotype: $F(2, 25) = 2.267$, $p = 0.125$, Sex: $F(1, 25) = 1.331$, $p = 0.260$). $n =$ wildtype: 5M (sociability) and 4M (social novelty), 5F; *Cntnap2*^{+/-}: 5M, 6F; and *Cntnap2*^{-/-}: 5M, 6F. Data expressed as mean \pm SEM. * = $p < 0.05$.

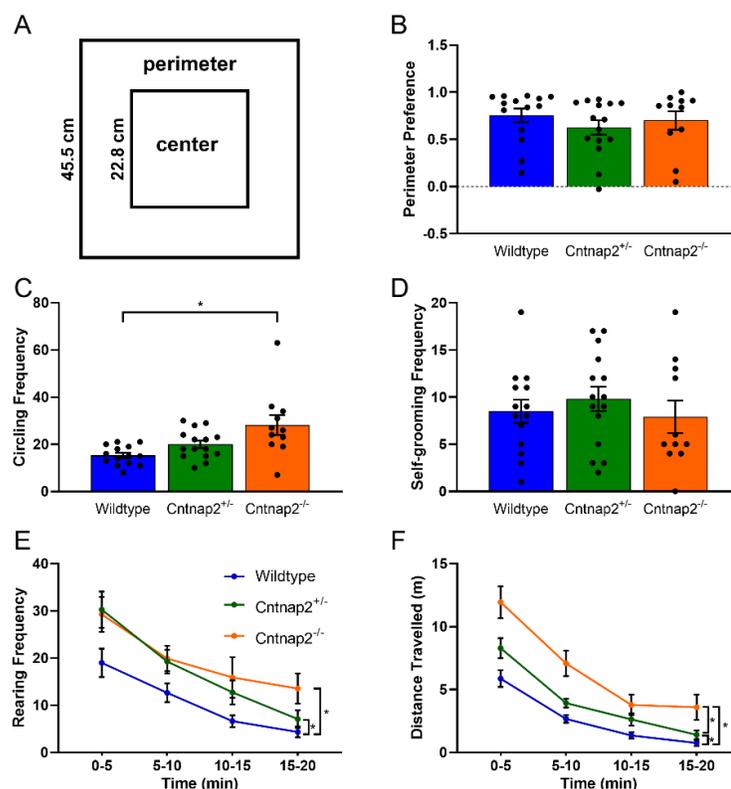


Figure 2-2: *Cntnap2*^{-/-} rats show greater stereotypic and exploratory behaviour, and are hyper-locomotive.

Rats were placed in an open-ceiling, plastic black chamber to freely explore for 20 min, and data was binned in 5 min segments for analysis (A). Perimeter preference (where zero = no preference; (B), circling frequency (C), self-grooming frequency (D), rearing frequency (E), and distance travelled (F) are depicted. Overall, *Cntnap2*^{-/-} rats did not show a difference in perimeter preference (B; 3-way RM ANOVA, Time vs. Genotype vs. Sex: $F(3.474, 59.052) = 1.003$, $p = 0.406$, Time vs. Genotype: $F(3.474, 59.052) = 1.089$, $p = 0.366$, Time vs. Sex: $F(1.737, 59.052) = 2.957$, $p = 0.067$, Genotype vs. Sex: $F(2, 34) = 0.018$, $p = 0.982$, Time: $F(1.737, 59.052) = 6.840$, $p = 0.003$, Genotype: $F(2, 34) = 0.996$, $p = 0.380$, Sex: $F(1, 34) = 12.828$, $p = 0.001$), and exhibited greater stereotypic behaviour through circling (C; 3-way RM ANOVA, Time vs. Genotype vs. Sex: $F(4.293, 72.985) = 0.901$, $p = 0.473$, Time vs. Genotype: $F(4.293, 72.985) = 0.513$, $p = 0.739$, Time vs. Sex: $F(2.147, 72.985) = 1.215$, $p = 0.304$, Genotype vs. Sex: $F(2, 34) = 1.147$, $p = 0.330$, Time: $F(2.147, 72.985) = 72.003$, $p < 0.001$, Sex: $F(1, 34) = 3.078$, $p = 0.088$, Genotype: $F(2, 34) = 6.922$, $p = 0.003$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.002$, WT vs. HET: $p = 0.411$, HET vs. KO: $p = 0.055$), but not grooming (D; 3-way RM ANOVA, Time vs. Genotype vs. Sex: $F(6, 102) = 0.890$, $p = 0.505$, Time vs. Genotype: $F(6, 102) = 0.874$, $p = 0.517$, Time vs. Sex: $F(3, 102) = 0.315$, $p = 0.815$, Genotype vs. Sex: $F(2, 34) = 0.190$, $p = 0.828$, Time: $F(3, 102) = 0.216$, $p = 0.885$, Genotype: $F(2, 34) = 0.473$, $p = 0.627$, Sex: $F(1, 34) = 3.492$, $p = 0.070$). Both *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats showed greater exploratory behaviour through rearing (E; 3-way RM ANOVA, Time vs. Genotype vs. Sex: $F(6, 102) = 1.019$,

to first reversal (Fig. 2-3E) or total number of reversals (Fig. 2-3F), suggesting intact reversal learning. In general, performance improved across the three days of testing for the win-stay ratio, trials to first reversal, and total reversals (all $p < 0.050$).

2.3.4 Spatial Learning and Memory

To assess spatial acquisition learning, the time to locate the hidden platform in the Morris water maze was measured across six learning trials (Fig. 2-4A). Although the rats' initial (trial 1) search performance did not differ amongst the genotypes (Fig. 2-4B), the ability to learn the platform location upon repeated exposure (i.e., trials 2-6) was modestly delayed in *Cntnap2*^{-/-} rats ($p = 0.035$; Fig. 2-4C). More specifically, wildtype and *Cntnap2*^{+/-} rats learned the platform location by trial 3 (in comparison to trial 6, wildtype: $p = 0.319$; *Cntnap2*^{+/-}: $p = 0.207$), whereas *Cntnap2*^{-/-} rats did not fully learn until trial 4 ($p = 0.172$; Fig. 2-4C). This delayed learning in *Cntnap2*^{-/-} rats was further evident in the overall greater amount of time they needed to find the platform across learning trials ($p = 0.035$; Fig. 2-4D). Importantly, these delays in spatial learning were not confounded by differences in swimming ability across the genotypes (Fig. 2-4E). To assess spatial reference memory (Fig. 2-4F), the time the rats spent in relevant areas of the maze were compared during the probe test conducted 24 h after the learning sessions. Overall, there were no differences found in the time to enter the platform location (Fig. 2-4G), time spent in the platform quadrant (NE, Fig. 2-4H), time in the maze perimeter (Fig. 2-4I), or in swimming speed (Fig. 2-4J). Finally, the rats also performed a cued version of the task in

Figure 2-2 continued... $p = 0.414$, Time vs. Genotype: $F(6, 102) = 1.056$, $p = 0.392$, Time vs. Sex: $F(3, 102) = 0.851$, $p = 0.469$, Genotype vs. Sex: $F(2, 34) = 2.648$, $p = 0.085$, Time: $F(3, 102) = 42.676$, $p < 0.001$, Sex: $F(1, 34) = 5.896$, $p = 0.021$, Genotype: $F(2, 34) = 4.999$, $p = 0.012$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.011$, WT vs. HET: $p = 0.050$, HET vs. KO: $p = 1.000$), and were hyper-locomotive (F; 3-way RM ANOVA, Time vs. Genotype vs. Sex: $F(3.743, 63.639) = 0.958$, $p = 0.433$, Time vs. Genotype: $F(3.743, 63.639) = 2.427$, $p = 0.061$, Time vs. Sex: $F(1.872, 63.639) = 3.952$, $p = 0.026$, Genotype vs. Sex: $F(2, 34) = 2.429$, $p = 0.103$, Time: $F(1.872, 63.639) = 103.701$, $p < 0.001$, Sex: $F(1, 34) = 13.750$, $p = 0.001$, Genotype: $F(2, 34) = 21.622$, $p < 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p < 0.001$, WT vs. HET: $p = 0.039$, HET vs. KO: $p < 0.001$). $n =$ wildtype: 7M, 7F; *Cntnap2*^{+/-}: 7M, 8F; and *Cntnap2*^{-/-}: 5M, 6F. Data expressed as mean \pm SEM. * = $p \leq 0.05$.

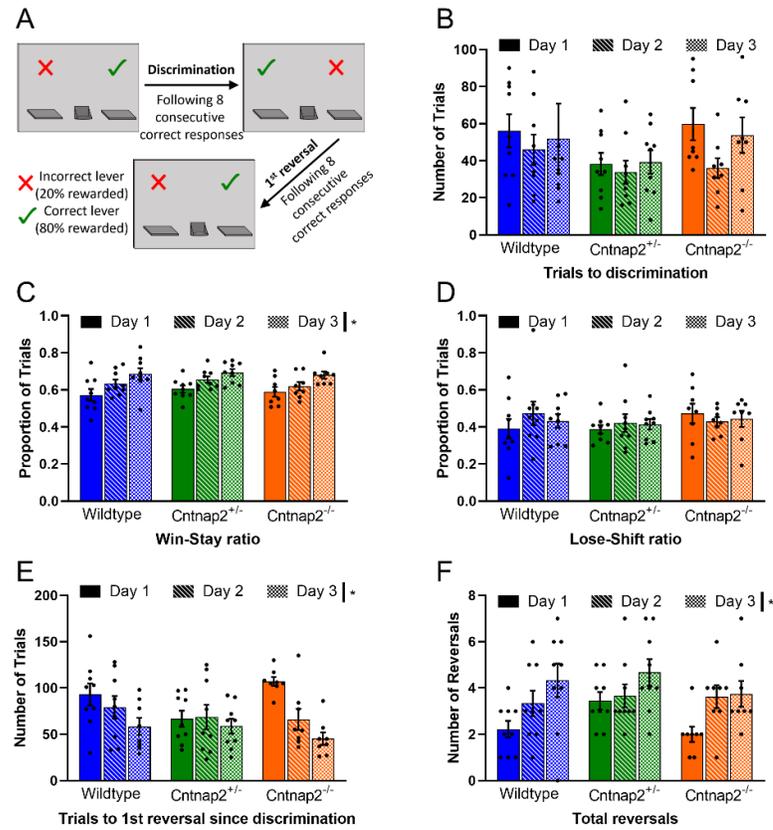


Figure 2-3: *Cntnap2*^{-/-} rats have typical probabilistic learning and reversal learning abilities.

Rats were trained to lever press to receive a food reward, and then were tested using an 80/20 probabilistic reward scheme. In the testing phase (A), rats first had to discriminate the correct lever, upon which reward contingencies switched, and rats had to reverse their learned correct-lever association to select the newly-correct, opposing lever (1st reversal). Once learned, the contingencies again switched, and the testing continued in this fashion for a total of 200 trials. No genotypic differences existed in the number of trials to criterion performance for initial discrimination (B; 3-way RM ANOVA, Day vs. Genotype vs. Sex: $F(4, 40) = 1.363$, $p = 0.264$, Day vs. Genotype: $F(4, 40) = 0.292$, $p = 0.881$, Day vs. Sex: $F(2, 40) = 0.128$, $p = 0.881$, Genotype vs. Sex: $F(2, 20) = 0.445$, $p = 0.647$, Day: $F(2, 40) = 1.680$, $p = 0.199$, Genotype: $F(2, 20) = 1.785$, $p = 0.194$, Sex: $F(1, 20) = 0.091$, $p = 0.766$), or in the win-stay (C; 3-way RM ANOVA, Day vs. Genotype vs. Sex: $F(4, 40) = 0.855$, $p = 0.499$, Day vs. Genotype: $F(4, 40) = 0.155$, $p = 0.959$, Day vs. Sex: $F(2, 40) = 0.043$, $p = 0.958$, Genotype vs. Sex: $F(2, 20) = 0.282$, $p = 0.757$, Genotype: $F(2, 20) = 0.458$, $p = 0.639$, Sex: $F(1, 20) = 0.093$, $p = 0.763$) and lose-shift (D; 3-way RM ANOVA, Day vs. Genotype vs. Sex: $F(4, 40) = 1.351$, $p = 0.268$, Day vs. Genotype: $F(4, 40) = 0.608$, $p = 0.659$, Day vs. Sex: $F(2, 40) = 1.562$, $p = 0.222$, Genotype vs. Sex: $F(2, 20) = 0.426$, $p = 0.659$, Day: $F(2, 40) = 0.472$, $p = 0.627$, Genotype: $F(2, 20) = 0.472$, $p = 0.631$, Sex: $F(1, 20) = 1.892$, $p = 0.184$) behaviours. Probabilistic learning generally improved with time, as depicted in the win-stay ratio (C; Day: $F(2, 40) = 16.653$, $p < 0.001$). The number of trials to criterion performance during the first reversal phase was equivalent for all genotypes (E; 3-way

which the platform was visually marked above the surface of the water (Fig. 2-4K). No genotypic differences existed in time to reach the cued platform location (Fig. 2-4L), regardless of its location in the maze (Fig. 2-4M), or in swimming speed (Fig. 2-4N). In all of the maze tasks, *Cntnap2*^{+/-} rats performed equivalent to the wildtype rats.

2.3.5 Pre-attentive Sound Processing

The acoustic startle response (Fig. 2-5A) and prepulse inhibition of startle (Fig. 2-5D) were used to assess pre-attentive sound processing to moderate and loud intensities. *Cntnap2*^{-/-} rats displayed a significant monotonic increase in their acoustic startle response (ASR) compared to wildtype rats in response to both moderate ($p < 0.001$ for 83 and 89 dB SPL) and loud ($p = 0.024$ for 105 dB SPL) sounds, whereas *Cntnap2*^{+/-} rats showed an increased ASR only to the loud startling stimulus ($p = 0.040$ for 105 dB SPL; Fig. 2-5B). The exaggerated acoustic reactivity in the *Cntnap2*^{-/-} rats to moderate stimuli was further evident when the startle magnitude for a given stimulus intensity was normalized to each rat's maximum startle response magnitude. As seen in Fig. 2-5C, an 83 and 89 dB SPL stimulus elicited a greater relative percent of the maximal startle response in *Cntnap2*^{-/-} rats compared to wildtype ($p < 0.001$ for 83 and $p = 0.003$ for 89 dB SPL) and *Cntnap2*^{+/-} rats ($p = 0.005$ for 83 dB SPL). Collectively, these results suggest that the *Cntnap2*^{-/-} rats have both an upward and leftward shift in their startle curve when compared to the wildtype rats, whereas the *Cntnap2*^{+/-} rats only showed an upward shift. Sensorimotor gating was assessed by measuring the percent inhibition of the ASR elicited by the presentation of a

Figure 2-3 continued... RM ANOVA, Day vs. Genotype vs. Sex: $F(4, 40) = 0.688$, $p = 0.604$, Day vs. Genotype: $F(4, 40) = 1.402$, $p = 0.251$, Day vs. Sex: $F(2, 40) = 0.303$, $p = 0.740$, Genotype vs. Sex: $F(2, 20) = 1.141$, $p = 0.340$, Genotype: $F(2, 20) = 2.263$, $p = 0.130$, Sex: $F(1, 20) = 3.047$, $p = 0.096$). Overall, the number of reversals performed by the *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats did not differ from wildtypes (F; 3-way RM ANOVA, Day vs. Genotype vs. Sex: $F(4, 40) = 0.554$, $p = 0.697$, Day vs. Genotype: $F(4, 40) = 0.734$, $p = 0.574$, Day vs. Sex: $F(2, 40) = 0.023$, $p = 0.978$, Genotype vs. Sex: $F(2, 20) = 0.690$, $p = 0.513$, Genotype: $F(2, 20) = 1.535$, $p = 0.240$, Sex: $F(1, 20) = 0.462$, $p = 0.505$), and all genotypes showed an improved ability to reverse with repeated testing (E; Day: $F(2, 40) = 4.384$, $p = 0.019$. F; Day: $F(2, 40) = 8.660$, $p = 0.001$). $n =$ wildtype: 5M, 4F; *Cntnap2*^{+/-}: 5M, 4F; and *Cntnap2*^{-/-}: 3M, 5F. Data expressed as mean \pm SEM. * = $p < 0.05$.

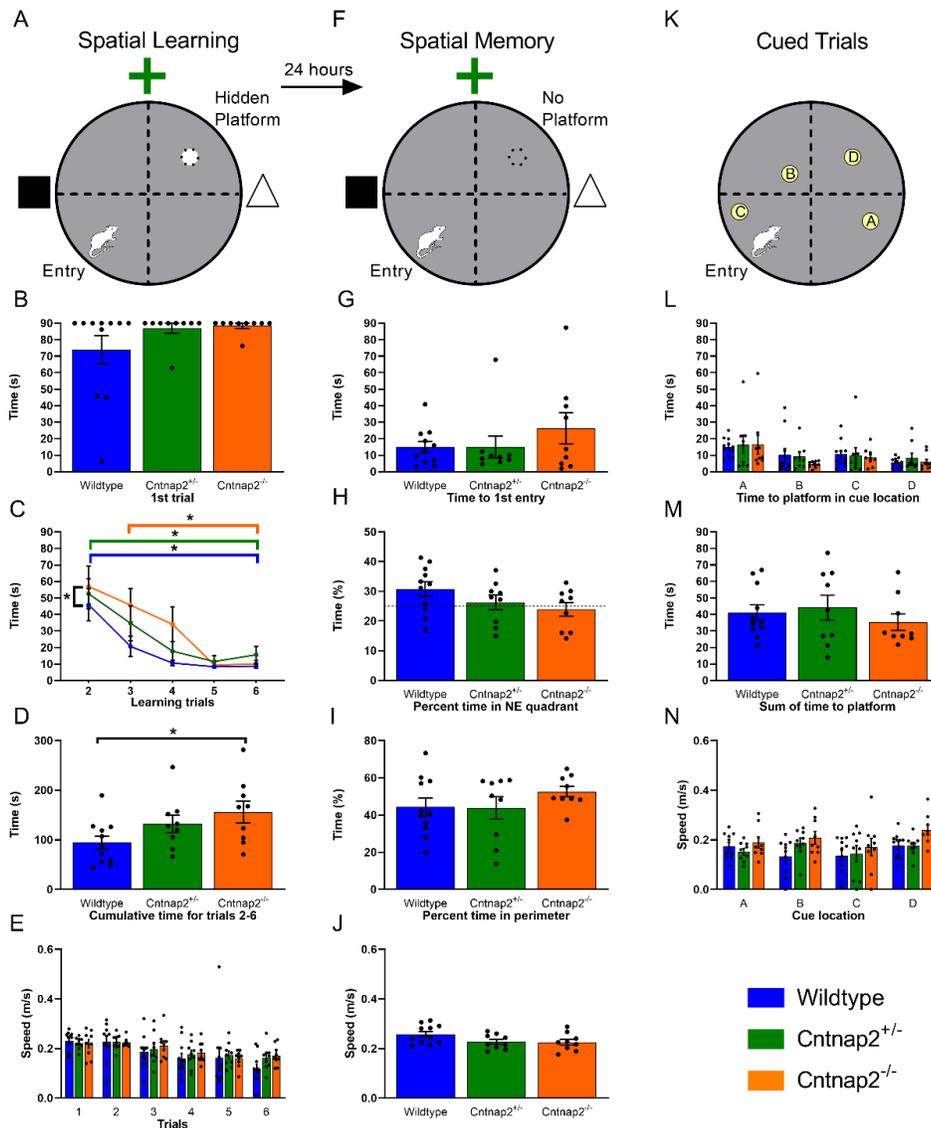


Figure 2-4: *Cntnap2*^{-/-} rats have modestly delayed spatial learning but intact spatial memory.

Using spatial cues surrounding the Morris water maze, rats had to locate a submerged platform across six learning trials (Left column; A). Although the initial search ability was equivalent amongst the genotypes (B; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 0.511$, $p = 0.607$, Genotype: $F(2, 23) = 2.123$, $p = 0.143$, Sex: $F(1, 23) = 01.742$, $p = 0.200$), *Cntnap2*^{-/-} rats required an additional trial to learn the platform location (by trial 4; 1-way ANOVA, Trial: $F(4, 40) = 6.002$, $p = 0.001$, post-hoc Dunnet's t-test, 2 vs. 6: $p = 0.002$, 3 vs. 6: $p = 0.021$, 4 vs. 6: $p = 0.172$, 5 vs. 6: $p = 1.000$) compared to wildtype t-test, 2 vs. 6: $p < 0.001$, 3 vs. 6: $p = 0.319$, 4 vs. 6: $p = 0.997$, 5 vs. 6: $p = 1.000$. HET: Trial: $F(4, 40) = 5.543$, $p = 0.001$, post-hoc Dunnet's t-test, 2 and *Cntnap2*^{+/-} rats (by trial 3; 1-way ANOVA, WT: Trial: $F(4, 50) = 9.242$, $p < 0.001$, post-hoc Dunnet's t-test, 2 vs. 6: $p < 0.001$, 3 vs. 6: $p = 0.319$, 4 vs. 6: $p = 0.997$, 5 vs. 6: $p = 1.000$. HET: Trial: $F(4, 40) = 5.543$, $p = 0.001$, post-hoc Dunnet's t-test, 2 vs. 6: $p = 0.003$, 3 vs. 6: $p = 0.207$, 4 vs. 6: $p = 0.998$, 5 vs. 6: $p = 0.984$),

brief non-startling acoustic stimulus prior to the startling stimulus (i.e., prepulse inhibition; PPI). *Cntnap2*^{+/-} showed typical PPI, whereas *Cntnap2*^{-/-} had reduced PPI across the various prepulse intensities ($p \leq 0.003$ for 74, 83, and 89 dB SPL when compared to wildtype or *Cntnap2*^{+/-} rats; Fig. 2-5E).

Figure 2-4 continued... and they took longer to find the platform on learning trials 2-6 (C; 3-way RM ANOVA, Trial vs. Genotype vs. Sex: $F(5.396, 62.057) = 1.316$, $p = 0.267$, Trial vs. Genotype: $F(5.396, 62.057) = 0.824$, $p = 0.583$, Trial vs. Sex: $F(2.698, 62.057) = 1.641$, $p = 0.171$, Genotype vs. Sex: $F(2, 23) = 3.395$, $p = 0.051$, Trial: $F(2.698, 62.057) = 20.434$, $p < 0.001$, Sex: $F(1, 23) = 0.261$, $p = 0.615$, Genotype: $F(2, 23) = 3.841$, $p = 0.036$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.035$, WT vs. HET: $p = 0.322$, HET vs. KO: $p = 0.964$. D; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 3.395$, $p = 0.051$, Sex: $F(1, 23) = 0.261$, $p = 0.615$, Genotype: $F(2, 23) = 3.841$, $p = 0.036$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.035$, WT vs. HET: $p = 0.322$, HET vs. KO: $p = 0.964$). Swimming ability did not confound these findings (E; 3-way RM ANOVA, Trial vs. Genotype vs. Sex: $F(5.829, 67.032) = 1.322$, $p = 0.261$, Trial vs. Genotype: $F(5.829, 67.032) = 0.487$, $p = 0.811$, Trial vs. Sex: $F(2.914, 67.032) = 1.107$, $p = 0.351$, Genotype vs. Sex: $F(2, 23) = 0.806$, $p = 0.459$, Trial: $F(2.914, 67.032) = 8.351$, $p < 0.001$, Sex: $F(1, 23) = 4.195$, $p = 0.052$, Genotype: $F(2, 23) = 0.765$, $p = 0.477$). Center column (F-J) depicts 24-hour spatial reference memory of platform location (F). *Cntnap2*^{-/-} rats' spatial reference memory did not differ from wildtype rats' for time to platform location (G; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 1.283$, $p = 0.296$, Sex: $F(1, 23) = 0.092$, $p = 0.764$, Genotype: $F(2, 23) = 0.781$, $p = 0.470$), percent time in NE quadrant (H; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 1.216$, $p = 0.315$, Sex: $F(1, 23) = 0.707$, $p = 0.409$, Genotype: $F(2, 23) = 1.946$, $p = 0.166$), percent time in perimeter (I; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 1.410$, $p = 0.264$, Sex: $F(1, 23) = 0.305$, $p = 0.586$, Genotype: $F(2, 23) = 0.881$, $p = 0.428$), or swimming speed (J; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 0.896$, $p = 0.422$, Sex: $F(1, 23) = 0.479$, $p = 0.496$, Genotype: $F(2, 23) = 2.656$, $p = 0.092$). To confirm typical visual acuity, rats had to locate a visually-cued platform, placed in different locations over four trials (Right column; K). *Cntnap2*^{-/-} rats showed no visual impairment; they did not take more time (L; 2-way MANOVA Cues 1-4: Genotype vs. Sex: $F(8, 40) = 1.017$, $p = 0.439$, Wilks' $\Lambda = 0.690$, Sex: $F(4, 20) = 0.069$, $p = 0.991$, Wilks' $\Lambda = 0.986$, Genotype: $F(8, 40) = 0.367$, $p = 0.932$, Wilks' $\Lambda = 0.868$, M; 2-way ANOVA, Genotype vs. Sex: $F(2, 23) = 0.655$, $p = 0.529$, Sex: $F(1, 23) = 0.091$, $p = 0.765$, Genotype: $F(2, 23) = 0.534$, $p = 0.593$) or swim slower (N; 2-way MANOVA Cues 1-4: Genotype vs. Sex: $F(8, 40) = 0.952$, $p = 0.486$, Wilks' $\Lambda = 0.952$, Sex: $F(4, 20) = 0.366$, $p = 0.830$, Wilks' $\Lambda = 0.932$, Genotype: $F(8, 40) = 2.116$, $p = 0.057$, Wilks' $\Lambda = 0.494$) to the cued platform. $n =$ wildtype: 5M, 6F; *Cntnap2*^{+/-}: 5M, 4F; and *Cntnap2*^{-/-}: 4M, 5F. Data expressed as mean \pm SEM. * = $p < 0.05$.

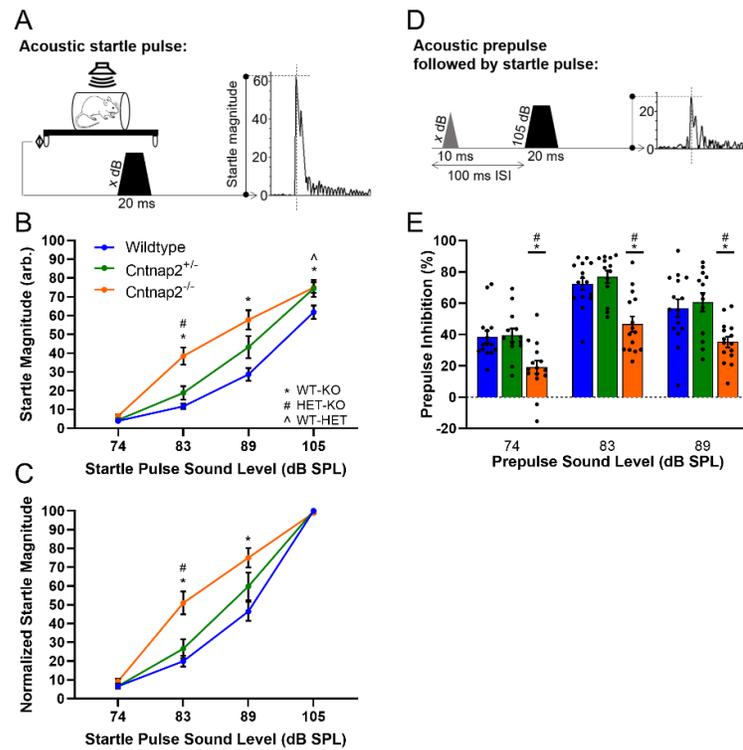


Figure 2-5: *Cntnap2*^{-/-} rats show an increased acoustic startle response (ASR) to moderate and loud intensities, and reduced sensorimotor gating.

Left column depicts the acoustic startle response (A-C). Rats were placed in a movement-limiting tube on a motion-sensitive platform. A startle pulse stimulus (black) of varying intensities was presented, which elicited the startle response, shown by the representative raw ASR trace from an adult wildtype male (A). *Cntnap2*^{-/-} rats exhibit a monotonic increase in the ASR magnitude to both moderate (83, 89 dB SPL) and loud intensities (105 dB SPL), whereas *Cntnap2*^{+/-} rats show greater ASR magnitudes only to the loudest startle stimulus (105 dB SPL; B; 3-way RM ANOVA, Sound level vs. Genotype vs. Sex: $F(4.922, 91.064) = 0.743$, $p = 0.591$, Sound level vs. Sex: $F(2.461, 91.064) = 1.938$, $p = 0.140$, Genotype vs. Sex: $F(2, 37) = 0.248$, $p = 0.782$, Sound level vs. Genotype: $F(4.922, 91.064) = 5.041$, $p < 0.001$, post-hoc simple main effect 1-way ANOVA for genotype, 74 dB: $F(2, 37) = 3.496$, $p = 0.041$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.050$, WT vs. HET: $p = 1.000$, HET vs. KO: $p = 0.181$, 83 dB: $F(2, 37) = 15.393$, $p < 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p < 0.001$, WT vs. HET: $p = 0.515$, HET vs. KO: $p = 0.002$, 89 dB: $F(2, 37) = 8.869$, $p = 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p < 0.001$, WT vs. HET: $p = 0.142$, HET vs. KO: $p = 0.145$, 105 dB: $F(2, 37) = 4.923$, $p = 0.013$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.024$, WT vs. HET: $p = 0.040$, HET vs. KO: $p = 1.000$, Sex: $F(1, 37) = 0.669$, $p = 0.419$). *Cntnap2*^{-/-} rats' greater reactivity to moderate intensities, which is not present in *Cntnap2*^{+/-} rats, was further evidenced in their normalized startle response magnitude (C; 3-way RM ANOVA, Sound level vs. Genotype vs. Sex: $F(3.742, 69.222) = 0.644$, $p = 0.623$, Sound level vs. Sex: $F(1.871, 69.222) = 1.311$, p

2.3.6 Sound Intensity Categorization

Given the above-described differences in acoustic reactivity, we next determined whether knockout rats, compared to wildtype rats, differentially categorized moderate-intensity sounds as more similar to high-intensity sounds (i.e., subjectively “loud”). We trained the rats to discriminate between a loud (89 dB SPL) and quiet (71 dB SPL) stimulus, and then tested how they categorized novel intermediate-intensity stimuli (Fig. 2-6A). Psychometric curves were generated from the percentage of trials that rats reported a given stimulus as loud (i.e., left feeder selection). Analysis of the unfitted curves allowed us to compare overall performance, with no genotypic differences present (Fig. 2-6B). Next, by fitting the data for each rat (Fig. 2-6C), we determined the subjective category boundary for each rat; confirming both *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats categorized sound intensity consistent with the wildtype rats (Fig. 2-6D).

Figure 2-5 continued... = 0.275, Genotype vs. Sex: $F(2, 37) = 0.499$, $p = 0.642$, Sound level vs. Genotype: $F(3.742, 69.222) = 6.505$, $p < 0.001$, post-hoc simple main effect 1-way ANOVA for genotype, 74 dB: $F(2, 37) = 1.192$, $p = 0.315$, 83 dB: $F(2, 37) = 10.708$, $p < 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p < 0.001$, WT vs. HET: $p = 1.000$, HET vs. KO: $p = 0.005$, 89 dB: $F(2, 37) = 6.184$, $p = 0.005$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.003$, WT vs. HET: $p = 0.348$, HET vs. KO: $p = 0.215$, 105 dB: $F(2, 37) = 2.885$, $p = 0.068$, Sex: $F(1, 37) = 2.066$, $p = 0.159$). Sensorimotor gating (D-E) occurred when a prepulse stimulus (grey) was presented prior to a startling stimulus (black), leading to a reduction in ASR magnitude (i.e., prepulse inhibition; D). *Cntnap2*^{-/-} rats showed reduced prepulse inhibition, regardless of prepulse stimulus intensity (E; 3-way RM ANOVA, Sound level vs. Genotype vs. Sex: $F(4, 74) = 2.676$, $p = 0.038$, post-hoc 2-way ANOVA for Genotype vs. Sex, 74 dB: Genotype vs. Sex: $F(2, 37) = 0.939$, $p = 0.400$, Sex = $F(1, 37) = 7.007$, $p = 0.012$, Genotype = $F(2, 37) = 8.518$, $p = 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.002$, WT vs. HET: $p = 1.000$, HET vs. KO: $p = 0.002$, 83 dB: Genotype vs. Sex: $F(2, 37) = 1.443$, $p = 0.249$, Sex = $F(1, 37) = 0.206$, $p = 0.653$, Genotype = $F(2, 37) = 14.354$, $p < 0.001$, post-hoc Bonferroni's t-test, WT vs. KO: $p < 0.001$, WT vs. HET: $p = 1.000$, HET vs. KO: $p < 0.001$, 89 dB: Genotype vs. Sex: $F(2, 37) = 1.441$, $p = 0.250$, Sex = $F(1, 37) = 1.894$, $p = 0.177$, Genotype = $F(2, 37) = 7.465$, $p = 0.002$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.009$, WT vs. HET: $p = 1.000$, HET vs. KO: $p = 0.003$). n = wildtype: 8M, 7F; *Cntnap2*^{+/-}: 7M, 6F; and *Cntnap2*^{-/-}: 7M, 8F. Data expressed as mean \pm SEM. *, #, ^ = $p < 0.05$.

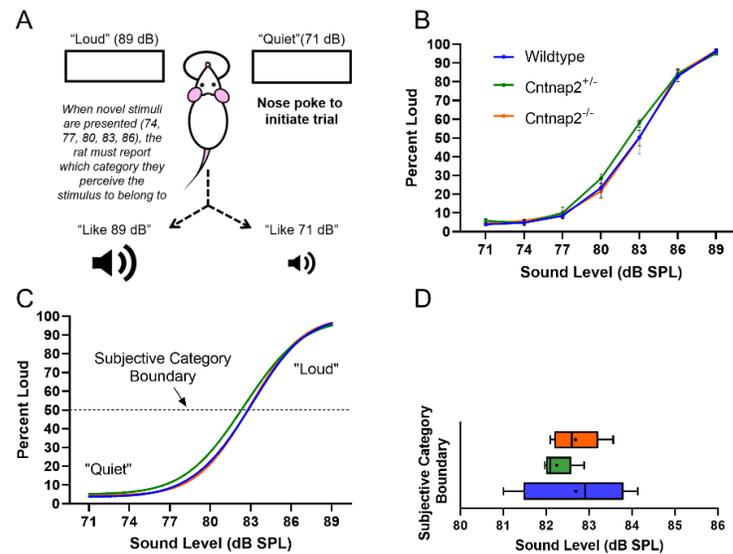


Figure 2-6: *Cntnap2*^{-/-} rats have intact sound intensity categorization.

Rats were trained using a two-alternative forced choice paradigm to discriminate a loud (89 dB SPL) versus quiet (71 dB SPL) stimulus by selecting a left or right feeder trough, respectively. Each trial was initiated by a center nose poke and a 2-s hold, after which a noiseburst was presented, and the rat selected a feeder trough. On testing days, rats were presented trials with novel sound intensities (74 – 86 dB SPL), and they categorized whether they perceived the stimulus to be “loud” or “quiet” by selecting a given feeder trough (A). Assessment of the percentage of trials the rats reported a given stimulus as loud, using raw (B; 3-way RM ANOVA, Sound level vs. Genotype vs. Sex: $F(4.300, 19.349) = 0.382, p = 0.935$, Sound level vs. Genotype: $F(4.300, 19.349) = 0.502, p = 0.747$, Sound level vs. Sex: $F(2.15, 19.349) = 1.072, p = 0.366$, Genotype vs. Sex: $F(2, 9) = 0.178, p = 0.839$, Sound level: $F(2.15, 19.349) = 485.895, p < 0.001$, Sex: $F(1, 9) = .015, p = 0.906$, Genotype: $F(2, 9) = 0.351, p = 0.713$) or fitted (C) data, revealed *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats have typical sound intensity categorization. The subjective category boundary, defined as the sound level at which 50% of a rat’s responses were reported as loud (C, dashed line), also revealed no genotypic differences (D; 1-way ANOVA, Genotype: $F(2, 12) = 0.483, p = 0.628$). n = wildtype: 3M, 2F; *Cntnap2*^{+/-}: 3M, 2F; and *Cntnap2*^{-/-}: 3M, 2F. (B) expressed as mean \pm SEM. (D) box center represents the median and extends to the 25th and 75th percentiles, whiskers represent extremes, and + represents the mean.

2.3.7 Active Sound Avoidance

To assess the rats’ aversion to moderate-intensity sounds, we used an active sound avoidance paradigm (adapted from Manohar et al., 2017), and measured the genotypic differences in the time the rats chose to spend in the dark region of the apparatus when

sounds of varying intensities (60 to 89 dB SPL) were presented therein (Fig. 2-7Ai). As expected, when the intensity of the sound increased, the rats spent significantly less time in the dark region ($p < 0.001$; Fig. 2-7Aii). Specifically, *Cntnap2*^{+/-} ($p = 0.019$) and *Cntnap2*^{-/-} ($p < 0.001$) rats showed a greater avoidance when the sound intensity was increased to 74 dB SPL in the dark box than was evident for the 60 dB SPL condition, whereas the sound intensity had to reach 83 dB SPL before a significant shift occurred in wildtype rats ($p = 0.002$; Fig. 2-7B). These genotypic effects were further evident by the significant difference in relative time spent in the dark box between *Cntnap2*^{-/-} and wildtype rats at 74 dB ($p = 0.018$); *Cntnap2*^{+/-} rats did not differ from either genotype (Fig. 2-7C).

2.3.8 Perceptual Audiovisual Processing

To determine if audiovisual temporal acuity and rapid temporal recalibration align with what is observed in individuals with ASD, we trained rats to perform a temporal order judgment (TOJ) task in which they learned to differentiate between trials when the auditory stimulus either preceded or followed the visual stimulus (Fig. 2-8A). No differences were found in the ability to judge the temporal order of stimuli between wildtype and *Cntnap2*^{-/-} rats' (Fig. 2-8B), and there were no genotypic differences in the point of subjective simultaneity (PSS; Fig. 2-8D-i) or just noticeable difference (JND; Fig. 2-8D-ii) metrics calculated from the fitted TOJ curves. Next, we determined if sensory priors (i.e., the internal representation of sensory event(s) occurring prior to the one of interest) are underutilized in *Cntnap2*^{-/-} rats, ultimately leading to reduced sensory adaptation, as has been reported in individuals with ASD (Noel et al., 2016; Turi et al., 2016). TOJ performance on a given trial (trial t) as a condition of whether the previous trial was auditory first or visual first (trial $t-1$) was calculated for wildtype and *Cntnap2*^{-/-} rats (Fig. 2-8E). As expected, we found a significant difference in the PSS of wildtype rats, depending if the prior trial was auditory-first or visual-first ($p = 0.001$; Fig. 2-8E-bottom inset). However, this effect was not present in the *Cntnap2*^{-/-} rats (Fig. 2-8E- top inset), and the difference in adaptation (Δ PSS) between the genotypes was statistically significant ($p = 0.015$; Fig. 2-8F).

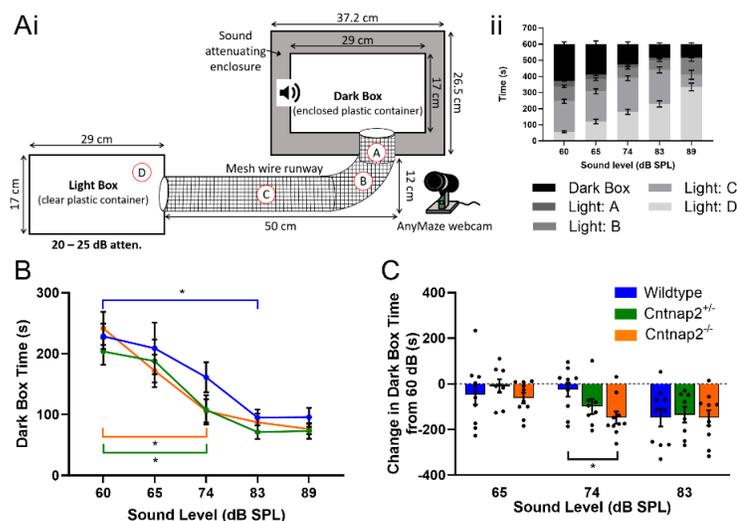


Figure 2-7: *Cntnap2*^{-/-} rats show sound avoidance behaviour at lower sound intensities.

Rats were placed into the dark box, and a sound stimulus was presented therein for 10 min. The time each rat spent in the dark box and illuminated areas (circled A-D) was measured (Ai). The change in average time spent in a given area across all groups as sound level increases shows task efficacy (Aii; 3-way RM ANOVA, Location vs. Sound level vs. Sex: $F(4.051, 105.330) = 1.434$, $p = 0.228$, Location vs. Sound level $F(4.051, 105.330) = 29.184$, $p < 0.001$), Sound level vs. Sex: $F(1, 26) < 0.001$, $p = 1.000$, Location vs. Sex: $F(2.190, 56.944) = 3.073$, $p = 0.019$). Testing occurred over 3 days, each day consisting of quiet (60 dB SPL), test (65, 74, or 83 dB SPL) and loud (89 dB SPL) conditions. Unlike the wildtype rats (1-way ANOVA, Sound level: $F(4, 45) = 5.888$, $p = 0.001$, post-hoc Dunnett's t-test, 60 vs. 65: $p = 0.951$, 60 vs. 74: $p = 0.205$, 60 vs. 83: $p = 0.002$, 60 vs. 89: $p = 0.002$), *Cntnap2*^{+/-} and *Cntnap2*^{-/-} rats (1-way ANOVA, Sound level: HET: $F(4, 35) = 7.692$, $p < 0.001$, post-hoc Dunnett's t-test, 60 vs. 65: $p = 0.966$, 60 vs. 74: $p = 0.019$, 60 vs. 83: $p = 0.001$, 60 vs. 89: $p = 0.001$. KO: $F(4, 45) = 11.488$, $p < 0.001$, post-hoc Dunnett's t-test, 60 vs. 65: $p = 0.065$, 60 vs. 74: $p < 0.001$, 60 vs. 83: $p < 0.001$, 60 vs. 89: $p < 0.001$) had already begun to avoid a sound intensity of 74 dB SPL to a greater extent than the 60 dB SPL condition (B; 3-way RM ANOVA, Sound level vs. Genotype vs. Sex: $F(4.261, 46.869) = 0.208$, $p = 0.971$, Sound level vs. Genotype: $F(4.261, 46.869) = 0.902$, $p = 0.475$, Sound level vs. Sex: $F(2.130, 46.869) = 5.381$, $p = 0.007$, Genotype vs. Sex: $F(2, 22) = 0.910$, $p = 0.417$, Sound level: $F(2.130, 46.869) = 41.325$, $p < 0.001$, Sex: $F(1, 22) = 0.213$, $p = 0.649$, Genotype: $F(2, 22) = 0.749$, $p = 0.484$). This difference in avoidance behaviour was further evident in the change in dark box time between the quiet and test stimulus condition on a given day (note: negative values = less time in dark box versus 60 dB SPL condition) where *Cntnap2*^{-/-} rats left the dark box for a greater amount of time in the 74 dB SPL condition in comparison to wildtype rats, with *Cntnap2*^{+/-} not being significantly different from either genotype (C; 1-way ANOVA, Sound level: 65 dB: $F(2, 25) = 0.612$, $p = 0.550$. 74 dB: $F(2, 25) = 4.580$, $p = 0.020$, post-hoc Bonferroni's t-test, WT vs. KO: $p = 0.018$, WT vs. HET: $p = 0.302$, HET vs. KO: $p = 0.805$. 83 dB: $F(2, 25) = 0.042$, $p = 0.959$). $n =$ wildtype: 5M, 5F; *Cntnap2*^{+/-}: 4M, 4F; and *Cntnap2*^{-/-}: 4M, 6F. Data expressed as mean \pm SEM. * = $p < 0.05$.

2.3.9 Pre-attentive Audiovisual Processing

Finally, in order to assess pre-attentive audiovisual temporal processing, the modulation of the acoustic startle response by an auditory, visual, and audiovisual prepulse stimulus was measured using the metric of percent prepulse inhibition, %PPI (Fig. 2-8G, H). Under these experimental conditions, *Cntnap2*^{-/-} rats exhibited typical PPI, with the audiovisual prepulse eliciting a greater reduction of the startle response compared to the auditory ($p < 0.001$) or visual ($p < 0.001$) prepulse cues alone.

2.4 Discussion

To better understand the consequences of a loss-of-function of *CNTNAP2* on the manifestation of ASD-related behaviours, we compared the performance of rats with a homozygous or heterozygous knockout of *Cntnap2* to their wildtype littermates across a comprehensive test battery, which included assessments of social interactions, behavioural flexibility and intellectual ability. Furthermore, we investigated if *Cntnap2*-related changes in low-level sound processing are accompanied by higher-level alterations in auditory perception and audiovisual integration.

Reminiscent of individuals with ASD, who generally orient less to social cues and prefer solitary activities (Vivanti et al., 2018), we found that *Cntnap2*^{-/-} rats did not preferentially orient to a conspecific rat versus an inanimate object, or prefer new social interactions. Similar findings were reported for *Cntnap2* knockout mice performing the 3-chamber interaction test (Brunner et al., 2015; Penagarikano et al., 2011; cf. R. Scott et al., 2019; Xing et al., 2019), although no differences in the play behaviour of *Cntnap2*^{-/-} rats, another task assessing social behaviour, was previously reported (Thomas, Schwartz, Saxe, & Kilduff, 2016). Ultimately, because impaired social interactions are present in a variety of genetically-modified rodent models of ASD (for review see Kazdoba et al., 2016; Möhrle et al., 2020), it would be informative to determine the extent that molecular/cellular changes in relevant brain regions (e.g., the limbic system) of these rodents are consistent to those in *Cntnap2*^{-/-} rats, as this comparative approach could help reveal the common

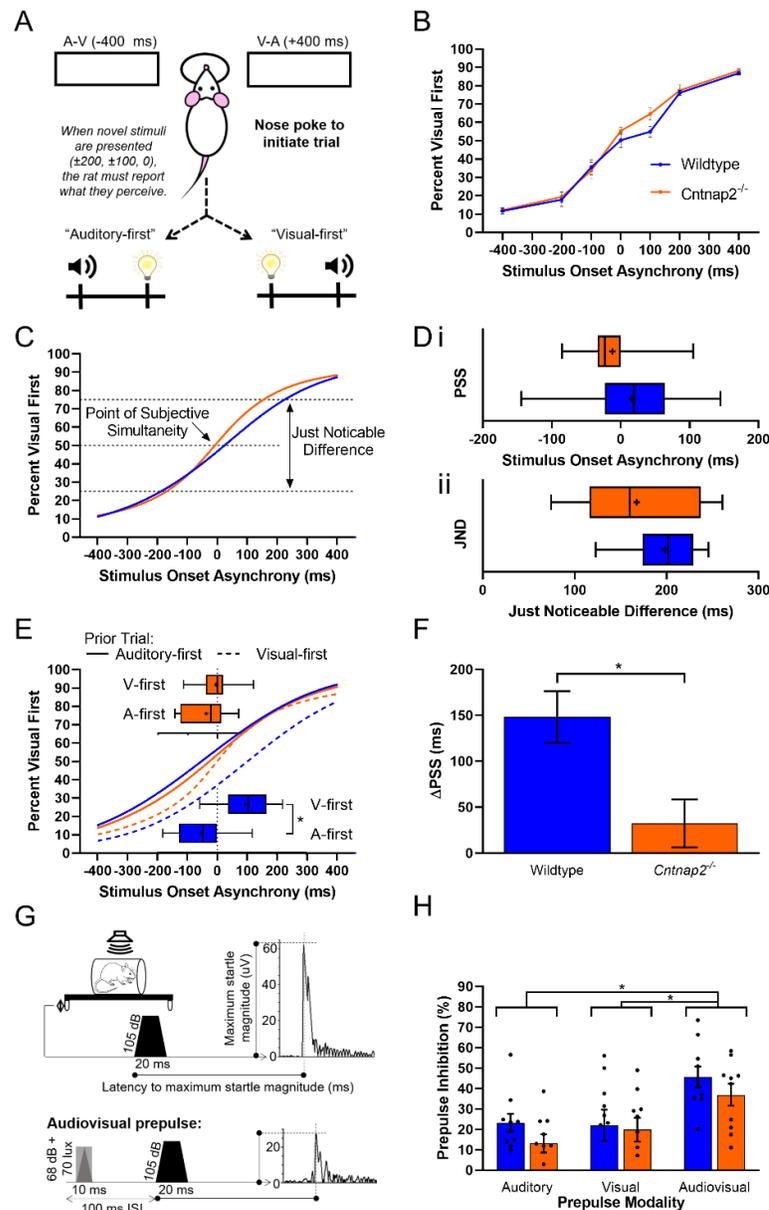


Figure 2-8: *Cntnap2*^{-/-} rats have typical audiovisual perception and filtering but lack rapid audiovisual recalibration.

Rats' perception of the relative timing of auditory and visual stimuli was assessed using a temporal order judgement task (A). Raw data (B; 3-way RM ANOVA, SOA vs. Genotype vs. Sex: $F(2.988, 47.813) = 1.698$, $p = 0.180$, SOA vs. Genotype: $F(2.988, 47.813) = 1.972$, $p = 0.131$, SOA vs. Sex: $F(2.988, 47.813) = 3.704$, $p = 0.018$, Genotype vs. Sex: $F(1, 16) = 0.123$, $p = 0.730$, SOA: $F(2.988, 47.813) = 349.401$, $p < 0.001$, Sex: $F(1, 16) = 0.477$, $p = 0.500$, Genotype: $F(1, 16) = 0.954$, $p = 0.343$) and its fitted psychometric function (C) revealed typical audiovisual temporal perception in *Cntnap2*^{-/-} and wildtype rats, with no genotypic differences in the point of subjective simultaneity

mechanisms by which social interaction deficits emerge in animals with distinct genetic disruptions.

As behavioural flexibility is often reduced in ASD (Chebli et al., 2016; Jiujiias et al., 2017), we screened the *Cntnap2*^{-/-} rats for stereotypic behaviours, as well as cognitive inflexibility using a probabilistic learning task. Consistent with a previous study on *Cntnap2*^{-/-} rats (Thomas et al., 2016), we found that a functional loss of *Cntnap2* caused hyper-locomotion. We also documented increased circling and rearing, but no differences in self-grooming. In contrast, mouse models of *Cntnap2* disruption have observed over-grooming as well as hyper-locomotion (Penagarikano et al., 2011; R. Scott et al., 2019; Thomas et al., 2016; Xing et al., 2019; cf. Brunner et al., 2015). As other ASD-related models have also found disparate grooming results between rats and mice (e.g. Shank3; Möhrle et al., 2020; Song et al., 2019) or that rats do not exhibit robust self-grooming behaviour (Thomas et al., 2016), it seems that caution should be exercised when attempting to use over-grooming as a putative metric of increased stereotypy in genetically-modified rats. Finally, we did not find any differences in probabilistic reversal learning across the genotypes; it

Figure 2-8 continued... (Di; 2-tailed independent samples t-test, $t(18) = 0.974$, $p = 0.343$) or the just noticeable difference (Dii; 2-tailed independent samples t-test, $t(18) = 1.328$, $p = 0.201$). When the prior trial was classified as auditory-first (solid line) or visual-first (dashed-line; E), rapid audiovisual temporal recalibration was observed in wildtype but not *Cntnap2*^{-/-} rats; exemplified by a leftward shift in the curve, and the PSS on auditory-first trials in wildtype rats (E- bottom inset; 2-tailed paired t-test, $t(9) = 5.256$, $p = 0.001$) but not *Cntnap2*^{-/-} rats (E- top inset; 2-tailed paired t-test, $t(9) = -1.246$, $p = 0.244$). *Cntnap2*^{-/-} rats' reduced adaptation was quantified as Δ PSS (F; 2-tailed independent samples t-test, $t(18) = 2.678$, $p = 0.015$). During the audiovisual sensorimotor gating paradigm (G), the auditory + visual prepulse elicited the largest inhibition in both genotypes (H; 3-way RM ANOVA, Prepulse Modality vs. Genotype vs. Sex: $F(2, 32) = 0.603$, $p = 0.553$, Prepulse Modality vs. Genotype: $F(2, 32) = 0.473$, $p = 1.415$, $p = 0.252$, Sex: $F(1, 16) = 0.067$, $p = 0.799$, Genotype: $F(1, 16) = 1.549$, $p = 0.231$, Prepulse Modality: $F(2, 32) = 22.976$, $p < 0.001$, post-hoc Bonferroni's t-test, A vs. V: $p = 1.000$, A vs. AV: $p < 0.001$, V vs. AV: $p < 0.001$). $n =$ wildtype: 6M, 4F and *Cntnap2*^{-/-}: 6M, 4F. Data in B, G, H expressed as mean \pm SEM. Data in D, F box center represents the median and extends to the 25th and 75th percentiles, whiskers represent extremes, and + represents the mean. * = $p < 0.05$.

therefore appears that this form of behavioural flexibility is unaltered following a functional loss of *Cntnap2*.

Although intellectual disability is genetically-linked to and often diagnosed comorbid with ASD (DSM-5; reviewed in Matson & Shoemaker, 2009; Srivastava & Schwartz, 2014), whether or not specific impairments in spatial learning and memory are observed ASD seems to greatly depend on the actual behavioural task performed (Edgin & Pennington, 2005; Smith, 2015). For example, individuals with ASD had similar performance to neurotypicals when a computerized version of a task akin to the Morris water maze for rodents was used (Edgin & Pennington, 2005). In the present study, the *Cntnap2*^{-/-} rats showed a modest delay in spatial acquisition learning, yet intact reference memory; findings similar to *Cntnap2* knockout mice performing the Morris water maze (Penagarikano et al., 2011) and radial-arm maze (Rendall, Truong, & Fitch, 2016). It is interesting that *Cntnap2*^{-/-} rat performance was largely spared on these spatial learning and memory tasks which are heavily reliant on hippocampal function given that CASPR2 is expressed throughout the brain (Gordon et al., 2016; Penagarikano et al., 2011), and that its functional loss results in impaired inhibitory neurotransmission in the hippocampus (Jurgensen & Castillo, 2015). Looking forward, the *Cntnap2*^{-/-} rats could provide a useful model to determine why it is that some brain regions and their associated behaviours (e.g., limbic system and social interactions) seem to be more affected than others (e.g., hippocampus and spatial learning/memory).

An estimated 75% - 90% of children with ASD show sensory processing abnormalities (Crane, Goddard, & Pring, 2009; Schoen, Miller, Brett-Green, & Nielsen, 2009; Talay-Ongan & Wood, 2000; Tomchek & Dunn, 2007). At the pre-attentive level, sound processing is altered in some individuals with ASD and fragile X syndrome, such that their acoustic startle response is increased, while their sensorimotor gating (measured as prepulse inhibition) is reduced (Chamberlain et al., 2013; Frankland et al., 2004; Kohl et al., 2014; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016; Takahashi, Komatsu, Nakahachi, Ogino, & Kamio, 2016). Interestingly, rats, but not mice, with disrupted *Cntnap2* show a similarly altered profile of low-level sound processing (Brunner et al.,

2015; Penagarikano et al., 2011; K. E. Scott et al., 2018). *Cntnap2* knockout rats exhibit a greater ASR and impaired prepulse inhibition which is consistent with our first study (K. E. Scott et al., 2018), despite a new breeding colony and breeding scheme. This replication increases the significance and confidence level with which we can conclude altered pre-attentive auditory processing is present in the *Cntnap2*^{-/-} rat. Perceptually, individuals with ASD find lower intensity sounds to be louder and more uncomfortable, although their ability to discriminate between sound intensities remains intact (Bonnell et al., 2010; Khalifa et al., 2004). Likewise, we found that *Cntnap2*^{-/-} rats exhibited a greater aversion to sounds at moderate intensities during an active sound avoidance paradigm in comparison to wildtype rats. To further investigate whether these moderate-intensity sounds would also be judged to be more similar to a “loud” stimulus, *Cntnap2*^{-/-} and wildtype rats performed a sound categorization task. Interestingly, we observed no differences in the perceptual judgment of sound intensity between the genotypes, indicating that a functional loss of *Cntnap2* did not alter the rats’ ability to differentiate these sounds. Ultimately, these collective findings highlight an important dichotomy in sound processing in this preclinical ASD model: *Cntnap2*^{-/-} rats had an unaltered ability to categorize which sounds were considered “loud”; however, this preserved perceptual judgment did not spare them from experiencing these sounds as more aversive or startle-eliciting. This dichotomy can be extended to the broader sensory behavioural profile observed; paradigms requiring learned associations did not reveal genotypic differences, whereas tasks relying on automatic/implicit behaviours did. As further evidence, similar to the autistic profile (Noel et al., 2016; Stevenson et al., 2014; Turi et al., 2016; Van der Burg, Alais, & Cass, 2013), the *Cntnap2*^{-/-} rats could accurately judge the temporal order of simple auditory and visual stimuli based on the learned associations, yet their rapid audiovisual temporal recalibration—a fast-acting, sensory effect—was impaired.

Apparent in the comparison of *Cntnap2* knockout rat and mouse phenotypes, no single model species is able to capture the full complexity of ASD (Möhrle et al., 2020; Vecchia et al., 2019). Indeed several reviews compare the utility of rats and mice on various behavioural tasks such as social behaviour, communication, learning, and cognition (Ellenbroek & Youn, 2016; Homberg, Wöhr, & Alenina, 2017; Kondrakiewicz, Kosteci,

Szadzińska, & Knapska, 2019; Wöhr & Scattoni, 2013). With respect to the *Cntnap2* knockout rodents, rats may be preferred when assessing social behaviours because mice exhibit greater aggression, stress, and variability (Homberg et al., 2017; Kondrakiewicz et al., 2019), as has been demonstrated in the *Cntnap2* mouse literature (Brunner et al., 2015; Penagarikano et al., 2011; R. Scott et al., 2019; Xing et al., 2019), whereas repetitive behaviours are more clearly demonstrated in *Cntnap2* knockout mice than rats. Rats may also be preferred in tasks involving learning and cognition, such as the probabilistic reversal learning, sound intensity categorization, and audiovisual temporal order judgement tasks used in this study, because rats typically require shorter habituation periods and fewer training sessions to perform the task (Ellenbroek & Youn, 2016). Given that rats also have a more complex acoustic communication system (Wöhr & Schwarting, 2013), and our data shows multiple types of auditory-based behaviours are well recapitulated in the *Cntnap2*^{-/-} rat, studies of auditory processing may be more suited to rats. However, sensory processing extends to other domains, including nociception and olfaction, with studies in *Cntnap2*^{-/-} mice reporting increased reactivity to mechanical, heat, or chemical stimuli, and increased sensitivity to olfactory cues in a buried food assay (Dawes et al., 2018; Penagarikano et al., 2011). Despite preference for a given species dependent on the type of behaviour assessed, differences in the function of neurotransmitter systems, neurogenesis, and disorder progression (Ellenbroek & Youn, 2016) suggest that the greatest utility lies in a cross-species approach. Comparing similarities and differences between species is able to provide a greater understanding of the role of the *CNTNAP2* gene in autism.

Overall, the present study provides a comprehensive understanding of the importance of the autism-risk gene, *CNTNAP2*, for behaviours associated with ASD. Given that the functional loss of *Cntnap2* in the rat is sufficient to cause some of the ASD-related alterations in social interaction, stereotypic behaviour, and sensory processing, future studies could take advantage of the face validity of this preclinical model, and design experiments to directly test the working hypothesis that altered sensory processing is at the core of the more complex ASD-related behaviours (Baum et al., 2015; Burns, Dixon, Novack, & Granpeesheh, 2017).

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2.6 Supplemental Methods

Animals

Tissue from the pinna was collected between P18 and P35, and genotyping was performed using the following primers, Fwd: 5'-TTCCCACTACTCAGGAAGCAA-3', Rev: 5'-AAGAAGGAAGGAAAAGGGGC-3'. Rats were housed in a temperature-controlled room on a 12 h light/dark cycle, with ad libitum food and water. Animals from a minimum of three litters were used in all experiments. Where possible, the experimenters remained blinded to genotype.

Blinding Procedure

All rats were given a numerical identifier (animal ID) during tissue collection for genotyping. A single experimenter performed all genotyping, logged the genotype based on the animal ID, and organized which animals were to be tested on each task. The experimenters involved in conducting the behavioural experiments were only informed of the numerical ID of each rat. Upon data collection completion, the experimenters were unblinded to each animal's genotype in order to conduct analysis. Given that adult homozygous knockout rats may have spontaneous seizures, the experimenter conducting the behavioural tasks may have become aware of the genotype if an animal seized in their presence. In the event a seizure occurred while awaiting testing, rats were given a minimum of 30 minutes of recovery time before performing a task. If seizure occurred during task performance, the data point was removed from analysis.

Social Behaviours

Social behaviour testing was done in a custom-made, rectangular (120 x 60 x 40 cm), clear polycarbonate apparatus placed on a black platform. The apparatus was divided into three equal-sized chambers (40 x 20 x 13.3 cm) with two clear inner dividing walls. Openings on the inner dividing walls (10 cm) allowed the rats to have free access into the three chambers. Each outer chamber had an empty cylindrical plexiglass tube with holes (13 cm diameter, 20 cm long) into which the stranger rats were placed (Fig. 2-1A, D). These tubes

allowed nose contact between the rats. The test rat was first habituated to the empty 3-chamber apparatus (10 min). To test sociability, a stranger rat (stranger 1) was placed in one tube, and the test rat again explored the apparatus (10 min; Fig. 2-1A). To test social novelty, the test rat was re-exposed to the now-familiar stranger rat (familiar), and a novel stranger rat (stranger 2) was placed in the second tube (10 min; Fig. 2-1D). Testing blocks occurred sequentially. Stranger rats were previously habituated to the tubes, their locations were counterbalanced and randomized, and they were always age and sex-matched to the test rat. For each stage, the amount of time the test rat spent sniffing the tubes, and total distance travelled were recorded via ANY-Maze software (v4.29, Stoelting Co.). Wildtype: 5M (sociability) and 4M (social novelty), 5F; *Cntnap2^{+/-}*: 5M, 6F; and *Cntnap2^{-/-}*: 5M, 6F.

Stereotypic and Exploratory Behaviours

Stereotypic and exploratory behaviour testing was completed in a custom made, black plastic (Plexiglas) open-top chamber (45.5 x 45.5 x 45.5 cm) with 3 cm deep layer of blue animal bedding (Living World® Fresh 'N Comfy Small Animal Bedding) to optimize tracking (Fig. 2-2A). Bedding was changed and apparatus cleaned between animals. Test animals freely explored the apparatus for 20 min in the presence of background white noise. Rat full body rotations (entire rotation of 360°) and locomotor activity (time (s) and distance (m)) were recorded via ANY-Maze software (v4.29, Stoelting Co.), and a blinded observer scored self-grooming, defined as pawing or licking any part of the body, and vertical rearing, defined as having both forepaws off the ground. Perimeter preference was calculated as: $\text{Perimeter preference} = (\text{Time in perimeter zone (s)} - \text{Time in center zone (s)}) / (\text{Time in perimeter zone (s)} + \text{Time in center zone (s)})$. Wildtype: 7M, 7F; *Cntnap2^{+/-}*: 7M, 8F; and *Cntnap2^{-/-}*: 5M, 6F.

Cognitive Flexibility

Cognitive flexibility was tested using a lever-pressing task performed in an operant conditioning chamber (30.5 x 24 x 21 cm; Med-Associates; Fig. 2-3A) with two retractable levers positioned on either side of a central pellet receptacle which dispensed sucrose pellets (45 mg; Bio-Serv, Frenchtown, NJ). A house light was located on the opposite wall

of the chamber. Customized computer software (MED-PC IV, Med-Associates) controlled the operation of the test chamber. Five to seven days preceding the first training session, the rats were food restricted to 90% of their free-feeding weight. Weights were monitored daily, and food restriction was maintained for the duration of the lever-pressing training and testing. Training: First, rats were trained on a 100% reward rate to acquire the right and left lever press response to a criterion of 60 trials. Rats then performed 90 trials in which they needed to respond to an inserted lever within 10 s to receive a reward. Failure to respond was scored as an omission and the house light was extinguished. To move to the next stage, the performance criterion was ≥ 85 successful trials. Next, rats were familiarized with a probabilistic reward scheme with only 50% of correct trials rewarded. This occurred for a minimum of three days, the performance criterion to proceed was ≥ 80 successful trials. Lastly, lever preference was determined. Testing: The probabilistic reversal learning protocol consisted of 200 discrete choice trials that initiated with both levers extended. At the start of the test session, the disfavoured lever was selected by the experimenter to be correct. Correct responses were rewarded 80% of the time, whereas incorrect responses were rewarded 20% of the time. Rats successfully completed the initial discrimination phase of the entire protocol once the correct lever had been pressed on eight consecutive trials; after which the contingencies were reversed, and the opposite lever was designated as correct. This transition signified the beginning of the reversal learning phase of the task. This process of reversals continued until the 200 trials were completed (see Dalton et al., 2014 for more details). Trial numbers and proportions were tabulated. As no significant genotypic differences in omissions were observed, the total number of reversals were counted. The metrics of win-stay ratio and loose-shift ratio were calculated as described in Dalton et al., 2014. Briefly, the win-stay ratio was determined from the number of trials on which a rat was previously-rewarded for a correct choice and selected the correct lever, divided by the total number of rewarded correct choices. Conversely, the loose-shift ratio was calculated from the number of trials on which a rat was previously not rewarded for a correct choice and selected the incorrect lever, divided by the total number of unrewarded correct choices. Wildtype: 5M, 4F; *Cntnap2*^{+/-}: 5M, 4F; and *Cntnap2*^{-/-}: 3M, 5F.

Spatial Learning and Memory

To assess spatial learning and memory, a modified Morris Water Maze task was conducted. These protocols were based on a modified 2-day learning and recall version of the MWM (Roof, Schielke, Ren, & Hall, 2001), and followed the methods established by Levit et al., 2019. Rats are placed in a large metal water tank (144 cm diameter, dyed with black non-toxic acrylic paint, 19° C) and must locate a hidden submerged platform (Fig. 2-4A; white dashed circle; 12 cm diameter, 3 cm below surface of water), using spatial cues located on the walls (Fig. 2-4A, F; N – green cross; E – white triangle; S – no cue, W –black square). To learn this task, rats were given six 90 s trials, with a 1h inter-trial rest interval. Upon trial initiation, the rat was always placed in a fixed start location (Fig. 2-4A; entry), and it had 90 s to locate the hidden platform in the NE quadrant. If the rats were unable to find the platform in 90 s, the experimenters guided the rats to it. Once situated on the platform, rats remained for 30 additional seconds to allow time for the rats to encode the spatial cues around them (Fig. 2-4A). Twenty-four hours after the last learning trial, the platform was removed (Fig. 2-4F grey dashed circle) and each rat underwent a probe trial (90 s) to assess if they consolidated and could recall the spatial memory to find the location of the platform (Fig. 2-4F). To investigate the possibility of differences in visual acuity and/or swim speed, one hour after the probe trial, each rat underwent four visual cue tests spaced 1 h apart. Spatial cues were removed from the walls, and the platform with a marker positioned above its location was inserted into the maze in a unique position for each visual cue trial (Fig. 2-4K; yellow circles). Time to the platform, time spent in various maze regions, and swimming speed (m/s) were recorded using the ANY-Maze software (version 4.1) throughout all trials. Wildtype: 5M, 6F; *Cntnap2*^{+/-}: 5M, 4F; and *Cntnap2*^{-/-}: 4M, 5F.

Sound Processing: Pre-attentive & Perceptual

Pre-attentive Sound Processing. Acoustic reactivity and sensorimotor gating were assessed in a sound-attenuating startle box (LE116; Panlab), in which rats were placed within movement-limiting, custom made clear plastic tubes and on a pressure-sensitive platform (Fig. 2-5A). Over two days, rats were acclimated (3 x 10 min) to the test chambers and habituated to the pulse stimuli (105 dB SPL; 20 ms duration; 5 ms rise/fall time; white

noise presented 30 times with varying inter-trial interval of 15, 17.5 or 20 s) (see Scott et al., 2018 for more details). Commercial hardware (StartFear system, Panlab) and software (STARTLE module PACKWIN-CSST, PACKWIN version 2.0, Panlab) were used to measure each rat's acoustic reactivity in response to startle pulse stimuli of varying intensities (74, 83, 89 and 105 dB SPL; 20 ms duration; 5 ms rise/fall time; white noise). A total of 40 trials were performed, in which the each of the sound intensities were randomly presented 10 times each with varying inter-trial intervals (ITI: 15, 17.5, or 20 s). Quantification of the acoustic startle response occurred over a 500 ms period from the beginning of the acoustic startle stimulus. Next, sensorimotor gating was determined by presenting a prepulse stimulus (cued: 74, 83, and 89 dB SPL; 10 ms duration; 5 ms rise/fall time; white noise, or uncued: no prepulse stimulus) 100 ms before the 105 dB startle pulse stimulus, and measuring the extent that the startle response was attenuated (i.e., prepulse inhibition; Fig. 2-5D). A total of 40 trials were performed, in which the each of the cued and uncued prepulse conditions were randomly presented 10 times each with varying inter-trial intervals (ITI: 15, 17.5, or 20 s). Overall, percent prepulse inhibition (% PPI) was calculated using the following formula:

$$\%PPI = \left(1 - \left(\frac{\text{ave startle magnitude (arb.) cued prepulse condition}}{\text{ave startle magnitude (arb.) uncued prepulse condition}} \right) \right) \times 100\%$$

Wildtype: 8M, 7F; *Cntnap2*^{+/-}: 7M, 6F; and *Cntnap2*^{-/-}: 7M, 8F.

Sound Intensity Categorization. Rats' ability to make a perceptual categorization of the sound intensity (subjectively, loudness) of noisebursts was assessed over a 1-2 month period. Five to seven days preceding the first training session, the rats were food restricted and approached 85% of their free-feeding weight. Weights were monitored daily, and food restriction was maintained for the duration of the training and testing. Behavioural training and testing were conducted in a standard modular test chamber (ENV-008CT; Med Associates Inc., St. Albans, VT) that was housed within a sound-attenuating box (29" W by 23.5" H by 23.5" D; Med Associates Inc.). The front wall of the behavioural chamber

included a nose poke as well as a left and right feeder trough, each fitted with an infrared detector to monitor the rat's performance. The test chamber was illuminated by a house light on the back wall. Custom real-time processing hardware (RZ6 and BH-32, Tucker Davis Technologies, Alachua, FL) were interfaced with the test chamber. Custom behavioural protocols running in MATLAB (EPsych Toolbox, <http://dstolz.github.io/epsych/>) monitored nose poke responses and controlled the presentation of the stimuli, as well as the positive reinforcement (i.e., sucrose pellet delivery) and punishment (i.e., turning off the house light). During training on a two-alternative forced choice paradigm, the rats learned to associate a “quiet” noiseburst (71 dB SPL; 25 ms; 1-32 kHz) with the right feeder trough, and a “loud” noiseburst (89 dB SPL; 25 ms; 1-32 kHz) with the left feeder trough (Fig. 2-6A). These stimuli were presented from a speaker (FT28D, Fostex, Tokyo) mounted on the ceiling of the behavioural chamber near the front wall. Each trial began with the rat placing its nose in the center nose poke, which triggered the acoustic stimulus after a 1.5 – 3 s delay. Correct feeder trough choices were reinforced with a sucrose pellet, whereas incorrect responses resulted in the house light extinguishing for up to 15 s, during which time a new trial could not be initiated. During their training to discriminate the two stimuli, the rats performed ~150-250 daily trials, which lasted ~30 min. Once the rats had achieved greater than 85% accuracy, they began to perform test sessions. To assess the rats' categorization of sound intensity, the test sessions introduced novel noiseburst intensities (i.e., 74, 77, 80, 83, 86 dB SPL; 25 ms; 1-32 kHz), such that 70% of the trials were the two training stimuli (i.e., 71 and 89 dB SPL), whereas the remaining 30% of the trials were made up of the random presentation of the novel stimuli. Furthermore, the trained stimulus conditions continued to be positively reinforced for correct responses with sucrose pellets and punished for incorrect responses with a 15 s timeout, whereas a sucrose pellet was delivered following each novel stimuli regardless of which feeder trough was chosen. The rats performed a minimum of 25 trials at each of the novel sound intensities across a maximum of three testing sessions, with data collapsed across all tests for analyses. Performance across all seven noiseburst conditions was measured as the proportion of trials in which the rat perceived the stimuli as “loud” (i.e., it responded to the left feeder trough). Consistent with rodent behavioural testing, a

psychophysical profile was generated for each rat (Caras & Sanes, 2015; von Trapp, Buran, Sen, Semple, & Sanes, 2016). These distributions were fit with a cumulative Gaussian using the maximum likelihood procedure of the open-source package *psignifit* 4 for MATLAB (Schütt, Harmeling, Macke, & Wichmann, 2016), from which the subjective category boundary (i.e., the intensity at which 50% of the responses were reported “loud”) was obtained. 3M, 2F; *Cntnap2*^{+/-}: 3M, 2F; and *Cntnap2*^{-/-}: 3M, 2F.

Active Sound Avoidance: The active sound avoidance paradigm was performed in a custom-made apparatus (see Fig. 2-7A for specifications), in which the dark box (clear plastic cage) was enclosed in a sound-attenuating thermoplastic cabinet, and the light box (clear plastic cage) was open to the testing room. The boxes were connected by a runway (bottom: PVC; top: wire mesh). A loudspeaker (FT28D, Fostex, Tokyo, Japan) was mounted on the wall of the dark box and presented endpoint sound stimuli (89 dB SPL; no sound i.e., ~60 dB ambient noise) or test stimuli (65, 74, 83 dB SPL). Prior to testing, rats underwent two acclimation days to familiarize with apparatus. Each day consisted of two 20 min sessions, starting first in the dark box, and next in the light box to ensure the entire apparatus was explored; separated by a 1.5 h inter-trial rest interval. During these four sessions, the light box was illuminated (550 lux) and no sound was presented in the dark box. On the next three days, the rats underwent three 10 minute sound sessions per day, separated by a minimum of 1.5 h; starting each session in the dark box. On each day, the no sound (~ 60 dB SPL) and 89 dB SPL conditions were presented as the first and last stimuli, respectively. The middle stimulus was 65, 74, and 83 dB SPL on days 1-3, respectively. For all of the sound trials, the time a rat spent in the four sections of the apparatus was key coded using ANY-Maze software (version 4.1). The actual time spent in the dark box for the 60 and 89 dB SPL conditions were averaged across the three days for each rat. The relative time spent in the dark box during the middle stimulus condition on a given day was calculated as Middle Stimulus time – No Sound time. Wildtype: 5M, 5F; *Cntnap2*^{+/-}: 4M, 4F; and *Cntnap2*^{-/-}: 4M, 6F.

Perceptual and Pre-attentive Audiovisual Processing

Audiovisual Temporal Order Judgement Task. The rats' perceptual ability to judge the relative timing of the audiovisual stimuli was assessed in the same operant conditioning chambers used for the sound intensity categorization task. In addition to the speaker mounted on the ceiling to deliver the auditory stimulus (50 ms noiseburst; 75 dB SPL; 1-32 kHz), the visual stimulus (50 ms flash; 27 lux) was presented from an LED (ENV-229M; Med Associates Inc.) mounted above the center nose poke. Over the course of several stages of training (see Schormans et al., 2017, 2018, 2019 for details), the rats learned to nose poke to initiate a trial, and ultimately associate a given audiovisual stimulus condition with a specific feeder trough (i.e., auditory-first (-) = left trough; visual-first (+) = right trough; Fig. 2-8A). Correct feeder trough choices were reinforced with a sucrose pellet, whereas incorrect responses resulted in the house light extinguishing for up to 15 s, during which time a new trial could not be initiated. During their training to discriminate the two trial conditions (i.e., ± 400 ms stimulus onset asynchrony, SOA), the rats performed ~ 200 -300 daily trials, which lasted ~ 30 min. Once the rats had achieved greater than 85% accuracy, they began to perform test sessions. Experimental test sessions were introduced in which seven SOAs were randomly delivered (i.e., 0, ± 100 , ± 200 and ± 400 ms), with 70% of the trials being the training stimuli (i.e., ± 400 ms SOA), and the remaining 30% of the trials made up of the random presentation of the novel (0, ± 100 , and ± 200) SOAs. Throughout the test session, the trained stimulus conditions continued to be positively reinforced for correct responses with sucrose pellets and punished for incorrect responses with a 15 s timeout, whereas a sucrose pellet was delivered following each novel SOA regardless of whether a correct or incorrect response was made. Rats performed a minimum of 25 trials at each of the novel SOAs across a maximum of 3 testing sessions, with data collapsed across all tests for analyses. Performance across all 7 SOAs was measured as the proportion of trials in which the rat perceived the stimuli as visual-first (i.e., it responded to the right feeder trough), and a psychophysical profile was generated for each rat. These distributions were fit with a cumulative Gaussian using the maximum likelihood procedure of the open-source package *psignifit* 4 for MATLAB (Schütt et al., 2016), from which the just noticeable difference (JND: the difference between the SOAs at which 25% and 75% of the responses were considered “visual- first”, divided by two) (Vroomen &

Stekelenburg, 2011) and point of subjective simultaneity (PSS: the SOA at which 50% of the responses were “visual first”) (Vatakis, Bayliss, Zampini, & Spence, 2007) were obtained. It is worth noting that the JND provides a measure of the smallest interval between the separately presented auditory and visual stimuli that can be detected reliably, whereas the PSS represents the actual timing of the audiovisual stimuli when the observer is most unsure of the temporal order. Lastly, we determined whether the rats demonstrated rapid temporal recalibration by carrying out a one-back analysis (analyzing trial t 's response as a conditional of trial $t-1$'s) with a focus on if the prior trial had a negative SOA (i.e., auditory-first) or a positive SOA (i.e., visual-first) and again fit the psychometric function with a cumulative Gaussian, from which the PSS, and the amount of change in PSS as a function of the prior trial ($\Delta\text{PSS} = \text{PSS auditory-first} - \text{PSS visual-first}$), also known as the adaptation effect, were obtained (Noel, De Nier, Stevenson, Alais, & Wallace, 2016; Turi, Karaminis, Pellicano, & Burr, 2016; Van der Burg, Alais, & Cass, 2013). Wildtype: 6M, 4F; and *Cntnap2*^{-/-}: 6M, 4F.

Audiovisual Sensorimotor Gating. An assessment of the rats' audiovisual sensorimotor gating was performed using the hardware and software to measure acoustic reactivity (described in section Pre-attentive Sound Processing). Over two days, rats were acclimated (3 x 10 min) to the test chambers, and habituated to the pulse stimuli (30 x). In the audiovisual version of the sensorimotor gating task, the prepulse stimuli consisted of either an acoustic cue (A; 68 dB SPL; 10 ms duration; 5 ms rise/fall time; white noise), a visual cue (V; 70 lux; 10 ms duration, LED), or an audiovisual cue (AV; simultaneous presentation of both A and V), which preceded the startle pulse stimulus (105 dB SPL; 20 ms duration; 5 ms rise/fall time; white noise) by 100 ms (Fig. 2-8G). A total of 40 trials were performed, in which the four conditions (cued: A, V, AV; uncued: no prepulse stimulus) were randomly presented 10 times each with varying inter-trial intervals (ITI: 12, 15 or 18 s). Quantification of the acoustic startle response occurred for a 500 ms period from the beginning of the acoustic startle stimulus. Ultimately, using the same formula described above, the relative percentage of prepulse inhibition was calculated using the average startle magnitudes for a given trial type. Wildtype: 6M, 4F; and *Cntnap2*^{-/-}: 6M, 4F.

Statistics

All statistical analyses and significant results are presented in the figure legends. In cases when the Mauchly test reported a violation of the assumption of sphericity, the degrees of freedom were corrected using the Greenhouse Geisser (if $\epsilon < 0.75$) or the Huynh-Feldt method (if $\epsilon > 0.75$). Post-hoc simple main effect analysis and corrected t-tests were used to further investigate differences of interest. Differences were considered statistically significant when p-values (adjusted) were smaller than $\alpha = 0.05$. If there was no genotype x sex interaction, data were plotted to include both sexes, with a main effect of sex reported if present. Unless otherwise stated, *Cntnap2*^{+/-} rats did not differ from wildtypes. Data analyses were performed with Microsoft Excel 2010 (Microsoft Corp.) or MATLAB (R2016a; MathWorks), and graphs were generated with GraphPad (Prism 8.3.0 for Windows, GraphPad Software, San Diego, CA, USA). IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, N.Y., USA) was used for statistical analysis.

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Chapter 3

3 Altered auditory processing, filtering, and reactivity in the *Cntnap2* knockout rat model for neurodevelopmental disorders²

Sensory processing, and auditory processing in particular, is altered in individuals with neurodevelopmental disorders such as autism spectrum disorder (ASD). A possible disturbance in the normal maturation of the auditory system during early development may underlie altered auditory reactivity that persists in later life. Of the many genes that regulate the auditory system development, loss-of-function mutations in the *CNTNAP2* gene are strongly associated with language processing deficits and ASD. Therefore, using a novel *Cntnap2* knockout rat model, we tested the impact of *Cntnap2* loss on auditory processing, filtering, and reactivity throughout development and young adulthood in male and female animals. While hearing thresholds were not altered in *Cntnap2* knockout animals, we found a reduction in response amplitudes and a delay in response latency of the auditory brainstem response (ABR) in juvenile *Cntnap2* knockout rats compared to age-matched wildtype animals. Amplitudes and latency of the ABR largely normalized by adulthood, indicating a delayed maturation of auditory processing pathways in *Cntnap2* knockout rats. Despite the reduced ABR amplitudes, adolescent *Cntnap2* knockout animals displayed increased startle reactivity, accompanied by disruptions in sensory filtering and sensorimotor gating across various conditions, most of which persisted in adulthood. All these observations show striking parallels to disruptions reported in ASD. Our results also imply that developmental disruptions of sensory signal processing are associated with persistent changes in neural circuitries responsible for implicit auditory evoked behaviour,

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emphasizing the need for interventions that target sensory processing disruptions early during development in ASD.

3.1 Introduction

The auditory system undergoes tremendous remodeling and plasticity in early development which has a profound effect on how the adult brain handles acoustic information. The typical maturation is perturbed in individuals with neurodevelopmental disorders, such as autism spectrum disorder (ASD), which can ultimately lead to long-term auditory processing deficits (see Sinclair et al., 2016 for review). Apart from varying degrees of language impairment (Kjelgaard and Tager-Flusberg, 2001; Deriziotis and Fisher, 2017), individuals with ASD have shown deficits in how their central auditory system processes the basic features of sound (Hitoglou, Ververi, Antoniadis, & Zafeiriou, 2010), including delayed neurotransmission throughout the successive relay nuclei of the brainstem (Fujikawa-Brooks, Isenberg, Osann, Spence, & Gage, 2010; Gonçalves, Wertzner, Samelli, & Matas, 2011; Kwon, Kim, Choe, Ko, & Park, 2007; Magliaro, Scheuer, Assumpcao Junior, & Matas, 2010; Miron et al., 2016; Rosenhall, Nordin, Brantberg, & Gillberg, 2003; Roth, Muchnik, Shabtai, Hildesheimer, & Henkin, 2012; Tas et al., 2007; Wong & Wong, 1991). Furthermore, self-report questionnaires (Danesh et al., 2015) and psychoacoustic testing (Khalifa et al., 2004) have provided evidence of increased sensitivity to sound in the autism population. Related to this hyperacusis, the implicit (reflexive) reactivity to acoustic stimuli—a behavioural measure reliant on auditory brainstem function—is greater in some individuals with ASD, as revealed by exaggerated responses to sudden sounds (Chamberlain et al., 2013; Kohl et al., 2014; Takahashi, Komatsu, Nakahachi, Ogino, & Kamio, 2016). At present, however, the developmental trajectory of these electrophysiological and behavioural indices of auditory brainstem dysfunction have not been fully elucidated since they are difficult to study longitudinally in patient populations. It is therefore not clear if these deficits are already present in early life, if they

improve/worsen with age, or how auditory processing disruptions impact auditory reactivity.

To date, a limited number of preclinical studies have examined ASD-related auditory processing deficits using rodents with gene mutations linked to ASD. In 2011, Penagarikano and colleagues first characterized a mutant mouse model with a loss-of-function of the contactin associated protein-like 2 gene (*Cntnap2*). Homozygous loss-of-function mutations in *CNTNAP2* are a rare single gene cause for ASD (Strauss et al., 2006; Poot et al. 2017), and multiple studies have identified various other *CNTNAP2* mutations being associated with, or a risk factor for, ASD and language-related disorders (Alarcón et al., 2008; Arking et al., 2008; Rodenas-Cuadrado et al., 2014, 2016; Murphy and Benítez-Burraco, 2017; Poot, 2017). *CNTNAP2*, which codes for the neurexin CASPR2, is known to be important in language development in humans, with its structure and biological functions appearing to be conserved (Abrahams et al., 2007; Newbury et al., 2011; Poot, 2015; Whalley et al., 2011; Whitehouse, Bishop, Ang, Pennell, & Fisher, 2011). CASPR2 is highly expressed throughout the mammalian auditory pathway, within brainstem structures including the spiral ganglion cells of the auditory nerve, cochlear nucleus, lateral superior olive, paralemniscal nucleus, and the inferior colliculus in mice (Gordon et al., 2016). In a series of studies, *Cntnap2* mutant mice showed reduced vocalizations and an impaired auditory temporal processing (Penagarikano et al., 2011; Truong, Rendall, Castelluccio, Eigsti, & Fitch, 2015). Despite the high construct validity of these *Cntnap2* mutant mice, it remains unknown how a deficiency in *Cntnap2* affects the time-course of maturation of brainstem-mediated auditory processing and behaviour.

In the present study, we used genetically-modified rats to investigate the developmental trajectory of *Cntnap2*-related deficits in electrophysiological and behavioural measures of brainstem function in male and female juvenile, adolescent, and adult *Cntnap2* homozygous (*Cntnap2*^{-/-}) and heterozygous knockout (*Cntnap2*^{+/-}) animals compared to wildtype controls (*Cntnap2*^{+/+}). Consistent with electrophysiological testing in humans, the four characteristic waves of the rat auditory brainstem response (ABR) to acoustic stimuli were used to assess hearing sensitivity (i.e., hearing threshold), neural responsivity

(i.e., ABR wave amplitude), and speed of neurotransmission (i.e., ABR wave latency) across development, which provided an index of the reliability of auditory information processing. Behaviourally, reflexive responses to startle-eliciting sounds were used to determine how *Cntnap2* dysfunction affected the maturation of acoustic reactivity, sensory filtering (i.e., habituation), and sensorimotor gating (i.e., prepulse inhibition). Overall, the present study provides the first comprehensive investigation of the direct contribution of the autism-linked gene, *CNTNAP2*, to the development of brainstem-mediated auditory processing and behaviour, and in doing so, has validated a new rat model for studying auditory brainstem dysfunction with high relevance to neurodevelopmental disorders.

3.2 Materials and Methods

3.2.1 Animals

Male and female Sprague-Dawley wildtypes, heterozygous (*Cntnap2*^{+/-}) and homozygous knockout (*Cntnap2*^{-/-}) rats were used in this study. Mutant breeders were obtained from Horizon Discovery (Boyertown, PA), and wildtype breeders from Charles River Laboratories (Wilmington, MA). Experimental animals were obtained from the following crossings: *Cntnap2*^{-/-} rats from homozygous knockout crossings; *Cntnap2*^{+/-} rats from crossings of wildtype and *Cntnap2*^{-/-} rats; and wildtype rats from wildtype crossings. Animals from a minimum of three litters of a given genotype were used in all experiments. Date of birth was designated as post-natal day zero (P0). Rats were weaned on P21, and sexes were separated on P35. Rats were housed in a temperature-controlled room on a 12h light/dark cycle, with *ad libitum* food and water. The electrophysiological and behavioural testing was performed during the light-phase of the cycle (lights on at 07:00h) and across age in order to gain insight into developmental changes in sensory processing. The electrophysiological assessment of the auditory brainstem responses (ABR) was performed at three time-points in each rat: juvenile (P28 or P29; referred to as P28), adolescent (P42 or P43; referred to as P42), and adulthood (P70 or P71; referred to as P70). The behavioural assessment of auditory brainstem function, as well as spontaneous locomotor activity, was assessed when rats were between P36 – P41 (referred to as P38), and P72-P85 (referred to as P78). All experimental procedures were approved by the University of Western Ontario

Animal Care Committee and were in accordance with the guidelines established by the Canadian Council on Animal Care.

3.2.2 Auditory Brainstem Responses (ABR)

The level of sound-evoked electrical activity in the brainstem was measured using an established protocol (Schormans et al., 2017) in order to assess hearing sensitivity, neural responsivity, and speed of neurotransmission in juvenile, adolescent, and adult rats of the three genotypes (wildtype: 10 males, 7 females; *Cntnap2*^{+/-}: 11 males, 11 females; and *Cntnap2*^{-/-}: 12 males, 10 females). Rats were anesthetized with ketamine (P28: 40 mg/kg or P42 & P70: 80 mg/kg; i.p.) and xylazine (P28: 2.5 mg/kg or P42 & P70: 5 mg/kg; i.p.), and placed in a double-walled sound-attenuating chamber. Subdermal electrodes (27 gauge; Rochester Electro-Medical, Lutz, FL) were positioned at the vertex (active electrode), over the right mastoid process (reference electrode) and on the mid-back (ground electrode; Fig. 3-1A). Throughout the electrophysiological assessment, body temperature was maintained at ~37 °C using a homeothermic heating pad (507220F; Harvard Apparatus, Kent, UK).

The acoustic stimuli used in the ABR assessment consisted of a click (0.1 ms) and two tones (4 kHz and 20 kHz; 5 ms duration and 1 ms rise/fall time), which were generated using a Tucker-Davis Technologies RZ6 processing module sampled at 100 kHz (TDT, Alachua, FL). A magnetic speaker (MF1; TDT) positioned 10 cm from the animal's right ear was used to deliver the stimuli, while its left ear was blocked with a custom foam plug. The acoustic stimuli were each presented 1000 times (21 times/second) at decreasing intensities from 90 to 40 dB sound pressure level (SPL) in 10 dB SPL steps, and at 5 dB SPL steps from 40 dB SPL to 5 dB SPL. At the lower sound intensities, each stimulus was presented twice. Consistent with previous studies, each rat's hearing sensitivity (i.e., ABR threshold) for the click and tonal stimuli was determined using the criterion of just noticeable deflection of the averaged electrical activity within a 10-ms window (Popelar et al., 2008; Abitbol et al., 2016; Schormans et al., 2017; Fig. 3-1B). Prior to the ABR assessment, the acoustic stimuli were calibrated with custom Matlab software (Mathworks,

Natick, MA) using a 1/4-inch microphone (2530; Larson Davis, Depew, NY) and pre-amplifier (2221; Larson Davis).

The sound-evoked activity associated with the ABR assessment was collected using a low-impedance headstage (RA4L1; TDT), pre-amplified and digitized (RA16SD Medusa preamp; TDT), and sent to a RZ6 processing module via a fiber optic cable. The signal was filtered (300 - 3000 Hz) and averaged using BioSig software (TDT). The peak amplitude of each of the characteristic positive waves of the rat ABR were measured in microvolts in reference to the baseline (0 μ V; Fig. 3-1C), and the latency of each of these peaks was determined from the stimulus onset (Fig. 3-1D). Since ABR waves IV/V are often described as a complex, with wave V riding on wave IV (Alvarado, Fuentes-Santamaría, Jareño-Flores, Blanco, & Juiz, 2012), for a wave peak to be analyzed, it must have a preceding and following trough less than its maximum. This resulted in the consistent presence of peaks for waves I – IV at 90 dB SPL. Inter-peak latencies were calculated by subtracting the timing of the respective peaks (e.g., wave IV minus wave II). The experimenter was blinded to the animal's genotype for all analysis associated with the ABR assessment.

3.2.3 Acoustic Startle Responses

To investigate the developmental maturation of brainstem-mediated responses to startle-eliciting sounds, rats of the three genotypes were tested at P38 and P78.

The assessment of acoustic reactivity, sensory filtering and sensorimotor gating was conducted in sound-attenuating startle boxes (LE116, Panlab, Spain) using the StartFear system (Panlab, Spain) and STARTLE software module (PACKWIN-CSST, PACKWIN V2.0, Spain). Animals were placed into large plastic tubes and set on a weight transducing platform in the sound-attenuating chamber. Prior to the behavioural procedures associated with the acoustic startle response (i.e., acoustic reactivity, sensory filtering and sensorimotor gating) animals were handled and acclimated to the startle boxes over three 10-minute sessions. During these acclimation sessions, only background noise (60 dB sound pressure level, SPL, white noise) was presented to the animals.

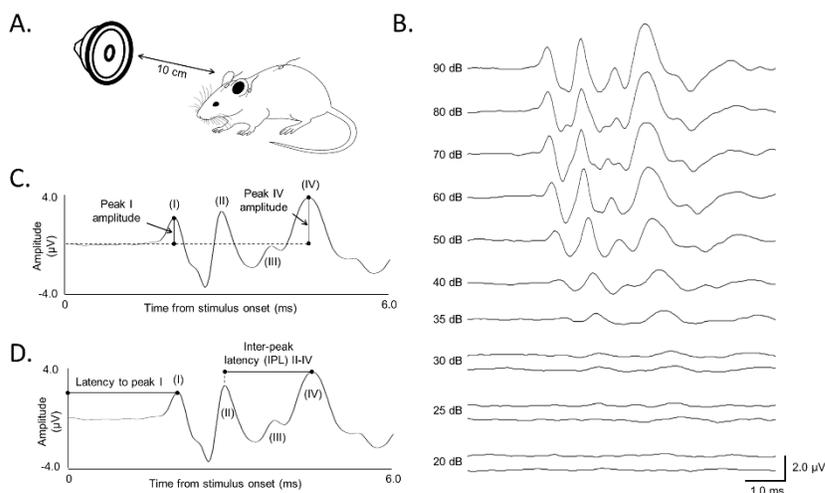


Figure 3-1: Auditory brainstem response methodology.

(A) Positioning of magnetic speaker and subdermal electrodes for the recording of the auditory brainstem response. (B) A representative example of an adult wildtype rat's ABR to a click stimulus used to determine hearing threshold. The last noticeable deflection of the averaged electrical activity can be seen at 25 dB SPL. (C & D) A representative ABR trace from a wildtype animal in response to a 90 dB SPL click stimulus depicts the four characteristic ABR waves in rats, and the measurement for peak amplitudes and latencies (black bars).

Following acclimation, behavioural procedures were conducted over three days. On day one, each animal's acoustic reactivity was assessed by determining the relationship between the intensity of a given acoustic stimulus and the magnitude of the elicited motor response. By exposing rats to 11 acoustic stimuli of increasing intensity from 65 - 115 dB SPL in 5 dB SPL steps (unrandomized, 20 ms white noise with 5 ms rise/fall time, every 60 s; presented on top of white background noise) the startle threshold was determined, as well as the maximum startle reactivity for the three genotypes of rats at the two ages (P38 and P78; (wildtype: 12 males, 10 females; *Cntnap2*^{+/-}: 16 males, 16 females; *Cntnap2*^{-/-}: 8 males, 12 females). For each animal, the peak amplitude of the startle response was recorded at each sound level, and the results averaged across females or males for each genotype (Fig. 3-5A).

On days two and three, the rats were acclimated to the startle boxes for five minutes. To determine the impact of *Cntnap2* knockout on sensory filtering, the rats were then

repeatedly presented a startle-eliciting stimulus, and the degree that their startle response habituated was compared across the genotypes. Thirty startle stimuli (20 ms white noise at 105 dB SPL; 5 ms rise/fall time) were presented with a randomly varying inter-trial interval (ITI: 12, 15, or 18 s) during a continuous background noise (60 dB SPL white noise). Habituation was assessed from the first 8 trials on day two in all three genotypes at both P38 (wildtype: 11 males, 11 females; *Cntnap2*^{+/-}: 14 males, 15 females; *Cntnap2*^{-/-}: 12 males, 14 females) and P78 (wildtype: 12 males; 8 females; *Cntnap2*^{+/-}: 15 males, 16 females; *Cntnap2*^{-/-}: 8 males, 12 females; Fig. 3-6A). A habituation score was calculated for each animal using the following formula:

$$\text{Habituation score} = \frac{(\text{maximum startle magnitude trial 7} + \text{maximum startle magnitude trial 8})/2}{\text{maximum startle magnitude trial 1}}$$

Next, sensorimotor gating was assessed by measuring the amount that each rat's startle response was attenuated (i.e., prepulse inhibition) when the startle stimulus (pulse) was preceded by a brief, non-startling stimulus (prepulse). Subsequently to habituation, prepulse inhibition was assessed: over a total of 50 additional trials, the startle stimulus (20 ms white noise at 105 dB SPL; 5 ms rise/fall time) was presented alone or following an acoustic prepulse stimulus (10 ms white noise at either 75 dB SPL or 85 dB SPL; 5 ms rise/fall time). During a continuous background noise (60 dB SPL white noise), prepulses were presented 30 or 100 ms (i.e., the inter-stimulus interval, ISI) before the startle stimulus for maximum prepulse inhibition (Graham, 1975; Graham & Murray, 1977; Hoffman & Ison, 1980; Ison, McAdam, & Hammond, 1973; Pinnock et al., 2015; Typlt et al., 2013; Valsamis & Schmid, 2011; Zaman et al., 2017). In total, the animals were randomly presented 10 trials of each stimulus type (startle alone; 75 dB at 30 ms ISI; 75 dB at 100 ms ISI, 85 dB at 30 ms ISI, and 85 dB at 100 ms ISI), with the trials separated by a randomly varying ITIs (12, 15 or 18 s; Fig. 3-7A). The relative percentage of PPI was calculated using the maximum startle amplitudes as follows:

$$\% \text{ PPI} = \left(1 - \left(\frac{\text{prepulse pulse}}{\text{pulse alone}}\right)\right) \times 100\%$$

In addition to measuring changes in startle magnitude to assess PPI, the latency of the startle response was also measured in trials with/without the prepulse as an increased latency to the maximum startle amplitude is indicative of sensorimotor gating (Hoffman & L, 1970; Ison et al., 1973). The relative changes in latency were calculated as the time to reach the maximum startle magnitude on startle pulse alone trials subtracted from that during prepulse trials (i.e., positive values represented an increase in latency on prepulse trials; Lyall et al., 2009; Marriott et al., 2016; Fig. 3-8A). The amplitude and latency measures of PPI from days two and three were grouped based on trial type, and averaged to obtain a single value for all five prepulse conditions per animal. Ultimately, to assess the developmental changes in sensorimotor gating, PPI was measured at P38 and P78 in the three genotypes (wildtype: 10 males, 9 females; *Cntnap2*^{+/-}: 15 males, 16 females; *Cntnap2*^{-/-}: 8 males, 12 females).

3.2.4 Locomotor Activity

Rats (wildtype: 12 males, 10 females; *Cntnap2*^{+/-}: 16 males, 16 females; *Cntnap2*^{-/-}: 8 males, 12 females) were tested at P38 and P78. Locomotor testing took place on day two at least 1 h prior to acoustic startle testing (described above) in a dimly lit room to which the animals were acclimated. Rats were placed in a 20 cm x 20 cm locomotor box (Versamax, Columbia, OH, USA) to freely explore for 20 minutes. Total distance travelled (m) and velocity (m/s) were used as measures of hyperactivity, and the proportion of time spent in center of the locomotor box was used as an index of anxiety. Locomotor data were tabulated, parsed into 5-minute blocks for each rat, and then averaged for the respective experimental groups.

3.2.5 Immunohistochemistry

Wildtype male animals at ages P28 (n = 3), P42 (n = 3), and P70 (n = 3) were euthanized by i.p. injections of an overdose of sodium pentobarbital (Euthanyl Forte: Bimeda-MTC Animal Health Inc. Cambridge, ON, CAN) and intracardially perfused with 0.9 % saline followed by 4% paraformaldehyde (PFA). The brains were harvested, post fixed in PFA for one hour and stored in 30% sucrose until sliced into 40 μ m slices using a freezing

microtome (KS34S, Thermo Fischer Scientific, Waltham, MA, USA). Slices were divided into 4 parallel series and stored at -20°C in cryoprotectant solution (30% sucrose, 30% ethylene glycol, and 5% of 0.01% sodium azide in 0.1M Phosphate Buffer (PB)). To assess the expression of CASPR2, the *Cntnap2* gene protein product, immunolabeling was carried out on free-floating tissue sections. Prior to free-floating immunohistochemistry, as well as in between all incubations with antibodies, all slices were thoroughly rinsed in 0.1M Phosphate Buffered Saline (PBS). Slices were pre-treated with a 1% H_2O_2 in 0.1M PBS for 10 min, then blocked for 1 hour in 10% Normal Goat Serum (NGS) (ThermoFisher Scientific # 50197Z) before incubation with primary antibody overnight (Anti-Caspr2, clone K67/25(1:100, mouse, EMD Millipore, Tamecula, CA, USA) in a solution of 0.1M PBS with 1% NGS. Next, sections were incubated with biotinylated secondary antibody (1:500; anti-mouse, Vector, AB_2336171, MJS BioLynx, Brockville, ON)) in 1% NGS solution for 1 hour at room temperature. Sections were then processed using Avidin-Biotin Complex solution in PBS (1 hour at room temperature, 1:1000; Vectastain Elite ABC Kit, pk 6100 (AB_2336819), and labeling was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (0.04% H_2O_2 , 0.2mg/ml DAB; D4293, Sigma-Aldrich, Oakville, ON, CAN)). Tissue was then mounted onto positively-charged glass slides, dried overnight, dehydrated in increasing concentration of alcohol, cleared in xylene, and cover-slipped with DPX mounting medium (EMD Millipore, HX55746679). Imaging was performed using a Nikon Eclipse Ni-U upright microscope with a DS-Qi2 high definition color camera and imaging software NIS Elements Colour Camera (Nikon Instruments, Melville, NY, USA).

3.2.6 Experimental Design and Statistical Analysis

The main objective of the present study was to investigate the developmental trajectory of auditory deficits in electrophysiological and behavioural measures of brainstem function in juvenile, adolescent and adult *Cntnap2* homozygous (*Cntnap2*^{-/-}) and heterozygous knockout (*Cntnap2*^{+/-}) and wildtype rats of both sexes. Therefore, electrophysiological and behavioural testing was performed on at least seven rats from each genotype and sex at P28, P42 and P70 (electrophysiology) or at P38 and P78 (behaviour). Various types of

split-plot randomized complete block designs with repeated measures were used (Altman & Krzywinski, 2015). To compare differences between genotypes across age for all experiments, general linear model repeated measures (GLM RM) analyses (3- or 4-way factorial design, with multiple within-subject and between-subject variables) were performed using a univariate model approach. More specifically, age (P28, P42 and P70; P38 and P78) and, in some experiments, stimulus type (various levels), were included as within-subject factors, whereas genotype (*Cntnap2*^{-/-}, *Cntnap2*^{+/-} and wildtype) and sex (male and female) represented the between-subject factors. The Mauchly test was used to determine whether the data violated the sphericity assumption. In the case of a violation, the degrees of freedom were corrected using the Greenhouse Geisser (if $\epsilon < 0.75$) or the Huynh-Feldt method (if $\epsilon > 0.75$). Main effects and interactions were assessed, followed by *post hoc* simple-main effect analysis for the overall effect of genotype, and t-tests using the Bonferroni correction to further investigate specific differences between wildtype animals and either *Cntnap2*^{-/-} or *Cntnap2*^{+/-} rats at a given age. Differences were considered statistically significant when p-values (adjusted) were smaller than $\alpha = 0.05$. In all experiments, interactions involving genotype or a main effect of genotype were of utmost interest. For most measures collected, heterozygous rats did not differ significantly from wildtypes, and thus, they were not included in figures. Main effects of sex are reported in text, but data is presented collapsed across sex since no genotype x sex interaction is present. Data analyses were performed with Microsoft Excel 2010 (Microsoft Corp.), and graphical display was completed with GraphPad (Prism 6.01, GraphPad Software, La Jolla, CA, USA) and Inkscape (Inkscape 0.92.1). SAS/STAT was used for statistical analysis (SAS Institute, Version 9.4).

3.3 Results

3.3.1 Animals

Prior to the ABR testing, rats were weighed, and the differences in body mass was analyzed (age \times sex \times genotype). A three-way interaction was observed ($F_{2,9,81.3} = 3.92$, $p = 0.005$), and a simple-main effect analysis for genotype revealed that only males' body mass differed between genotypes at P42 ($F_{2,110} = 18.3$, $p < 0.0001$, $\eta^2 = 0.25$) and P70 ($F_{2,110} =$

39.0, $p < 0.0001$, $\eta^2 = 0.42$). Ultimately, *post hoc* t-tests revealed that *Cntnap2*^{-/-} males had a lower body mass than wildtypes in adolescence (P42, wildtype: 232.2 ± 3.17 g; *Cntnap2*^{-/-}: 205.6 ± 3.47 g; $p < 0.0001$) and adulthood (P70, wildtype: 447.1 ± 3.17 g; *Cntnap2*^{-/-}: 412.5 ± 3.47 g; $p < 0.0001$). In contrast, the body mass of *Cntnap2*^{+/-} rats did not differ from wildtypes (data not shown).

3.3.2 Auditory Brainstem Responses—Hearing Thresholds

To determine whether hearing sensitivity throughout development differed between genotypes, the ABR thresholds to a click, 4 kHz, and 20 kHz stimulus were compared at P28, P42 and P70 (age \times sex \times sound type \times genotype; Fig. 3-2). This analysis revealed a significant interaction of sound type \times genotype ($F_{4,110} = 3.41$, $p = 0.011$), a main effect of age ($F_{2,110} = 3.25$, $p = 0.043$) and no effect of sex ($F_{1,55} = 1.07$, $p = 0.31$). Surprisingly, *post hoc* tests revealed that heterozygous (*Cntnap2*^{+/-}) rats had a slightly lower hearing threshold (i.e., better hearing sensitivity) to the 20 kHz stimulus in adulthood compared to wildtypes ($p < 0.001$; data not shown). In contrast, the ABR thresholds for the three stimuli (i.e., click, 4 kHz and 20 kHz) were not significantly different between *Cntnap2*^{-/-} rats versus wildtypes at the three age groups tested (P28, P42 and P70; Fig. 3-2), indicating that the homozygous deletion of *Cntnap2* did not affect hearing levels throughout development.

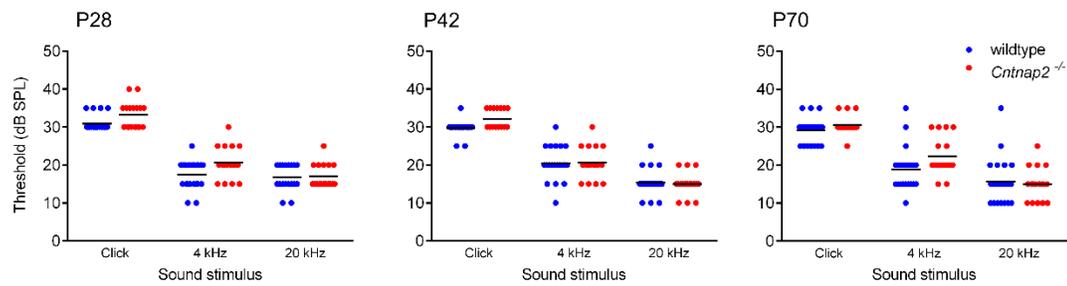


Figure 3-2: *Cntnap2*^{-/-} rats have typical hearing thresholds.

Scatter plot of click, 4 kHz and 20 kHz stimulus thresholds for individual wildtype (blue) and *Cntnap2*^{-/-} (red) animals as juveniles (P28), adolescents (P42), and adults (P70). Mean is represented by a horizontal line. ABR thresholds showed no differences between wildtype and knockout animals for all stimuli tested.

3.3.3 Auditory Brainstem Responses—Peak Amplitudes & Latencies.

The rodent ABR consists of four prominent waves, which are thought to represent synchronized neural activity in the auditory nerve, cochlear nucleus, superior olivary complex and lateral lemniscus, respectively (Alvarado et al., 2012; Church & Kaltenbach, 1993; Popelar et al., 2008). In humans, the ABR waves approximating the auditory nerve (wave I), cochlear nucleus (wave III), and lateral lemniscus (wave V)—corresponding to the rat waves I, II and IV—are of importance when studying individuals on the autism spectrum (Wong and Wong, 1991; Rosenhall et al., 2003; Kwon et al., 2007; Tas et al., 2007; Fujikawa-Brooks et al., 2010; Magliaro et al., 2010; Gonçalves et al., 2011; Picton, 2011; Roth et al., 2012; Miron et al., 2016). Therefore, using data derived from the 90 dB SPL click stimulus, we analyzed the peak amplitude of waves I and IV (age × sex × peak number × genotype), absolute latency of waves I through IV (age × sex × peak number × genotype), and inter-peak latencies (IPLs) between waves I and II (I-II IPL) as well as II and IV (II-IV IPL, age × sex × genotype). In addition, wave III peak amplitudes were assessed since they visually appeared to differ between genotypes.

When comparing neural response of ABR waves I and IV, a three-way interaction was found between age × peak number × genotype ($F_{4,110} = 5.76$, $p = 0.0003$), suggesting differential central gain changes over development between the genotypes. A main effect

of sex ($F_{1,55} = 9.44$, $p = 0.003$) was also found. Subsequently, a significant genotype \times age interaction was found for both peak I and IV amplitude (peak I: $F_{8,110} = 15.5$, $p < 0.0001$, $\eta^2 = 0.53$; peak IV: $F_{8,110} = 40.5$, $p < 0.0001$, $\eta^2 = 0.75$). Taken further, the simple main effect of genotype followed by post-hoc t-tests revealed the effect of *Cntnap2* on ABR peak amplitudes throughout development. Indeed, the wave IV peak amplitudes, but not wave I peak amplitudes, were smaller in *Cntnap2*^{-/-} rats compared to wildtypes at all ages (P28: $p < 0.0001$, P42: $p < 0.0001$, P70: $p = 0.023$, Fig. 3-3). This reduction of the wave IV peak amplitude in the homozygous knockout rats was not sex-dependent, as there was no genotype \times sex interaction ($F_{2,55} = 1.04$, $p = 0.36$). Interestingly, this reduction of wave IV peak amplitude in the *Cntnap2*^{-/-} rats decreased as they aged, as evidenced by the smaller effect size found in adults (P70: $F_{2,110} = 13.2$, $p < 0.0001$, $\eta^2 = 0.19$) compared to adolescents (P42: $F_{2,110} = 37.7$, $p < 0.0001$, $\eta^2 = 0.41$) and juveniles (P28: $F_{2,110} = 72.5$, $p < 0.0001$, $\eta^2 = 0.57$). This lower genotype effect observed with age was also reflected in the ratio of peak IV/peak I amplitude, where the ratio in the *Cntnap2*^{-/-} rats approached that of the wildtypes in adulthood (Fig. 3-3), indicating a normalization of ABR amplitudes upon adulthood. ABR peak amplitudes in *Cntnap2*^{+/-} rats did not differ from the wildtypes (data not shown).

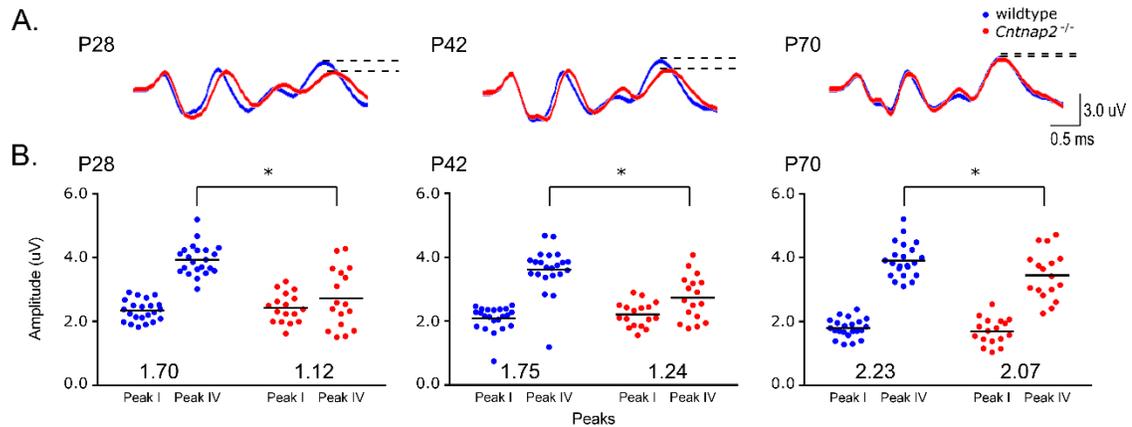


Figure 3-3: *Cntnap2*^{-/-} rats exhibit a region-specific reduction in neural responsivity in the auditory brainstem across development

(A) Raw averaged ABR waveforms of wildtype (blue) and *Cntnap2*^{-/-} (red) animals to a 90 dB click stimulus. Dashed lines depict the difference in wave IV amplitude between genotypes. (B) Scatter plot of absolute peak amplitudes of waves I and IV of the evoked response at P28, P42, and P70. Individual data are plotted and means are represented by a horizontal line. *Cntnap2*^{-/-} rats show a persistent reduction in the amplitude of wave IV, representing activity of neurons in the lateral lemniscus terminating at the inferior colliculus. The peak IV:I ratio is presented above the x-axis to provide an indication of central gain change across age, illustrating the near recovery of *Cntnap2*^{-/-} rats wave IV amplitude through development.

Though the wave representing the superior olive is not often assessed in human ASD literature, we observed wave III peak amplitude differences, and therefore included them in our analysis. A significant genotype \times age interaction was found ($F_{4,110} = 4.38$, $p = 0.003$), with no effect of sex ($F_{1, 55} = 0.46$, $p = 0.5$). The simple main effect of genotype followed by post-hoc t-tests (see Table 3-1) revealed that the loss of *Cntnap2* affected peak III amplitudes throughout development (P28: $F_{2,110} = 36.8$, $p < 0.0001$, $\eta p^2 = 0.40$; P42: $F_{2,110} = 32.7$, $p < 0.0001$, $\eta p^2 = 0.37$; P70: $F_{2,110} = 14.3$, $p < 0.0001$, $\eta p^2 = 0.21$).

Table 3-1: Statistical table for ABR wave III peak amplitudes and corrected *post hoc t-test*. Amplitudes are presented in microvolts relative to baseline. t test for a given age compare either the heterozygous knockout or homozygous knockout animals with wild-types. Heterozygous *Cntnap2^{+/-}* are different from wild-types in young ages, but not in adulthood, whereas differences from wild-types persist in homozygous *Cntnap2^{-/-}*; suggesting a potential gene-dose effect.

Genotype	Age	Mean (μ V)	SEM (μ V)	<i>p</i> -value
Wildtype	28	0.58	0.065	---
<i>Cntnap2^{+/-}</i>	28	1.21	0.065	< 0.0001
<i>Cntnap2^{-/-}</i>	28	1.35	0.075	< 0.0001
Wildtype	42	0.10	0.065	---
<i>Cntnap2^{+/-}</i>	42	0.46	0.065	0.008
<i>Cntnap2^{-/-}</i>	42	0.91	0.075	< 0.0001
Wildtype	70	-0.33	0.065	---
<i>Cntnap2^{+/-}</i>	70	-0.21	0.065	---
<i>Cntnap2^{-/-}</i>	70	-0.18	0.075	< 0.0001

In addition to the amplitude of the ABR waves, the latency to reach each of the prominent peaks was analyzed (age \times sex \times peak number \times genotype). We observed a genotype effect on peak latency that was dependent on both age and peak number (three-way interaction between peak \times age \times genotype; $F_{6.5, 181} = 5.52$, $p < 0.0001$), but there was no main effect of sex ($F_{1,55} = 0.25$, $p = 0.622$). As evidenced with the effect size calculations, the influence of genotype on the peak latencies appeared to be compounded in the ABR trace such that the later waves showed greater and more persistent slowing across age than the earlier waves (age \times genotype interaction, peak I: $F_{8, 330} = 11.4$, $p < 0.0001$, $\eta^2 = 0.22$; peak II: $F_{8, 330} = 74.6$, $p < 0.0001$, $\eta^2 = 0.64$; peak III: $F_{8, 330} = 209.8$, $p < 0.0001$, $\eta^2 = 0.84$; peak IV: $F_{8, 330} = 338.6$, $p < 0.0001$, $\eta^2 = 0.89$). Put simply, the genotype effect on latency was most prominent in young animals at the later-occurring peaks, and this effect lessened with age (see Table 3-2).

Table 3-2: Statistical table for the simple-main effect of genotype on ABR peak latency for a given peak and age. Effect sizes are greater in the later peaks and overall decrease with age. Degrees of freedom: 2; error degrees of freedom: 330.

Peak	Age	F-statistic	p-value	Effect size
1	28	4.42	0.010	.03
	42	2.41	0.090	---
	70	6.55	0.002	.04
2	28	178.25	<0.0001	.32
	42	9.09	0.0001	.05
	70	3.51	0.03	.02
3	28	173.63	<0.0001	.51
	42	43.95	<0.0001	.21
	70	4.64	0.010	.03
4	28	262.98	<0.0001	.61
	42	44.43	<0.0001	.21
	70	1.85	0.160	---

As shown in figure 3-4A and B, post-hoc t-tests revealed *Cntnap2*^{-/-} rats, in particular, had increased peak latencies compared to wildtypes when young (peak II, P28: $p < 0.0001$; peak II, P42: $p = 0.02$; peak III, P28: $p < 0.0001$; peak III, P42: $p < 0.0001$; peak IV, P28: $p < 0.0001$; peak IV, P42: $p < 0.0001$), which disappeared upon maturation so that there were no longer any latency differences in adulthood. Finally, this delayed maturation of the speed of neurotransmission throughout the auditory brainstem was only observed in the homozygous knockout rats, ABR peak latencies were not increased in the *Cntnap2*^{+/-} rats. In fact, compared to wildtypes, the *Cntnap2*^{+/-} rats showed modestly shorter latencies (i.e., faster neurotransmission) for ABR waves III and IV as juveniles (P28, $p < .0001$; data not shown).

The effect of the loss of *Cntnap2* on the inter-peak latencies (IPL) between wave I-II and II-IV were examined separately (age \times sex \times genotype). Age was found to influence the effect of genotype ($F_{4,110} = 5.40$, $p = 0.0005$) such that the strength of the genotype effect on I-II IPL decreased with age (P28: $F_{2,110} = 67.0$, $p < 0.0001$, $\eta^2 = 0.55$; P42: $F_{2,110} = 23.9$, $p < 0.0001$, $\eta^2 = 0.30$; P70: $F_{2,110} = 17.0$, $p < 0.0001$, $\eta^2 = 0.24$) with no main effect of sex ($F_{1,55} = 3.46$, $p = 0.07$). Whereas the *Cntnap2*^{+/-} rats were again not different from wildtypes (data not shown), the *Cntnap2*^{-/-} rats had a longer wave I-II IPL than

wildtypes at all ages (P28: $p < 0.0001$; P42: $p < 0.0001$; P70: $p < 0.0001$; Fig. 3-4C). Similarly, an age \times genotype interaction was found for IPL II-IV ($F_{4,110} = 6.45$, $p = 0.0001$), with post hoc tests revealing that *Cntnap2*^{-/-} animals had a significantly longer IPL at only P28 ($p = 0.0096$, Fig. 3-4D). Interestingly, *Cntnap2*^{+/-} animals had a slightly shorter IPL than wildtypes at P28 ($p = 0.002$; data not shown).

3.3.4 Acoustic reactivity

To assess acoustic reactivity throughout development, startle response magnitudes to a series of startle pulses of increasing volume (65 to 115 dB in 5 dB SPL increments) were measured and analyzed (age \times sex \times startle pulse level \times genotype). All of the three-way interactions involving genotype were found to be significant (age \times startle pulse level \times genotype: $F_{13,37,454.44} = 2.35$, $p = 0.004$; sex \times startle pulse level \times genotype: $F_{10,20,373.73} = 2.35$, $p = 0.008$; age \times sex \times genotype: $F_{1,68} = 3.23$, $p = 0.046$). Since an animal's body mass can affect its startle response magnitude (and body mass was found to vary with age and sex in the present study), we subsequently analyzed the simple-main effect of genotype for males and females at both ages P38 and P78. Collapsing across startle pulse level ultimately revealed genotype differences in adulthood for both females ($F_{2,68} = 8.46$, $p = 0.0005$, $\eta^2 = 0.20$) and males ($F_{2,68} = 27.4$, $p < 0.0001$, $\eta^2 = 0.45$; Fig. 3-5B). To summarize the results, *Cntnap2*^{-/-} rats of both sexes showed increased acoustic reactivity compared to wildtypes (female: $p = 0.007$; male: $p < 0.0001$), which could be visualized as a leftward shift in the relation between startle response magnitude and intensity; Fig. 3-5. Interestingly, this increased acoustic reactivity in the *Cntnap2*^{-/-} rats became more robust as animals aged, evidenced by the increased effect size of the startle pulse level \times genotype interaction found in adulthood compared to adolescence for both females (P72: $F_{32,680} = 37.7$, $p < 0.0001$, $\eta^2 = 0.64$; P38: $F_{32,680} = 16.0$, $p < 0.0001$, $\eta^2 = 0.43$) and males (P72: $F_{32,680} = 58.3$, $p < 0.0001$, $\eta^2 = 0.73$; P38: $F_{32,680} = 17.6$, $p < 0.0001$, $\eta^2 = 0.45$). Most notably, adult male *Cntnap2*^{-/-} rats showed a considerable increase in acoustic reactivity compared to wildtypes at moderately loud sound intensities of 85 dB SPL ($p < 0.0001$), 90 dB SPL ($p < 0.0001$) and 95 dB SPL ($p = .019$; Fig. 3-5B). *Cntnap2*^{+/-} rats did not differ from wildtypes in acoustic reactivity (data not shown).

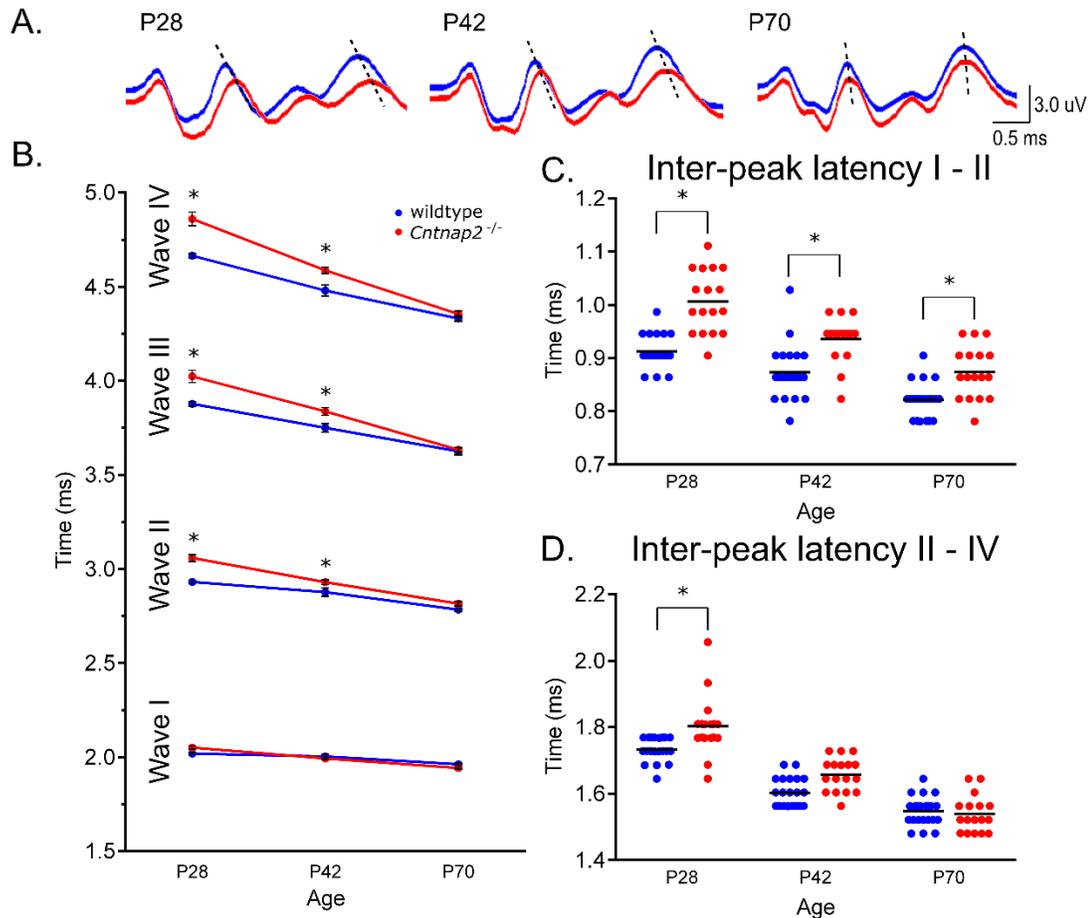


Figure 3-4: *Cntnap2*^{-/-} rats exhibit reduced brainstem neurotransmission speed when young, which matures by adulthood.

(A) Raw averaged traces of the ABR waveform to a 90 dB SPL click stimulus for wildtype (blue) and *Cntnap2*^{-/-} (red) animals at P28, P42 and P70. Dashed lines highlight the delay in response latency. (B) Averaged absolute peak latency from stimulus onset for waves I through IV at P28, P42, and P70 for wildtype and *Cntnap2*^{-/-} animals plotted as mean ± standard error. While wave I representing the auditory nerve shows no significant latency deficit, the later occurring waves II, III, and IV show a compounding delay in response time which normalizes with age. (C & D) Individual data are plotted and mean is represented by a horizontal line for inter-peak latency I-II and IPL II-IV. *Cntnap2*^{-/-} rats show a prolonged IPL I-II that only partially recovers with age, whereas the IPL II-IV delay fully recovers with age.

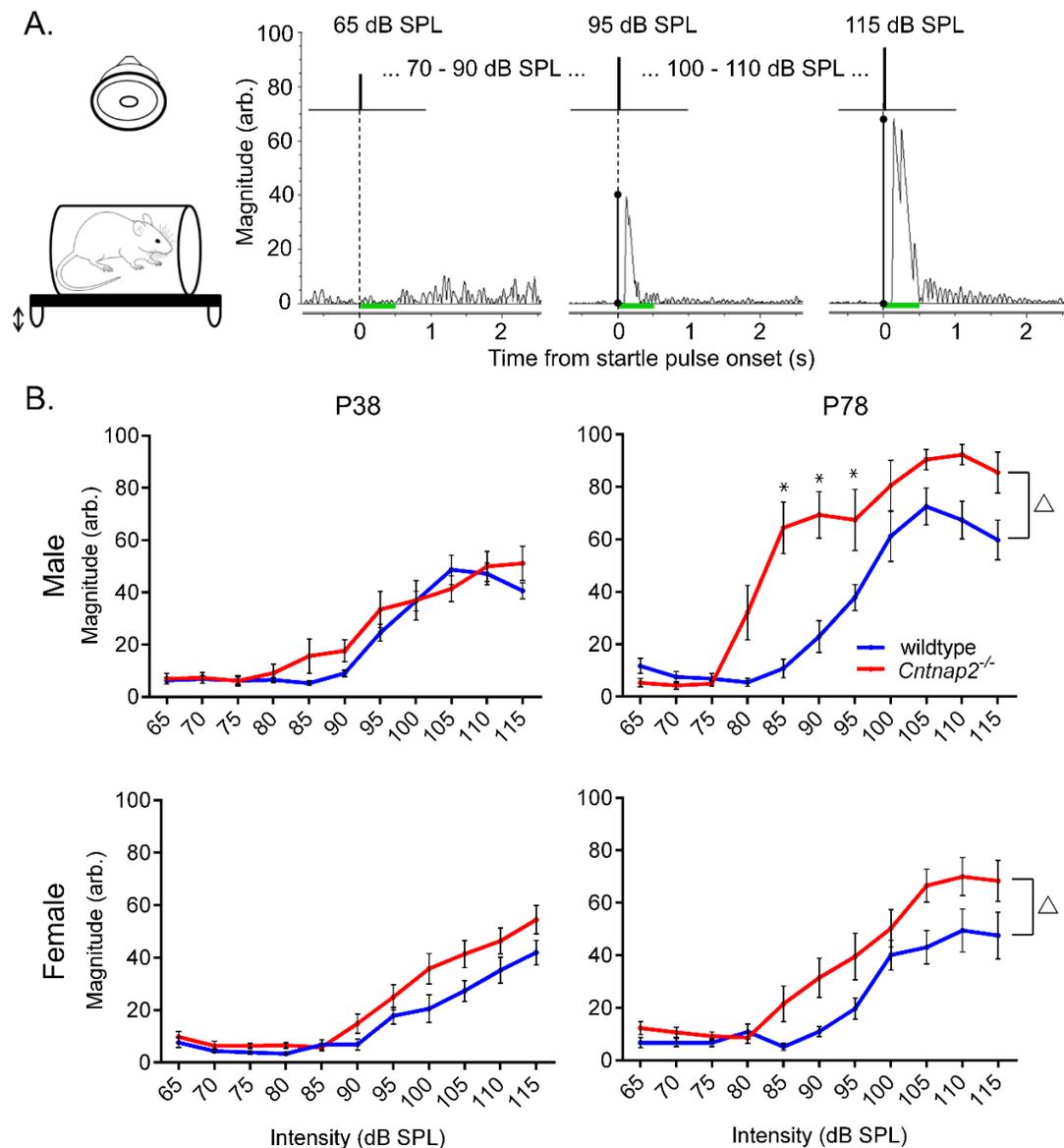


Figure 3-5: Acoustic reactivity is increased in adult *Cntnap2*^{-/-} rats compared to wildtypes.

(A) Positioning of rat in startle tube apparatus set on a movement sensitive platform with an overhead speaker emitting the acoustic startle stimulus (left). Right diagram depicts three representative raw acoustic startle stimuli (65, 95, and 115 dB SPL) and corresponding acoustic startle response (ASR) traces from an adult male wildtype rat as measured by the STARTLE software module. Black bars denote the ASR magnitude and green bars indicate the 500 ms ASR recording window. (B) Male (top) and female (bottom) animals' startle response magnitude to decibel levels from 65 dB SPL to 115 dB SPL in wildtype (blue) and *Cntnap2*^{-/-} (red) animals at P38 (left) and P78 (right) plotted as mean \pm standard error. Young *Cntnap2*^{-/-} animals are no different from wildtypes; however, a significant acoustic startle reactivity deficit appears in adult

3.3.5 Habituation

.To assess sensory filtering, short-term habituation of the startle response was measured across the first eight startle trials of the test day and analyzed (age \times sex \times genotype). A main effect of genotype was found at P38 ($F_{2,70} = 5.25$, $p = 0.008$), in which adolescent *Cntnap2*^{-/-} rats habituated significantly less than wildtype animals ($p = 0.011$; Fig. 3-6B). The extent of short-term habituation was further quantified by normalizing the average of the last two startle responses of each animal to its initial startle response to calculate a habituation score. A main effect of genotype was found for the habituation score ($F_{2,70} = 5.50$, $p = 0.006$), with post hoc t-tests revealing a significant difference only between *Cntnap2*^{-/-} and wildtype rats at P38 ($p = 0.012$; Fig. 3-6C). No differences were found between genotypes at P78 in habituation of startle magnitude across trials ($F_{2,64} = 0.83$, $p = 0.441$; Fig. 3-6B) or in the habituation score ($F_{2,64} = 0.09$, $p = 0.911$, Fig. 3-6C). Thus, sensory filtering was only impaired in young *Cntnap2*^{-/-} rats.

3.3.6 Prepulse inhibition

The effect of *Cntnap2* knockout on sensorimotor gating throughout development was assessed using prepulse inhibition of startle. The relative amount of prepulse inhibition (%PPI) elicited by two prepulse stimulus levels at two different ISIs was analyzed (prepulse type \times age \times genotype \times sex). This analysis revealed a four-way interaction ($F_{5.3,169.6} = 2.24$, $p = 0.049$), with genotype interacting with prepulse type and age, but not sex (prepulse type \times genotype: $F_{4.85,155.06} = 9.73$, $p < 0.0001$; age \times genotype: $F_{2,64} = 15.4$, $p < 0.0001$; genotype \times sex: $F_{2,64} = 0.80$, $p = 0.453$). These significant interactions were further explored by collapsing across sex to examine the effect of genotype across age for the four different prepulse types (i.e., 75 dB at 30 ms ISI, 75 dB at 100 ms ISI, 85 dB at 30 ms ISI, and 85 dB at 100 ms ISI). Using analyses of simple-main effects, it was found that the genotypes differed for the majority of the prepulse types used in the present study, with increased effect sizes in adulthood (Table 3-3). Ultimately, post hoc t-tests

Figure 3-5 continued... males and females. A leftward shift of the I/O curve is observed in *Cntnap2*^{-/-} rats, indicative of increased reactivity to the acoustic stimuli, is especially apparent at the 85, 90, and 95 dB SPL startle pulses in adult *Cntnap2*^{-/-} males.

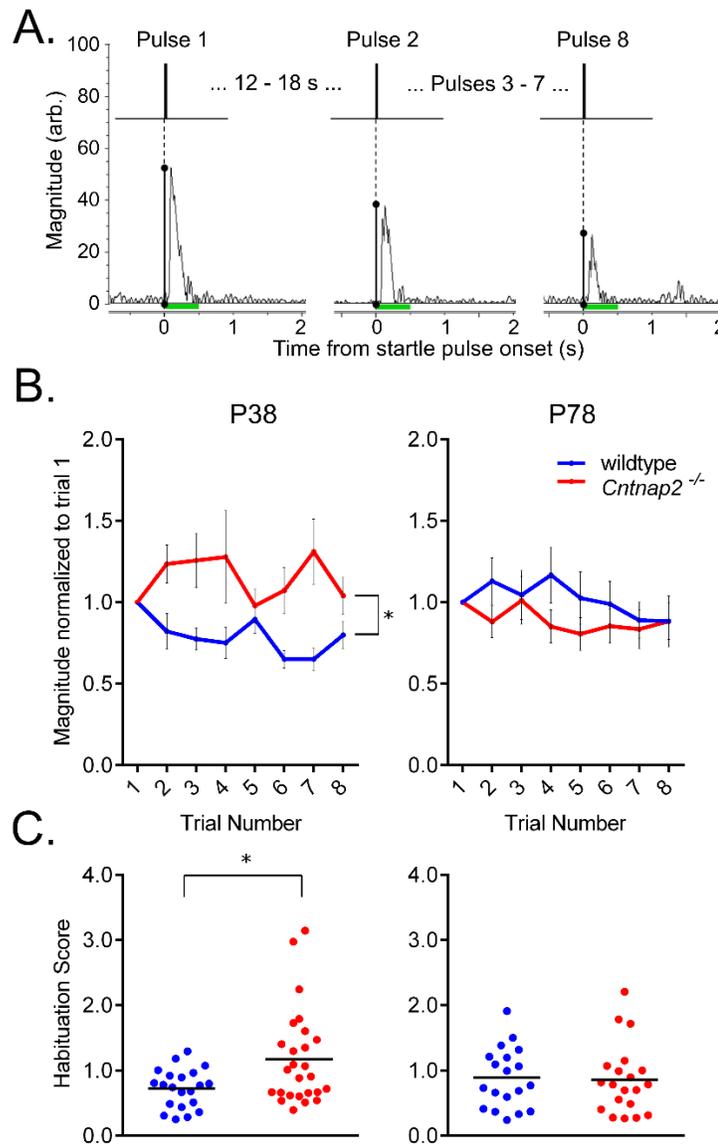


Figure 3-6: Sensory filtering as measured by short-term habituation of the acoustic startle response is perturbed in young knockout rats.

Representative raw acoustic startle response traces from an adult male wildtype rat depicts the decrease in ASR magnitude (black bars) to the repeated presentation of the startle pulse (105 dB SPL) as measured by the STARTLE software module. Green bars indicate the 500 ms ASR recording window. (B) Wildtype (blue) and *Cntnap2*^{-/-} (red) animals' normalized startle response magnitudes at P38 and P78 across eight subsequent trials. Values below 1.0 are indicative of habituation of the startle response. *Cntnap2*^{-/-} animals show no decline in response magnitude at P38. (C) Individual habituation scores, taken as the average of the last 2 trials divided by that of the first, at P38 and P78 of the respective genotypes are displayed with the horizontal line representing the mean score. A score below 1.0 is indicative of habituation of the startle response. *Cntnap2*^{-/-} animals do not habituate across trials compared to wildtype animals during adolescence.

confirmed that the *Cntnap2*^{-/-} rats showed a significant PPI deficit for the 75 dB, 100 ms condition early during development (P38: $p < 0.0001$), and that this deficit extended to all prepulse conditions by adulthood (P78: 75 dB SPL, 30 ms: $p < 0.0001$; 75 dB SPL, 100 ms: $p < 0.0001$; 85 dB SPL, 30 ms: $p = 0.0026$; 85 dB SPL, 100 ms: $p < 0.0001$; Fig. 3-7C). Thus, the homozygous knockout of *Cntnap2* impaired sensorimotor gating as assessed by the relative level of prepulse inhibition during youth, and this impairment worsened with age. *Cntnap2*^{+/-} rats were largely no different from wildtypes, and only showed slightly increased PPI in adulthood at the 85dB, 100ms prepulse condition ($p = 0.018$).

Table 3-3: Statistical table for the simple-main effect of genotype on % prepulse inhibition. Effect sizes for each prepulse stimulus type increased with age. Degrees of freedom: 2; error degrees of freedom: 192.

Prepulse Type	Age	F-statistic	p-value	Effect size
75 dB SPL 30 ms	38	6.65	0.002	0.06
	78	36.7	<0.0001	0.28
75 dB SPL 100 ms	38	35.0	<0.0001	0.27
	78	94.2	<0.0001	0.50
85 dB SPL 30 ms	38	0.29	0.752	---
	78	22.2	<0.0001	0.19
85 dB SPL 100 ms	38	3.43	0.034	0.03
	78	59.6	<0.0001	0.38

As a complement to the assessment of prepulse inhibition, we also examined the change in latency to the maximum startle response in trials with- versus without a prepulse. While there was no interaction of all four factors (prepulse type \times age \times genotype \times sex), there was a significant three-way interaction between prepulse type, age and genotype ($F_{6,192} = 2.47$, $p = 0.025$). As shown in Table 3-4, there was a simple-main effect of genotype for most of the prepulse types; however, there were no clear trends in the effect size across age. Overall, post hoc tests confirmed that, compared to wildtypes, *Cntnap2*^{-/-} rats' startle latencies did not increase in trials that included a prepulse; findings indicative of impaired sensorimotor gating. This lack of latency increase was apparent during the 75 dB, 30ms prepulse trials in the young *Cntnap2*^{-/-} rats ($p = 0.001$). Moreover, a persistent deficit was observed in both 85 dB conditions in the young and adult *Cntnap2*^{-/-} rats (85 dB SPL, 30

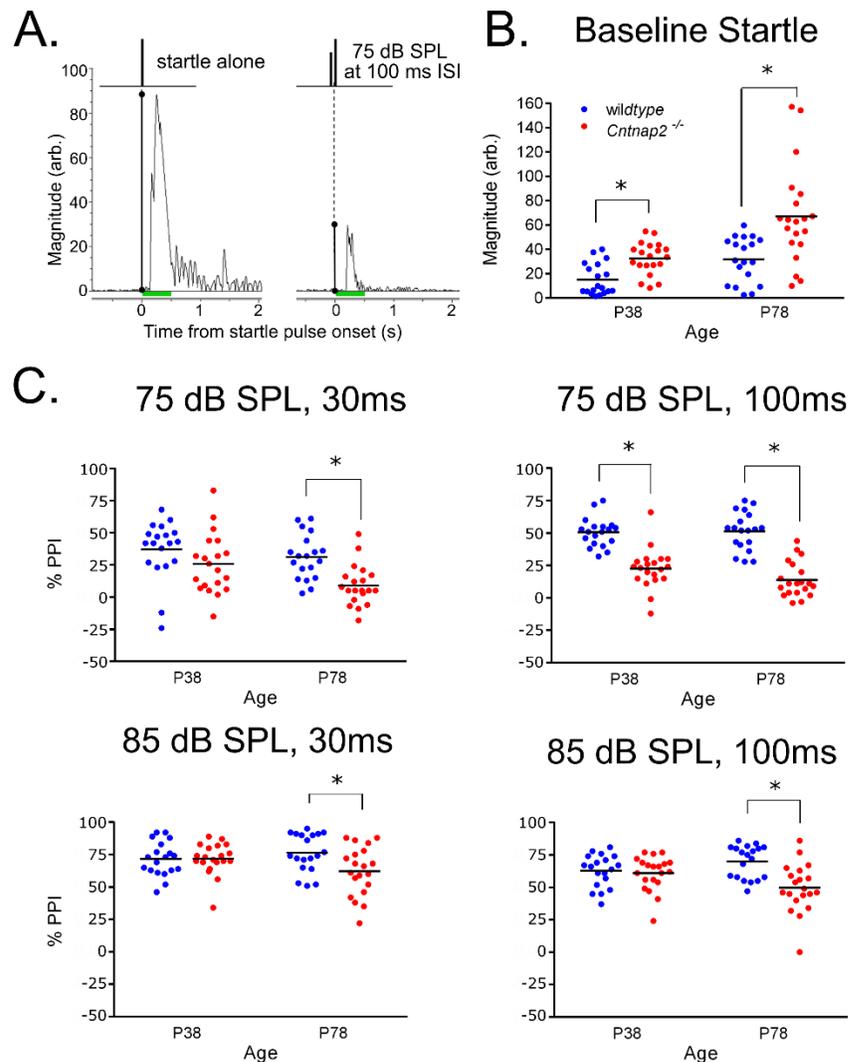


Figure 3-7: Sensorimotor gating measured as percent prepulse inhibition revealed a gating deficit that worsens with age.

(A) Representative raw acoustic startle response traces from an adult male wildtype rat depicts the acoustic startle response magnitude (black bars) to a startle pulse (105 dB SPL) alone or preceded by a prepulse stimulus (75 dB SPL, 100 ms ISI) as measured by the STARTLE software module. Green bars indicate the 500 ms ASR recording window. (B) Since startle reactivity can affect sensorimotor gating (Csomor et al., 2008), differences in baseline startle magnitude were calculated using the startle only trials during PPI blocks and analyzed (Age \times Genotype \times Sex). A three-way interaction was found ($F_{2,64} = 3.32$, $p = 0.043$). Since sex did not interact with genotype ($F_{2,64} = 2.76$, $p = 0.071$), age ($F_{2,64} = 3.88$, $p = 0.053$), or have a main effect ($F_{2,64} = 3.84$, $p = 0.054$), the simple-main effect of genotype and post hoc t-tests were analyzed at P38 and P78. *Cntnap2*^{-/-} rats (red) had a significantly greater startle response magnitude to the 105dB startle stimulus compared to wildtype rats (blue), despite being the same or less weight (P38, $p = 0.026$; P78, $p < 0.0001$; see above). Scatter plots depict individual data with the horizontal line representing the mean ASR magnitude. *Cntnap2*^{+/-} rats

ms, P38: $p < 0.0001$; 85 dB SPL, 30 ms, P78: $p = 0.018$; 85 dB SPL, 100 ms, P38: $p < 0.0001$; 85 dB SPL, 100 ms, P78: $p < 0.0001$; Fig. 3-8C). Heterozygous (*Cntnap2*^{+/-}) rats were not different from wildtypes in any condition (data not shown).

Table 3-4: Statistical table for the simple-main effect of genotype on the change in latency to maximum startle response for a prepulse stimulus type and age. Degrees of freedom: 2; error degrees of freedom: 192.

Prepulse Type	Age	F-statistic	p-value	Effect size
75 dB SPL 30 ms	38	12.5	<0.0001	0.12
	78	0.58	0.560	---
75 dB SPL 100 ms	38	3.01	0.051	---
	78	8.44	0.0003	0.08
85 dB SPL 30 ms	38	53.1	<0.0001	0.36
	78	19.8	<0.0001	0.17
85 dB SPL 100 ms	38	39.9	<0.0001	0.29
	78	39.5	<0.0001	0.29

3.3.7 Locomotor Activity

To evaluate the effect of *Cntnap2* on locomotion and anxiety-like behaviour, locomotor activity was evaluated in 5-minute time bins for a total of 20 minutes. The distance travelled and velocity were used as measures of activity (time bin \times age \times genotype \times sex). Although there were no four- or three-way interactions found involving the distance travelled, a significant interaction existed between genotype \times time bin ($F_{4,07,138.5} = 5.51$, $p = 0.0003$), as well as a main effect of age ($F_{1,68} = 4.36$, $p = 0.041$). To further explore this interaction, data were collapsed across sex, and the effect of genotype was considered with respect to time bin and age. Consequently, a simple main effect analysis revealed an effect of genotype, but only in the first time bin at both ages (P38: $F_{2,204} = 32.3$, $p < 0.0001$, $\eta^2 = 0.24$; P78: $F_{2,204} = 27.7$, $p < 0.0001$, $\eta^2 = 0.21$). Ultimately, post hoc tests showed

Figure 3-7 continued... did not differ from wildtypes at P38 or P72 (data not shown). (C) Scatter plots depict individual data for each prepulse condition with the horizontal line representing the mean % PPI in wildtype (blue) and *Cntnap2*^{-/-} (red) animals at P38 and P78. *Cntnap2*^{-/-} rats exhibit reduced prepulse inhibition in the 75 dB SLP, 100 ms ISI prepulse condition when young which extends to all conditions upon adulthood, indicative of a sensorimotor gating deficit which worsens with aging.

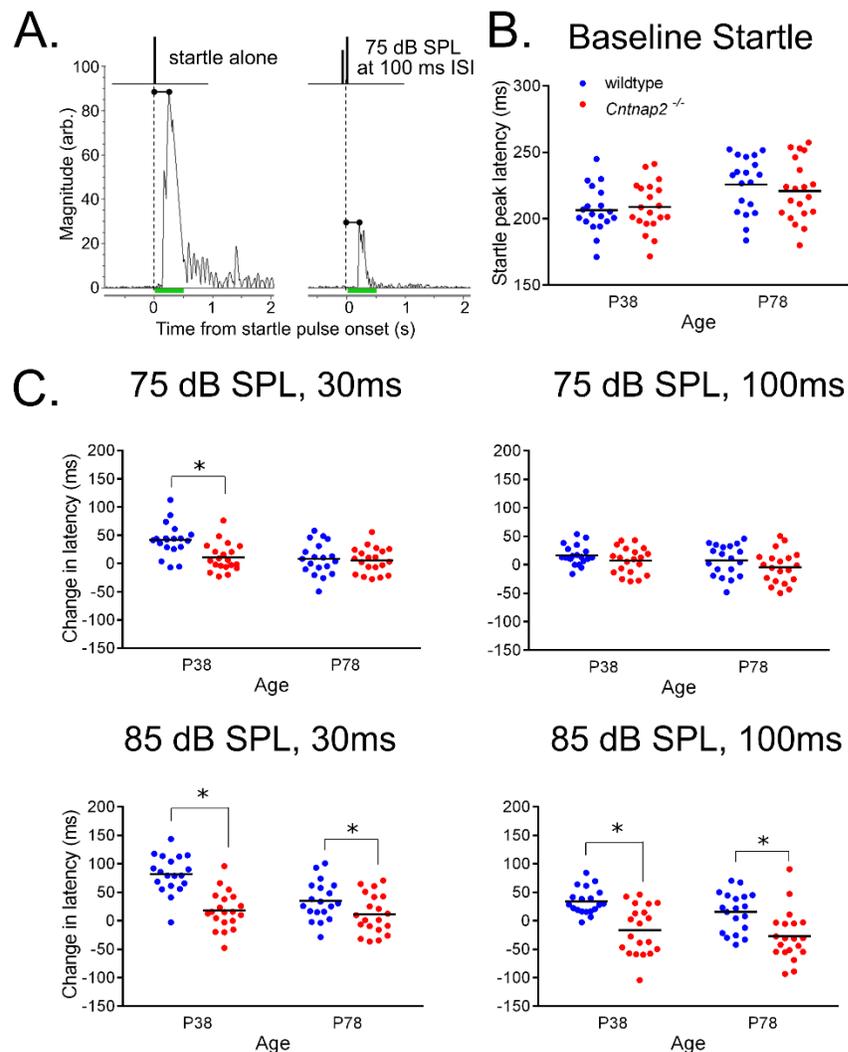


Figure 3-8: Sensorimotor gating measured by the latency to acoustic startle response

(A) Representative raw acoustic startle response traces from an adult male wildtype rat, as measured by the STARTLE software module, depicts the latency to acoustic startle response (black bars) elicited by a startle pulse of 105 dB SPL presented alone or preceded by a prepulse stimulus (75 dB SPL, 100 ms ISI). Green bars indicate the 500 ms ASR recording window. (B) No differences in the latency to baseline ASR peak were found as there was no main effect of genotype ($F_{2,64} = 0.28$, $p = 0.755$), nor any interactions involving genotype. Therefore, any latency effects observed when a prepulse was presented are not confounded by baseline startle differences. Scatter plots depict individual data with the horizontal line representing the mean ASR latency. (C) Values above 1 indicate an increased latency to the acoustic startle response compared to the startle pulse only condition. Scatter plots depict individual data for each prepulse condition with the horizontal line representing the mean latency change. *Cntnap2*^{-/-} rats' (red) ASR latency does not increase to the same degree as wildtype rats' (blue) in the 75 dB, 30 ms prepulse condition at P38 and this deficit can be seen in the 85dB conditions in young and adult animals.

that *Cntnap2*^{-/-} rats initially travelled a significantly greater distance in the first time bin during adolescence (P38: $p < 0.0001$) and adulthood (P72: $p < 0.0001$; Fig. 3-9A), similar to previous findings in this knockout rat (Thomas, Schwartz, Saxe, & Kilduff, 2016). No differences were found between *Cntnap2*^{+/-} and wildtype rats (data not shown).

Similar to the aforementioned results, a significant interaction between genotype and time bin was found for the velocity of movement during the locomotor testing ($F_{4,08,139} = 5.55$, $p = 0.0003$), as well as a main effect of age ($F_{1,68} = 4.50$, $p = 0.038$). Upon further analysis, there was an effect of genotype in the first time bin at both P38 ($F_{2,204} = 32.2$, $p < 0.0001$, $\eta^2 = 0.24$) and P78 ($F_{2,204} = 27.4$, $p < 0.0001$, $\eta^2 = 0.21$), and also in the second time bin at P78 ($F_{2,204} = 8.06$, $p = 0.0004$, $\eta^2 = 0.07$). Ultimately, post hoc tests showed that *Cntnap2*^{-/-} rats had a greater movement velocity than wildtypes in the first time bin at both P38 (wildtype: 0.046 ± 0.001 m/s; *Cntnap2*^{-/-}: 0.056 ± 0.001 m/s; $p < 0.0001$) and P78 (wildtype: 0.036 ± 0.001 m/s; *Cntnap2*^{-/-}: 0.024 ± 0.001 m/s; $p < 0.0001$; data not shown). There were no differences between *Cntnap2*^{+/-} and wildtype rats (data not shown).

The amount of time spent in center of the locomotor box versus the surrounding perimeter (% center) was used as a surrogate measure of anxiety-like behaviour. A main effect of age ($F_{1,68} = 22.90$, $p < 0.0001$) was found, with no main effect of genotype or interactions. There was, however, a trend for an interaction between genotype \times time bin ($F_{6, 204} = 2.03$, $p = 0.064$). We therefore examined the simple-main effect of genotype for young and adult animals in each time bin. In adolescence, the genotypes differed in their spent more time in the center in the last 15 minutes and adults in the last 5 minutes of the 20 min. testing sessions, although the effects were small (Table 3-5), and did not reach significance in corrected t-tests.

Table 3-5: Statistical table for the simple-main effect of genotype on the percent of time spent in the center of a locomotor box for a given time bin and age. Degrees of freedom: 2; error degrees of freedom: 204.

Time bin	Age	F-statistic	p-value	Effect size
0 – 300 ms	38	0.22	0.80	---
	78	1.36	0.26	---
300 – 600 ms	38	7.0	0.001	0.064
	78	0.49	0.61	---
600 – 900 ms	38	3.11	0.047	0.03
	78	1.31	0.27	---
900 – 1200 ms	38	6.23	0.002	0.058
	78	6.89	0.001	0.063

3.3.8 CASPR2 expression

To establish the presence of CASPR2 in the auditory and startle structures of interest, IHC was performed across age in wildtype animals (Fig. 3-10). At all three ages, CASPR2 staining can be observed in the dorsal cochlear nucleus (DCN; -11.04 to -11.28 bregma), the caudal pontine reticular nucleus (PnC; -10.08 to -10.20 bregma), the superior olivary complex (SOC; -10.08 to -10.20 bregma), the cochlear nerve (8n; -10.08 to -10.20 bregma), the ventral cochlear nucleus (VCN; -10.08 to -10.20 bregma), the pedunculopontine tegmental nucleus (PPT; -8.16 to -8.28 bregma), and the dorsal nucleus of the lateral lemniscus (DNLL; -8.16 to -8.28 bregma).

3.4 Discussion

To our knowledge, the present study represents the first systematic longitudinal investigation of brainstem auditory processing and auditory reactivity disruptions in an animal model for ASD with very high construct and face validity. It is also the first report on sensory processing and reactivity of the novel *Cntnap2* knockout rat model. Neural measures of hearing sensitivity, responsivity and speed of transmission, as well as behavioural measures of acoustic reactivity, filtering and sensorimotor gating were assessed to allow a broad understanding of auditory brainstem dysfunction and the behavioural consequences thereof. We found that the homozygous knockout rats have typical hearing sensitivity (threshold), but reduced auditory evoked neural responsivity and

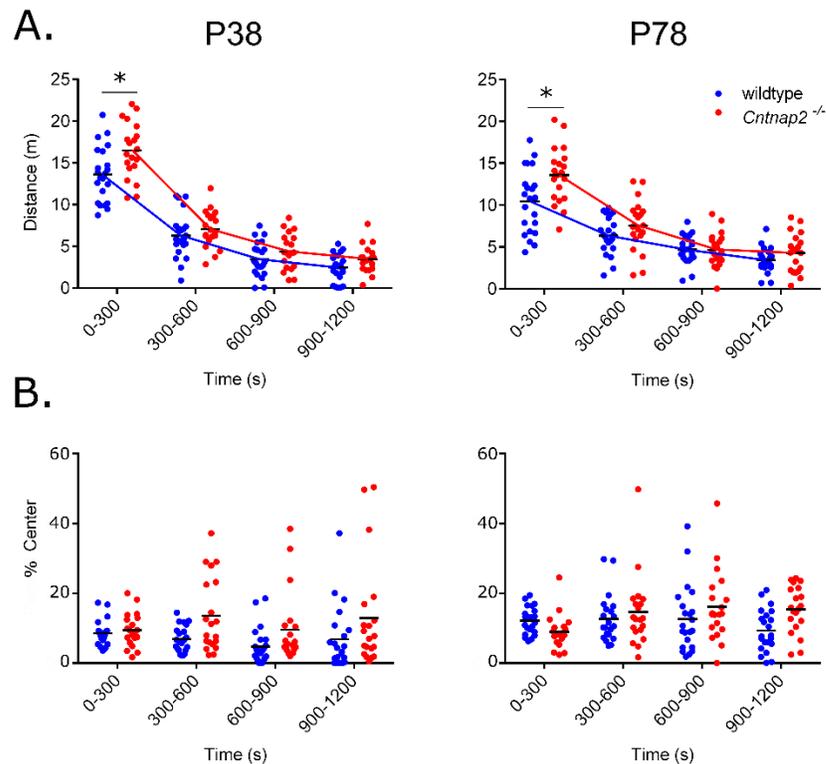


Figure 3-9: Locomotor measures of hyperactivity and anxiety.

(A) Exploratory behaviour was measured using 5 minute bins for a total time of 20 minutes in wildtype (blue) and *Cntnap2*^{-/-} (red) rats at P38 and P78. Scatter plot depicts individual data with horizontal line representing the mean distance traveled. At both P38 and P78 *Cntnap2*^{-/-} rats travel a greater distance in the first 5 minute epoch compared to wildtype rats, indicative of a hyperactive phenotype that does habituate over time. (B) Percent time spent in center was used as a measure of an anxiety-like phenotype. Scatter plot depicts individual data with horizontal line representing the mean distance traveled.

slowed signal transmission throughout different levels of the brainstem in adolescence. Behaviourally, animals showed increased reactivity to acoustic stimuli and disruptions in habituation and prepulse inhibition. Interestingly, the disruptions in auditory signal processing mostly disappeared by adulthood, indicating that they are caused by a delay in maturation of the auditory pathway, while increased behavioural reactivity and disruptions in sensorimotor gating persisted in adulthood. Of importance are also the minor differences in heterozygous knockout animals compared to wildtypes, since humans with disruptions in the *CNTNAP2* gene other than a complete loss-of-function present with minor language

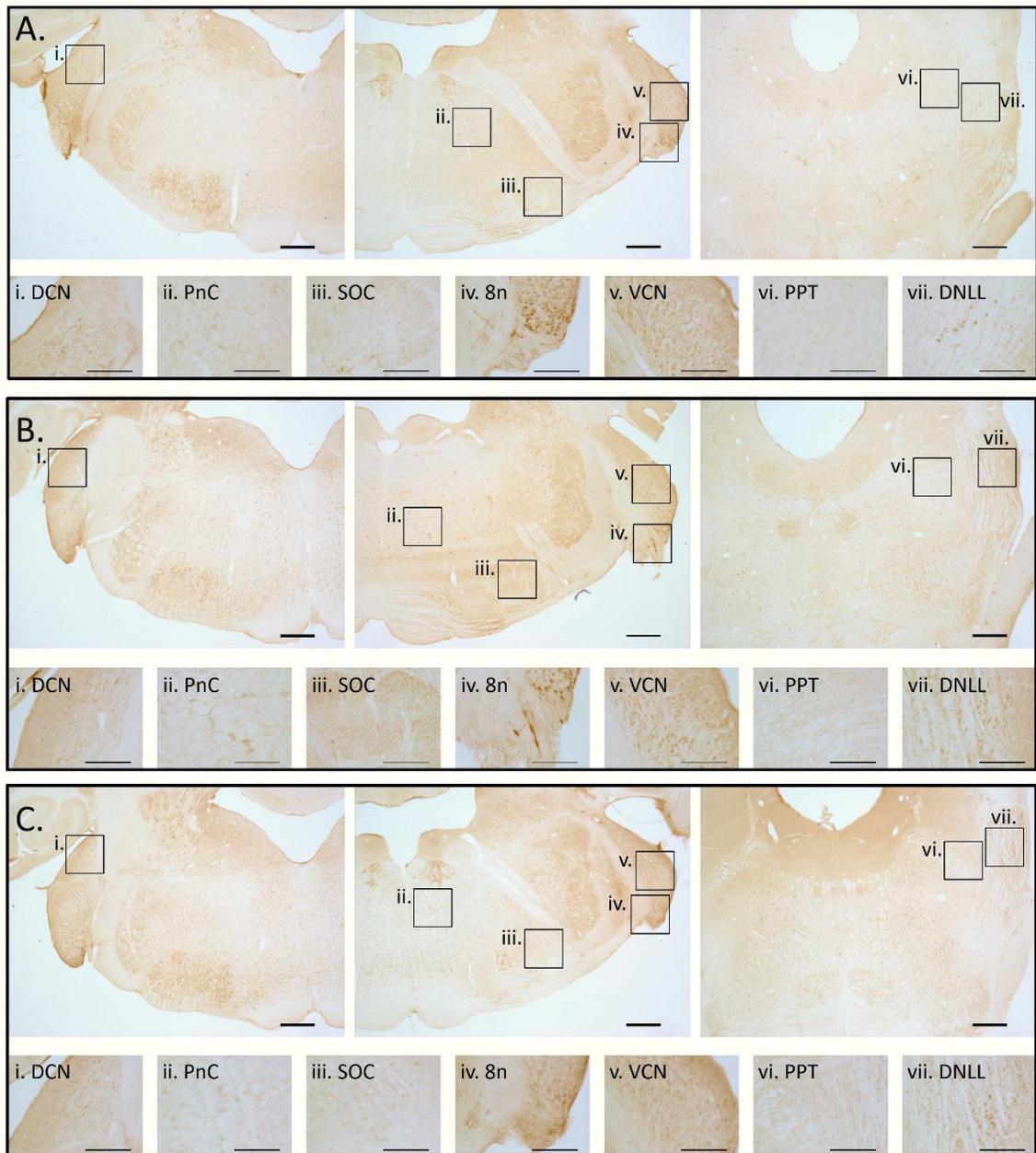


Figure 3-10: CASPR2 expression across development.

Representative images of CASPR2 expression in wildtype rats at P28 (A), P42 (B), and P70 (C) in the dorsal cochlear nucleus (DCN; i), caudal pontine reticular nucleus (PnC; ii), superior olivary complex (SOC; iii), cochlear nerve (8n; iv), ventral cochlear nucleus (VCN; v), pedunculopontine tegmental nucleus (PPT; vi), and the dorsal nucleus of the lateral lemniscus (DNLL; vii). Scale bars are 500 μ m in the large images and 200 μ m in the small images, respectively.

problems and milder forms of neurodevelopmental disorder (Poot, 2015; Whalley et al., 2011; Whitehouse et al., 2011).

3.4.1 Brainstem responsivity and reactivity

ABR wave amplitudes were assessed to determine if the peripheral (I: cochlear nerve) versus central brainstem (IV: lateral lemniscus terminating at the inferior colliculus) responsivity was impacted in *Cntnap2* knockout animals. In cases of peripheral deficits such as cochlear synaptopathy, loss of auditory nerve fibers, or noise induced hidden hearing loss, reduced wave I amplitudes have been reported (Bourien et al., 2014; Sergeenko et al., 2013; Shi et al., 2016). As we observed no differences in the amplitude of wave I between *Cntnap2*^{-/-} and wildtype animals, we suggest that peripheral auditory function is preserved in these animals (Fig. 3-3). This is further supported by the lack of ABR threshold differences (Fig. 3-2). However, acoustic startle reactivity, which is a behavioural read-out of brainstem auditory signaling (see Fig. 3-11), is increased in knockout animals, and this worsens with age, as indicated by the leftward shift of the startle reactivity curve (Fig. 3-5b). Therefore, the underlying mechanism for the over-reactivity in *Cntnap2* knockout animals must occur outside of the primary auditory pathway, possibly in the sensorimotor interface of the startle pathway—the caudal pontine reticular nucleus (PnC) - where cochlear root neurons synapse on pre-motor neurons (reviewed in Koch, 1999; Larrauri and Schmajuk, 2006; Simons-Weidenmaier et al., 2006; Fig. 3-11, green). Importantly, both the electrophysiological and behavioural phenotypic pattern reported here parallel those reported in individuals with ASD (reviewed in Sinclair et al., 2016).

Interestingly, while ASR magnitudes increase during development, habituation of this response improves with age. Startle habituation is a normalized measure, and therefore can be quantified independently from changes in baseline startle. Baseline startle responses rely on the glutaminergic excitation of PnC giant neurons, while habituation relies on synaptic depression at the axon terminals of the sensory afferents in the PnC, likely mediated by voltage- and calcium activated potassium channel function (Ebert and Koch, 1992; Weber et al., 2002; Simons-Weidenmaier et al., 2006; Zaman et al., 2017, see figure 3-11). Given CASPR2 presence in cochlear root neurons as well as the giant neurons of

the PnC (Fig. 3-10), and its association with potassium channels (Kv1.2) at the soma membrane or axon initial segment (Dawes et al., 2018; Inda, DeFelipe, & Muñoz, 2006), one can speculate that CASPR2 affects startle through influencing PnC excitability and that it directly interferes with startle habituation through its function in clustering potassium channels.

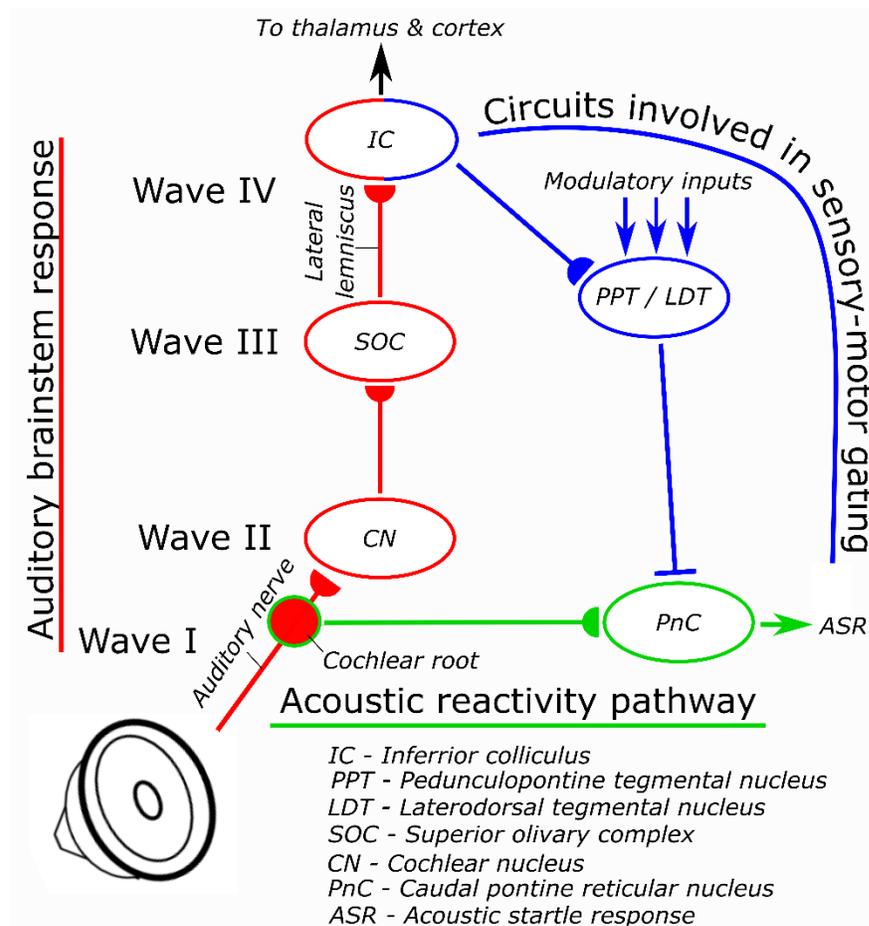


Figure 3-11: Generalized circuit outlining relevant auditory brainstem structures.

The measures of hearing sensitivity, neural responsiveness, and speed of neurotransmission were obtained from the auditory brainstem response. Acoustic reactivity and sensory filtering (i.e., habituation) rely on the acoustic reactivity pathway, and sensorimotor gating is dependent on the interplay of both circuits (Koch, 1999; Larrauri & Schmajuk, 2006; Yeomans, Lee, Yeomans, Steidl, & Li, 2006).

An analysis of the activity thought to arise from the superior olivary complex (ABR peak III) shows a clear effect of genotype across age, with differences persisting but decreasing

with development. The ABR wave representing the lateral lemniscus/inferior colliculus in humans is more variable and therefore is not often studied because of its lack of clinical applicability (Rosenhall et al., 2003). However, alterations in peak amplitude hold important information about the number, the individual contribution, and the synchronization of neuronal components, as alterations in any of these factors can lead to differences in wave amplitudes. It remains to be determined which of these aspects is disrupted in *Cntnap2* knockout rats. Differences in the wave IV:I ratio can provide an indication of gain changes in the brainstem, therefore the smaller ratio reported in young *Cntnap2*^{-/-} rats indicates that specifically the central brainstem is less responsive during development, but eventually normalizes with age (Fig. 3-3). A single paper in the ASD population has looked at a similar phenomenon and reported that children (2 - 6 yrs) with ASD and language delay exhibited higher amplitudes of wave I than wave V (35%) more frequently than the control group (Fernandes, Santos, Marques, Ana, & Coutinho, 2017). Amazingly, the *Cntnap2*^{-/-} rats show the same pattern at P28, such that 47% have a larger wave I than wave IV amplitude (resulting in a wave IV:I ratio less than 1.0) compared to 0% of wildtype (see Table 3-6).

Table 3-6: Auditory brainstem response descriptive table for the wave IV:I ratio analysis.

Genotype	Rats with ratio less than 1 (%)	Range	Mean	SEM
Wildtype	47.1	0.65 – 1.77	1.12	±0.08
<i>Cntnap2</i> ^{-/-}	0.0	1.30 – 2.18	1.70	±0.05

Importantly, sensorimotor gating relies on these central components of the auditory brainstem. For example, lesioning the inferior colliculus (IC) causes both a greater startle response, and prevents inhibition of startle by a prepulse (Leitner & Cohen, 1985). PPI is impaired in knockout rats (Fig. 3-7 & 3-10); therefore, the reduced PPI might directly reflect the reduced responsivity of wave IV, which may be caused by signal strength loss if synapses are not properly developed (Murphy & Benítez-Burraco, 2016; Poot, 2015). This is more evident in the 75 dB prepulse condition at both ages since at greater prepulse intensities (i.e., 85 dB) there might be a saturation of startle inhibition (ceiling effect).

Interestingly, the wave IV amplitudes improve with age while PPI deficits persist. This persistent PPI deficit may be caused by the increased baseline startle in knockout animals as the amount of PPI is influenced by baseline startle amplitudes, such that higher baseline startle is accompanied by poorer inhibition by prepulses (Csomor et al., 2008). However, significant correlations were, for the most part, not observed between PPI and baseline startle in wildtype or knockout animals in any prepulse condition (exception: *Cntnap2*^{-/-}, P78, 75 dB SPL 100 ISI condition: $R^2 = 0.203$, $p = 0.046$). It remains to be determined to what extent PPI deficits are caused by aberrant signal processing in the higher brainstem due to the disruptions during development as a result of the loss of *Cntnap2*, by the higher baseline startle, or by additional top-down modulatory effects from cortical areas. In any case, this same pattern has been described in humans with autism where increased startle reactivity is accompanied by a PPI deficit (Perry, Minassian, Lopez, Maron, & Lincoln, 2007). Furthermore, the top-down modulation by higher auditory areas via the pedunculopontine tegmental nucleus (PPT) have been shown to affect both the baseline magnitude of the ASR as well as its inhibition by prepulse stimuli (Fendt et al., 2001; Larrauri and Schmajuk, 2006; Fig. 3-10, blue). Therefore, the *Cntnap2*^{-/-} rats' PPI deficit is likely a result of aberrant neural brainstem responsivity to stimulus intensity and increased acoustic startle reactivity.

3.4.2 Speed of neurotransmission and startle response latency

The speed of neural transmission in the inner ear and auditory nerve has implications for latency to maximum startle response. Knockout animals show typical startle latencies, suggesting that the synapse from the inner hair cells to the auditory nerve, synapses in the cochlear root, axonal conduction, as well as synaptic transmission along the motor part of the startle pathway are not impacted in terms of speed (Fig. 3-10, green). This is consistent with our finding that the wave I latency was also normal in *Cntnap2* knockout animals. In humans with autism, prolonged startle response latencies have been reported; however, startle measures in humans normally measure an electromyogram of the eye-blink reflex, which is one component of the overall startle, and is dependent on a slightly different neural circuit (Takahashi et al., 2016, 2014; Yuhas et al., 2011). In contrast to these early auditory

processing stages that were not impacted in knockout animals, we found an increased latency between the auditory nerve (wave I) and cochlear nucleus (wave II) that seemed to at least partly persist into adulthood, suggesting deficits in either axonal conduction time and/or the synapse onto the cochlear nucleus (i.e., endbulb of Held; Rosenhall et al., 2003). Studies of *Cntnap2* knock-down mice showed no differences in the conduction velocity or refractory periods of the optic or sciatic nerves. Importantly, there is a close correspondence between synapse function and ABR maturation, and the endbulb of Held synapse has been shown to be crucial for temporal precision (Blatchley, Cooper, & Coleman, 1987; Poliak et al., 2003; Yu & Goodrich, 2015). This is particularly relevant given *Cntnap2*'s function in synapse development and maintenance, and thus synaptic transmission in developing neurons (Murphy & Benítez-Burraco, 2016; Poot, 2015). Importantly, Kv1 channels with which CASPR2 is associated contribute to the precise temporal pattern of synaptic transmission, are present in high concentrations in the soma of cells within the cochlear nucleus, and exhibit an age related increase in mRNA level expression before leveling off at P56 (Bortone, Mitchell, & Manis, 2006; Robbins & Tempel, 2012). Therefore, altered synaptic function is the more likely cause for the increased IPL I-II, as well as for the delay in subsequent ABR waves. Notably, the *Cntnap2*^{-/-} rat is first rodent model to recapitulate the slowed brainstem neurotransmission reported in ASD (Fujikawa-Brooks et al., 2010; Gonçalves et al., 2011; Kwon et al., 2007; Magliaro et al., 2010; Miron et al., 2016; Rosenhall et al., 2003; Roth et al., 2012; Tas et al., 2007; Wong & Wong, 1991). The slower IPL I-II partially, and both the prolonged IPL II-IV and absolute latencies of wave II, III, and IV of *Cntnap2*^{-/-} animals fully normalize by adulthood (Fig. 3-4; Table 1), suggesting that this reflects a delay in brainstem development, which again, has been reported to be associated with ASD (Amorim, Agostinho-Pesse, & Alvarenga, 2009; Fuess, Bento, & de Silveira, 2002). Studies have also found a delayed maturation of cortical auditory processing in autism (Edgar et al., 2015; Gage, Siegel, & Roberts, 2003). Given that cortical development relies on the brainstem, it is highly likely this phenotype is also present in *Cntnap2*^{-/-} animals and is an exciting future consideration.

Studies in rats exploring the neural substrates of sensorimotor gating have noted that prepulse effects on startle latency occur separately from prepulse inhibition (i.e., startle amplitude), therefore PPI deficits cannot simply reflect reduced prepulse detection since latency modulation may still remain intact (Ison et al., 1973; Swerdlow, Caine, Braff, & Geyer, 1992). Two previous studies support this conclusion since the presence of PPI deficits in animal models of schizophrenia was not associated with differences in startle latency (Lyall et al., 2009; Marriott et al., 2016). This is further exemplified in the present study where *Cntnap2*^{-/-} rats exhibit greater baseline startle responses without effects on latency (Fig. 3-8a & 3-9a). Startle latency typically increases in PPI trials with ISIs greater than 30 ms ISI (Ison et al., 1973; Hoffman and Ison, 1980; Fig. 3-8b). While mostly confirmed in our wildtype controls, *Cntnap2*^{-/-} animals did not only show deficient PPI (amplitudes), but also a lack of increased latencies in PPI trials; in fact, latency sometimes decreased. Since latency and amplitude manipulations by prepulse stimuli are thought to be independent phenomena (Hoffman & Ison, 1980; Hutchison, Niaura, & Swift, 2000), these results suggest robust deficits in sensorimotor gating in the knockout animals.

3.4.3 Comparison to other animal models of autism

Our collective results are in line with several important abnormalities observed in individuals with autism; however, they do differ from findings in the *Cntnap2* knockout mice. Behaviourally, no startle differences were reported in *Cntnap2* knockout mice and prepulse inhibition differences vary with experimental protocol (Brunner et al., 2015; Penagarikano et al., 2011; Truong et al., 2015). Our results also differ from studies on *Fmr1* knockout mice and rats, a model for fragile X syndrome. With respect to the ABR, fragile-X mice show smaller wave I and III peak amplitudes and no latency differences (Rotschafer, Marshak, & Cramer, 2015). Behaviourally, *Fmr1* knockout rats show typical startle responses and no significant PPI differences. However, results in the mouse literature vary, others show increased startle responses to low intensity sounds, like is found in our *Cntnap2* knockout rats, but reduced startle to high intensity sound (Hamilton et al., 2014; Nielsen, Derber, McClellan, & Crnic, 2002; Sinclair et al., 2016). Adult mice with a FOXP2 missense mutation, exhibit a similar pattern to the juvenile *Cntnap2* knockout rats,

with prolonged ABR latencies and reduced wave I and IV amplitudes (Kurt et al., 2009). This is promising since FOXP2 transcription factor regulates *CNTNAP2* gene expression (Poot, 2015; Rodenas-Cuadrado et al., 2014; Vernes et al., 2008) revealing a common path for auditory dysfunction. Lastly, the greater locomotor activity observed in this study is in line with previous studies in the *Cntnap2* knockout rats, as is the observed presence of motor seizures in the adult rats (Thomas et al., 2016). Interestingly, other rodent models with the knocked out Kv1.1 or Kv1.2 also have a seizure pathology, highlighting the potential importance of examining potassium channel function as a mechanism for altered excitability in ASD (Robbins & Tempel, 2012).

3.4.4 Conclusion

Overall, our findings show that alterations in sensory processing during early development due to the delayed maturation of the auditory pathway are associated with alterations in behavioural reactivity that persist in adulthood, emphasizing the need of early interventions targeting sensory processing in order to prevent potential maladaptive behavioural changes. Our results also validate a new rat model for studying auditory system dysfunction with high relevance to ASD (Poot, 2015, 2017; Servadio, Vanderschuren, & Trezza, 2015). Future studies need to explore cellular and molecular mechanisms that can be targeted to rectify altered auditory processing and behavioural reactivity.

3.5 References

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Chapter 4

4 Immature Neural Population Activity and Hyper-excitable Neurons in the Auditory Cortex of Rats with a Functional Loss of the Autism-linked Gene *Cntnap2*³

The contactin-associated protein-like 2 gene, *CNTNAP2*, is a highly penetrant risk gene thought to play a role in the genetic etiology of language-related disorders such as autism spectrum disorder (ASD) and developmental language disorder (DLD). Despite its candidacy for influencing language development, few preclinical studies have examined the role of *CNTNAP2* in auditory processing. Using a rat model with a loss-of-function mutation in the *Cntnap2* gene, we examined differences in auditory processing in adulthood by measuring (1) brainstem and cortical auditory evoked potentials (AEP), and (2) cortical multi-unit response dynamics and temporal processing. We show for the first time that, despite mature brainstem auditory evoked potentials in adulthood, cortical auditory function remains altered. Specifically, a loss of the *Cntnap2* gene function caused immature cortical evoked potentials, delayed multi-unit response latencies, impaired temporal processing, and resulted in a pattern of hyper-excitability in both the multi-unit response to acoustic stimuli. These results provide the first direct evidence that a constitutive loss of *Cntnap2* gene function in rats can cause auditory processing impairments similar to those seen in language-related human disorders, and that its contribution in maintaining cortical neuron excitability may underlie these abnormalities.

³ A version of this chapter is being prepared for submission to *eLife*

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4.1 Introduction

The genetic etiology of neurodevelopmental disorders involving altered speech and/or language ability is complex, with several genes thought to play a role (reviewed in Deriziotis & Fisher, 2017; Graham & Fisher, 2015; Kang & Drayna, 2011). Over the past two decades, shared genetic pathways have been implicated in disorders where language-related phenotypes are a core defining feature, such as autism spectrum disorder (ASD) and developmental language disorder (DLD; Deriziotis & Fisher, 2017; Kang & Drayna, 2011; Poot, 2015; Rodenas-Cuadrado, Ho, & Vernes, 2014). For example, the contactin-associated protein-like 2 (*CNTNAP2*) gene is a highly penetrant risk gene associated with both DLD and ASD (Arking et al., 2008; Newbury et al., 2011; Vernes et al., 2008). A homozygous loss-of-function in *CNTNAP2* causes cortical dysplasia, seizures, language impairment, and autistic features (Rodenas-Cuadrado et al., 2016; Strauss et al., 2006), with various heterozygous mutations leading to less severe phenotypes, typically including an impairment in speech and language, such as dysarthric language, language delay or absent speech/language (reviewed in Poot, 2015; Rodenas-Cuadrado et al., 2014). Moreover, genetic variation in *CNTNAP2* in neurotypicals is responsible for differences in language processing and development (Whalley et al., 2011; Whitehouse, Bishop, Ang, Pennell, & Fisher, 2011; Worthey et al., 2013). Despite the clear links between *CNTNAP2* and language development, it remains unresolved whether *CNTNAP2*-mediated differences in language processing manifest from altered response properties of neurons in the auditory cortex, such as difficulties in processing the rapidly-changing sounds associated with speech.

In general, neurophysiological studies on individuals with language-related disorders have reported differences in the processing of temporally manipulated sounds (Oram Cardy et al., 2004; Samson et al., 2011). Furthermore, sound-evoked cortical responses in individuals with ASD or DLD are often found to be delayed, reflected by slower response latencies (Berman et al., 2016; Edgar et al., 2015; Gage, Siegel, & Roberts, 2003; Matsuzaki et al., 2012; Port et al., 2016; Roberts et al., 2010, 2019; cf. Madsen et al., 2015; reviewed in O'Connor, 2012; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016). As human

maturation is typically associated with decreases in the latency (O'Connor, 2012; Sinclair et al., 2016; cf. Ruhnu, Herrmann, Maess, & Schröger, 2011), the activation pattern observed in ASD and DLD is thought to reflect that of an 'immature' cortex. Moreover, ASD-linked changes in the timing of neural activity in response to sensory stimuli (e.g. response onset and offset) has been suggested to result from altered cortical excitability (reviewed in Takarae & Sweeney, 2017). In addition to differences in response latency, autistic severity is associated with an altered morphology of sound-evoked activity thought to originate from the primary auditory cortex, specifically a larger temporal N1 (N1a) and a smaller frontal N1 (N1b) (Brandwein et al., 2015; Bruneau, Roux, Adrien, & Barthélémy, 1999; reviewed in Sinclair et al., 2016). In the case of *CNTNAP2*-related deficits, it is possible that altered cortical response properties underlie the significant impairments in language; however, no studies have investigated whether a loss-of-function of this autism-linked gene causes delayed cortical responses to sounds and/or an impaired ability for cortical neurons to accurately process rapidly-presented sounds.

In the present study, we used a *Cntnap2* knockout rat model, which has been shown to have considerable face validity for ASD-related behaviours (Scott et al., 2020), to assess if a loss of *Cntnap2* function results in immature auditory processing reflected by changes in the temporal control over the timing of neural activity. More specifically, auditory temporal processing and cortical excitability were examined by recording neural population activity (i.e., evoked potential and multi-unit firing rates) from the auditory cortex in response to temporally simple and complex acoustic stimuli using *in vivo* extracellular recordings with multi-channel microelectrode arrays. Furthermore, as cortical excitability can result from an altered readiness of neurons to produce an action potential, we used whole cell patch-clamp recordings to assess pyramidal neuron function in the auditory cortex of *Cntnap2* knockout and wildtype rats by comparing presynaptic and intrinsic neuron properties, as well as the resulting changes in the features and kinematics of generated action potentials

(Appendix A)⁴. Finally, as alterations in the inhibitory and excitatory systems have been hypothesized as a mechanism for altered cortical excitability in language-related disorders such as ASD or DLD, with the auditory cortex of autistic individuals having higher glutamate and lower GABA concentrations (Blatt & Fatemi, 2011; Brown, Singel, Hepburn, & Rojas, 2013; Chattopadhyaya & Di Cristo, 2012; Gaetz et al., 2014; Rojas, Singel, Steinmetz, Hepburn, & Brown, 2014), we investigated possible changes in the expression of markers for glutamatergic excitation and GABAergic inhibition. Overall, we show for the first time that, despite mature brainstem auditory evoked potentials in adulthood, the cortical ability to process simple and complex temporally-modulated sounds remains altered in *Cntnap2* knockout rats, characterized by immature cortical evoked potentials, delayed multi-unit latencies, impaired temporal processing, and a pattern of hyper-excitability in both multi-unit and single cell recordings.

4.2 Materials and Methods

4.2.1 Animals

Male Sprague-Dawley wildtype and homozygous knockout rats were used in this study. Homozygous knockout breeders were obtained from Horizon Discovery (Boyertown, PA; originally created at SAGE Laboratories, Inc. in conjunction with Autism Speaks; the line is now maintained by Envigo) and bred to obtain *Cntnap2*^{-/-} rats. The model contains a five base pair deletion in exon six of the *Cntnap2* gene, created using the zinc finger nuclease target site CAGCATTTCGCACC|aatgga|GAGTTTGACTACCTG. Wildtype breeders were obtained from Charles River Laboratories (Wilmington, MA). Experimental animals were obtained from the following crossings: *Cntnap2*^{-/-} rats from homozygous knockout crossings and wildtype rats from wildtype crossings. Animals from a minimum of three litters of a given genotype were used in all experiments. Date of birth was designated as

⁴R.S. Mann designed, oversaw, and conducted all *in-vitro* electrophysiological recordings, performed the associated data analysis, and assisted in writing the corresponding sections of the manuscript. Methods and results are presented in Appendix A.

post-natal day zero (P0). Rats were weaned on P21, and sexes were separated on P35. Rats were housed in a temperature-controlled room on a 12h light/dark cycle, with *ad libitum* food and water. All procedures were approved by the University of Western Ontario Animal Care Committee and were in accordance with the guidelines established by the Canadian Council on Animal Care.

4.2.2 Experimental Series 1

4.2.2.1 Hearing assessment and collection of BAEPs.

Hearing levels were assessed using the brainstem auditory evoked potential (BAEP) (Petrova, 2009) which was performed in a double-walled sound-attenuating chamber. Rats were anesthetized with ketamine (80 mg/kg; i.p.) and xylazine (5 mg/kg; i.p.), and subdermal electrodes (27 gauge; Rochester Electro-Medical, Lutz, FL) were positioned at the vertex, over the right mastoid process and on the mid-back in wildtype and homozygous knockout rats. The animal was not secured in a stereotaxic frame during the ABR testing. Body temperature was maintained at $\sim 37^{\circ}\text{C}$ using a homeothermic heating pad (507220F; Harvard Apparatus, Kent, UK). Sound stimuli were generated by a Tucker-Davis Technologies (TDT, Alachua, FL) RZ6 processing module at 100 kHz sampling rate and delivered by a magnetic speaker (MF1; TDT) positioned 10 cm from the animal's right ear. The left ear was occluded with a custom foam earplug. Sound stimuli for the BAEP and electrophysiological recording experiments were calibrated with custom Matlab software (The Mathworks, Natick, MA) using a 1/4-inch microphone (2530; Larson Davis, Depew, NY) and pre-amplifier (2221; Larson Davis). The auditory evoked activity was collected using a low-impedance headstage (RA4L1; TDT), preamplified and digitized (RA16SD Medusa preamp; TDT), and sent to a RZ6 processing module via a fiber optic cable. The signal was filtered (300-3000 Hz) and averaged using BioSig software (version 5.5, TDT). Auditory stimuli consisted of a click (0.1 ms) stimulus which was each presented 1000 times (21 times/second) at decreasing intensities from 90 to 20 dB sound pressure level (SPL) in 10 dB SPL steps. Near threshold, successive steps were decreased to 5 dB SPL, and each sound level was presented twice in order to best determine BAEP threshold using

the criteria of just noticeable deflection of the averaged electrical activity within the 10-ms window (Abitbol et al., 2016; Popelar, Grecova, Rybalko, & Syka, 2008; Schormans et al., 2016; K. E. Scott et al., 2018). Evoked potentials collected in response to the 90 dB SPL stimulus were used for BAEP analysis (Fig. 4-1A, 2). The peak amplitudes of each of waves I and IV were measured in microvolts in reference to the baseline (0 μ V), and the latency of each of these peaks was determined from the stimulus onset. Data analyses were performed with BioSig software (Tucker-Davis Technologies) and Microsoft Excel 2010 (Microsoft Corp.).

4.2.2.2 Surgical procedure.

Immediately following the BAEP measures, each rat was maintained under ketamine/xylazine anesthesia, the foam earplug was removed from the left ear, and the animal was fixed in a stereotaxic frame with blunt ear bars. Supplemental doses of ketamine/xylazine were administered (i.m.) as needed. A midline incision was made in the skin, and the underlying tissue was reflected from the skull. A headpost was fastened to the skull with dental acrylic, and a stainless steel screw was inserted into the right frontal bone to serve as an anchor for the headpost as well as electrical ground. A craniotomy (2.0 mm x 3.0 mm; 3.5 – 5.5 posterior to bregma and 0.0 to 3.0 medial the lateral ridge) was performed in the left parietal bone in order to expose the cortex. At the end of the surgical procedure, the right ear bar was removed to allow free-field auditory stimulation of the right ear during the electrophysiological recordings in the contralateral cortex. The rat remained securely positioned in the stereotaxic frame using the left ear bar and the headpost for the remainder of the experiment.

4.2.2.3 *In-vivo* electrophysiological recordings.

Recording equipment. Extracellular electrophysiological signals were acquired using a 32-channel microelectrode array which consisted of a single 50 μ m thick shank with 32 equally-spaced recording sites (50 μ m apart), spanning 1.55 mm in length (A1x32-10mm-50s-177-A32; NeuroNexus Technologies, Ann Arbor, MI). The electrode array was connected to a high-impedance headstage (NN32AC; TDT), and the neuronal activity was

preamplified and digitized (two RA16SD Medusa preamps; TDT), and sent to a RZ5 processing module via a fiber optic cable. For each of the 32 channels, multi-unit activity was digitally sampled at 25 kHz and bandpass filtered online at 3-300 Hz for local field potential (LFP) data and 300-3000 Hz for spiking data, using a voltage threshold for spike detection of three standard deviations above the noise floor. All LFP activity, and the timing of the detected spikes and their associated waveforms were stored for offline analyses.

Recording sites. Two recording penetrations were conducted in each rat targeting the auditory cortex between 3.7 and 4.5 mm caudal to bregma. Using a high-precision stereotaxic manipulator (World Precision Instruments, Sarasota, FL), the electrode array was inserted in the cortex through a small slit in the dura using a dorsomedial-to-ventrolateral approach (30° angle), with the electrode array entering the cortex targeting first 4.0 mm caudal to bregma and 1.0 mm medial to the temporal ridge of the skull (i.e., ~4.6 mm lateral to midline). The second penetration targeted 4.3 mm caudal to bregma and 1.0 mm medial to the temporal ridge of the skull. For both penetrations, the electrode array was advanced at the 30° angle until all recording sites were within the cortex (depth of 1.55 mm) based on visual confirmation using a surgical microscope equipped with a high-resolution camera. A hydraulic microdrive (FHC; Bowdoinham, ME) was then used to slowly advance the electrode array into the auditory cortex (~ 4800 um depth).

Acoustic stimulation paradigms. Overall, in each rat, acoustic stimulation paradigms (described below) were performed at two locations within the auditory cortex: 4.0 and 4.3 mm caudal to bregma. Before conducting electrophysiological recordings at each location, the electrode array was allowed to settle in place for 45 min. At each recording location, two auditory stimulation paradigms were presented using a RZ6 processing module (TDT; 100 kHz sampling rate) and custom Matlab (The Mathworks) software. For both paradigms, acoustic stimuli consisted of broadband noise bursts (1-32 kHz) presented at 90 dB sound pressure level (SPL) from a magnetic speaker (MF1; TDT) positioned 10 cm above the surface of the stereotaxic frame and 10 cm from the base of the right pinna on a 30° angle from midline in the contralateral space. In the first paradigm, a 50 ms noise burst

was presented every 3 – 5 s for a total of 50 presentations, which allowed for the quantification of the cortical auditory evoked potential (CAEP), and multi-unit cluster cortical response dynamics. To determine the ability of the cortex to respond to rapidly presented stimuli (i.e., temporal response dynamics), the second paradigm consisted of a train of six discrete 25 ms noise bursts, with each six-pulse train presented 25 times at each of six repetition rates (0.3, 0.9, 2.8, 5.2, 7.4, 9.2 pulses per second [pps]). Repetition rates were randomly interleaved to reduce adaptation effects, and a minimum of 2 s of silence separated each train.

Histological confirmation of penetrations. To allow for post-experiment histological reconstruction of the electrode penetrations, the electrode array was coated in DiI cell-labeling solution (V22885; Molecular Probes, Inc., Eugene, OR) prior to insertion into the cortex. At the completion of the electrophysiological experiment, the rat was injected with sodium pentobarbital (100 mg/kg; i.p.) in preparation for exsanguination via transcardial perfusion of 0.1 M phosphate buffer (PB; 300 ml) followed by 4% paraformaldehyde (400 ml). Next, the brain was removed and post-fixed in paraformaldehyde for 12 h, followed by storage in 30% sucrose/PB solution for cryoprotection. Using a microtome (HM 430/34; Thermo Fisher Scientific, Waltham, MA), frozen sections (40 μ m) were cut in the coronal plane and collected serially. The sections were mounted in fluorescent DAPI mounting medium to label DNA (F6057 Fluoroshield™ with DAPI; Sigma-Aldrich, St. Louis, MO), and coverslipped. Ultimately, fluorescent and brightfield images were obtained using an Axio Vert A1 inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) and ZEN lite imaging software (Carl Zeiss Microscopy). Each penetration was matched to its corresponding rostral-caudal location using the *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 2007), and the penetrations were reconstructed (Fig. 4-1B). Penetrations that fell between 3.72 and 4.56 caudal to bregma were used for analysis.

Offline analysis of cortical auditory evoked potentials (CAEPs). Cortical auditory evoked potentials were analyzed following the presentation of the 50 ms noise burst stimulus, in order to assess sound-evoked activity. For each penetration, auditory-evoked LFP activity was examined within 500 ms of the onset of the noise burst using custom

Matlab scripts (R2020a; The MathWorks). For each channel and recording location, mean LFPs were calculated by averaging the LFP recordings across all 50 trials. Using the mean LFP, the N1 and P2 amplitudes and latencies were determined, as these are well-established response measures of LFP recordings (Eggermont et al., 2011; Engineer, Centanni, Im, Borland, et al., 2014; Kurt, Moeller, Jeschke, & Schulze, 2008; Nagy, Featherstone, Hahn, & Siegel, 2015). The most negative peak occurring within 40 ms of the noise burst stimulus was taken as the N1 peak. The most positive peak occurring after the N1 peak and within 100 ms of the noise burst stimulus was taken as the P2 peak. Finally, to create a compound CAEP trace and average amplitude and latency measures for each genotype, mean LFPs as well as the corresponding amplitude and latency were averaged for across all channels (Fig. 4-3). Data analyses were performed with Matlab (R2020a; The MathWorks).

Offline analysis of multi-unit activity. Auditory responsiveness was examined for each multi-unit cluster in response to a single 50 ms noise burst. To do so, rasters and peristimulus time histograms (PSTH) were generated for each multi-unit cluster. Spontaneous activity was calculated by tallying the number of spikes within the last 500-ms of each trial, and then calculating the average spontaneous firing rate across all 50 trials (Hz/trial; Schormans, Typlt, & Allman, 2016). To provide consistency across recording sites and animals, the spiking activity of each multi-unit cluster was measured within a 35 ms time window which was time-locked to 5-40 ms from stimulus onset. The responsivity of each multi-unit was based on the average firing rate, determined by counting the number of spikes within the 35-ms time window for each trial, and then calculating the average firing rate across the 50 trials (Hz/trial; Schormans et al., 2016). For a multi-unit cluster to be considered responsive to the acoustic stimulus, it needed to show a significantly increased firing rate per trial compared to the spontaneous activity as determined with a paired t-test ($\alpha = 0.05$). Non-responsive multi-units were removed from analysis. To accurately assess multi-unit latency differences between the two genotypes, spiking activity within a 500-ms time window after stimulus onset was parsed into 2 ms bins (Fig. 4-1A). Onset latency was defined as the first time the firing rate within a 2 ms bin surpassed three standard deviations above the spontaneous firing rate (Engineer, Centanni, Im, Rahebi, et al., 2014; Tao et al., 2016). Offset latency was defined as the time at which the

firing rate returned to the spontaneous firing rate for two consecutive bins (i.e., 4 ms). Response duration was then calculated as the time between the response onset latency and

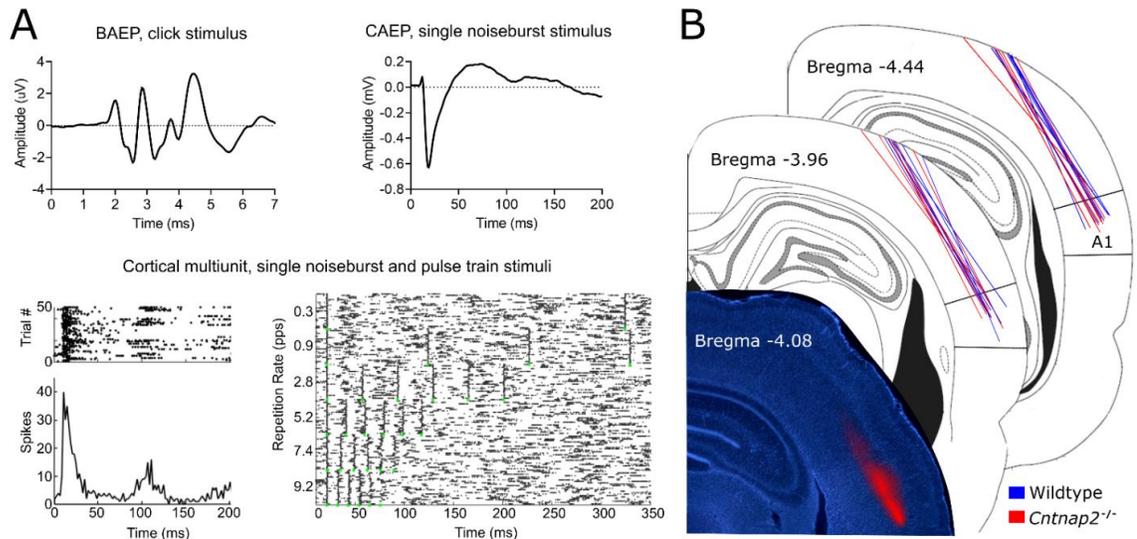


Figure 4-1: Responses to acoustic stimuli from central auditory areas in the rat.

(A) Representative responses to acoustic stimuli presented monaurally; brainstem auditory evoked potential (BAEP) from a single rat elicited by the presentation of click stimuli (90 dB SPL); averaged cortical auditory evoked potential (CAEP) from a single multi-unit cluster elicited by the presentation of a 50 ms noise burst stimuli (90 dB SPL); cortical spiking activity from a single multi-unit cluster elicited by the presentation of a 50 ms noise burst stimulus or from six-pulse train composed of 25 ms noise bursts presented at six different repetition rates (90 dB SPL). Dot raster plot (dot = 1 spike; row = 1 trial) and/or line peristimulus time histogram (PSTH; 2 ms bins) shown. (B) Representative recording penetration in the primary auditory cortex (A1) in the rat, accompanied by schematics of the locations of electrode penetrations reconstructed from histological sections for each of the rats that underwent in vivo electrophysiological recordings (wildtype rats, $n = 7$ and *Cntnap2*^{-/-} rats, $n = 8$). Electrodes were advanced into the cortex, falling between 3.72 mm and 4.56 mm caudal to bregma.

response offset latency, and ultimately defined as the response window. The response magnitude of each multi-unit was then determined by tallying the number of spikes within the response window for each trial, and dividing by the response duration, to determine the average firing across the 50 trials (Hz/trial). Lastly, the peak firing rate and latency were measured by determining the maximum firing rate within a 2 ms bin that was located within the response window, and the time at which it occurred. All described metrics were averaged across multi-unit clusters for each genotype (Fig. 4-4).

Auditory temporal processing was examined for each multi-unit cluster in response to 25 ms noise bursts presented at six different repetition rates. Similar to the analysis described above, spontaneous activity was calculated within the final 2 seconds of each trial and averaged across all trials. The response magnitude of each multi-unit cluster was determined for each noise burst in the six-pulse train by calculating the spiking activity in a 35 ms response window, which was time-locked to 5-40 ms from each stimulus onset (Kilgard et al., 2001; Schormans et al., 2016). For a multi-unit cluster to be considered responsive to a given pulse, it needed to show a significantly increased firing rate compared to the spontaneous activity as determined with a paired t-test ($\alpha = 0.05$). Multiunits without an acoustic response to the first pulse were removed from analysis. Unlike the auditory responsiveness analysis, spiking activity of each multi-unit in response to the six different repetition rates was parsed into 5 ms bins (Bao, Chang, Woods, & Merzenich, 2004; Zhou & Merzenich, 2008) for both the raster and PSTHs plots (Fig. 4-5). To determine the effects of increasing repetition rate and allow for cross-site and genotype comparisons, the repetition rate transfer function (RRTF; i.e., the normalized response as a function of temporal rate) was determined (Kilgard & Merzenich, 1998, 1999). The response magnitude evoked by the last five pulses in the train (pulses 2-6) was averaged and then compared to the average response magnitude to the first noise burst in the six-pulse train. Next, mean temporal modulation-transfer functions (tMTFs) were calculated as it provides the normalized cortical response as a function of the repetition rate (Bao et al., 2004; Chang, Bao, Imaizumi, Schreiner, & Merzenich, 2005; Zhou & Merzenich, 2008). More specifically, for each repetition rate, the average response magnitude evoked by the last five noise bursts in the six-pulse train (pulses 2-6) was divided by the response magnitude evoked by the first noise burst in the train. The ability of the cortex to follow the rapidly presented stimuli was then estimated for each multi-unit by interpolating the highest temporal rate at which the tMTF was at least half of its maximum, referred to as $f_{h1/2}$ (Fig. 4-6; Zhou & Merzenich, 2008). Lastly, temporal fidelity, or the capacity of cortical neurons to respond in a time-locked fashion to each pulse at each repetition rate, was quantified using vector strength and Rayleigh statistic, taking into account the total number of spikes generated in the full phase of each pulse (i.e., all spikes in response to a given noise burst

up to the onset of the following noise burst; Bao et al., 2004; Chang et al., 2005; Zhou & Merzenich, 2008). Vector strength and the Rayleigh statistic were calculated using the toolbox for circular statistics in Matlab (Berens, 2009). All described metrics were averaged across multi-unit clusters for each genotype (Fig. 4-7). Data analyses were performed with Matlab (R2020a; The MathWorks).

4.2.3 Experimental Series 2

4.2.3.1 Immunohistochemistry

The opposing auditory cortex of select animals in which *in vivo* electrophysiology was completed were used to stain for GAD67 (wildtype, n = 7; *Cntnap2*^{-/-}, n = 6) and VGluT1 (wildtype, n = 7; *Cntnap2*^{-/-}, n = 6). Before free-floating immunohistochemistry, as well as in between all incubations with antibodies, all slices were thoroughly rinsed in 0.1 M phosphate buffered saline (PBS). Slices were pretreated with a 1% H₂O₂ in 0.1 M PBS for 10 min. Sections were then blocked in a PBS solution containing 0.2% Triton-X (Thermo Fisher Scientific, Waltham, MA) and 0.1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) for 1 hour, before incubation in the blocking solution with the primary antibody, either mouse anti-GAD67 primary antibody (1:5000; MAB5406, Millipore, USA) or guinea pig anti-VGluT1 primary antibody (1:5000; AB5905, Sigma-Aldrich, St. Louis, MO) overnight at room temperature. Next, sections were incubated in the blocking solution with biotinylated secondary antibody (1:500; anti-mouse, BA-9200, Vector Labs, Burlingame, CA or 1:500; anti-guinea pig, 106-065-003, Jackson ImmunoResearch Lab; West Grove, PA) for 1 hour at room temperature, then processed using avidin-biotin complex solution (1:1000; Vectastain Elite ABC Kit; pk 6100; Vector Labs, Burlingame, CA) in PBS for 1 hour at room temperature. Labelling was visualized using 3,3'-diaminobenzidine (DAB) solution (0.04% H₂O₂, 0.2 mg/mL DAB; D4293, Sigma-Aldrich, St. Louis, MO) at room temperature. The slices being stained for GAD67 were incubated for 7 min, the slices being stained for VGluT1 were incubated for 3 min. Tissue was then mounted onto positively charged glass slides, air dried overnight, dehydrated in increasing

concentration of alcohol, cleared in xylene, and coverslipped with DPX mounting media (HX55746679, Millipore, USA).

4.2.3.2 Imaging and processing

Imaging was performed using a Nikon Eclipse Ni-U upright microscope with a DS-Qi2 high definition color camera and imaging software NIS Elements Color Camera (Nikon Instruments Inc., Melville, NY). The complete auditory cortex in a given slice was imaged by capturing a stitched image at 10x (VGluT1) or 20x (GAD67) magnification. Images were then processed using Fiji (Schindelin et al., 2012). Using rectangular rulers scaled via the specify tool, the auditory cortex (region of interest, ROI) was defined as the region spanning 1500 – 2500 μM dorsal to the rhinal fissure, from the inner edge of the cortex to the outer edge and outlined using the polygon tool. Images were converted to 8-bit, processed using the despeckle command, and then thresholded with a fixed grayscale cut-off value. Protein expression was determined by calculating the staining intensity (% area coverage), and density (cells/ mm^2) within the auditory cortex (Fig. 4-8). Using the ROI manager, area coverage (%) and total area (mm^2) were measured and recorded for each ROI. Cell count was determined using the analyze particles tool, and was divided by the total ROI area. Data analyses were performed with Fiji (Schindelin et al., 2012) and Microsoft Excel 2010 (Microsoft Corp.).

4.2.4 Data Presentation and Statistics

Graphs were generated with GraphPad (Prism 8.3.0 for Windows, GraphPad Software, San Diego, CA). Statistical analyses were conducted using IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, N.Y., USA). Statistical tests performed were based on the experimental design and included independent samples t-tests, one-way analysis of variance (ANOVA), and two-way, or three-way repeated measures analysis of variance (RM-ANOVA). In cases where independent samples t-test were performed, Levene's test was used to assess the equality of variances. In cases where a RM-ANOVA was performed, the Mauchly test was used to report a violation of the assumption of sphericity, such that the degrees of freedom were corrected using the Greenhouse Geisser (if $\epsilon < 0.75$) or the

Huynh-Feldt method (if $\epsilon > 0.75$). *Post hoc* one-way ANOVAs and paired-samples t-tests with a Dunnett's or Bonferroni-corrected significance levels were used to compare differences in the group means in the case of a significant interaction. Differences were considered statistically significant when p-values (adjusted) were smaller than $\alpha = 0.05$. Exact *p* values are reported, except in cases where $p < 0.001$. The following sections provide a summary of the various statistical tests performed in each of the experimental series. For complete statistical reporting, see 4.6 – Supplemental Statistics Table.

Animal Characteristics. To compare differences in the body mass, age, and hearing threshold between wildtype and *Cntnap2*^{-/-} rats, independent samples t-tests were performed.

Experimental Series 1: BAEP, CAEP, multi-unit response dynamics to a single noise burst. To determine the effect of genotype on brainstem auditory evoked potential amplitude and latency, a two-way RM-ANOVA was performed for peak (I, IV) \times genotype (wildtype, *Cntnap2*^{-/-}). Independent samples t-tests were used to compare the amplitude and latency of the N1 and P2 components of the cortical auditory evoked potential, and subsequent derived metrics, between wildtype and knockout rats. Furthermore, genotypic differences in the response features of multi-unit cluster spiking activity to a single 50 ms noise burst, including the response onset, response duration, firing rate (responsivity and response magnitude), peak latency, and peak firing rate, were compared using independent sample t-tests.

Experimental Series 1: multi-unit temporal processing and temporal fidelity. To assess whether auditory temporal processing ability differed between wildtype and *Cntnap2*^{-/-} rats, the multi-unit firing rate (i.e., within in a 35 ms window) in response to each pulse in a six-pulse train presented at six different repetition rates was compared using a three-way RM-ANOVA, performed for pulse number (1, 2, 3, 4, 5, 6) \times repetition rate (0.3, 0.9, 2.8, 5.2, 7.4, 9.2 pps) \times genotype (wildtype, *Cntnap2*^{-/-}). As a significant three-way interaction was found, *post hoc* Bonferroni-corrected t-tests were performed to assess the presence of genotypic differences at each pulse. A two-way RM-ANOVA was performed on the RRTF for repetition rate (0.3, 0.9, 2.8, 5.2, 7.4, 9.2 pps) \times genotype

(wildtype, *Cntnap2*^{-/-}) to further quantify differences in temporal processing and followed up with *post hoc* Bonferroni-corrected t-tests for genotype. To determine the pulse rate at which a given genotype's cortical response began to degrade, a one-way ANOVA for repetition rate (0.3, 0.9, 2.8, 5.2, 7.4, 9.2 pps) was performed on each genotype, and followed by *post hoc* Dunnett's t-test comparing each rate to 0.3 pps. Lastly, genotypic differences in the capacity of cortical neurons to process high-rate stimuli was determined using an independent samples t-test on $f_{hl/2}$. Using a two-way repeated measures ANOVA for repetition rate (0.3, 0.9, 2.8, 5.2, 7.4, 9.2 pps) × genotype (wildtype, *Cntnap2*^{-/-}), vector strength and Rayleigh statistics were assessed to compare the temporal fidelity of wildtype and knockout multi-unit clusters. As a significant interaction was found, *post-hoc* Bonferroni-corrected t-tests were completed for genotype.

Experimental Series 2: VGluT1 and GAD67 expression. To compare genotypic differences in the expression of excitatory and inhibitory markers, independent samples t-tests were performed for VGluT1 percent area coverage, GAD67 percent area coverage, and the number of GAD67 reactive cell bodies.

4.3 Results

4.3.1 Animal Characteristics

Prior to collection of subdermal and *in vivo* electrophysiological recordings, all wildtype (n = 7) and *Cntnap2*^{-/-} (n = 8) male rats were weighed and the differences in body mass analyzed, with no differences in body mass present (wildtype 503 ± 6 g, *Cntnap2*^{-/-} 500 ± 20 g; $p = 0.88$). Moreover, no genotypic differences exist in the age of rats used (wildtype 88 ± 1 days, *Cntnap2*^{-/-} 92 ± 2 days; $p = 0.09$). Lastly, no differences in hearing threshold existed between the genotype of rats used in BAEP, CAEP, and multi-unit *in-vivo* cortical recordings (wildtype 27.9 ± 1.0 dB SPL, *Cntnap2*^{-/-} 30.0 ± 0.9 dB SPL; $p = 0.15$).

4.3.2 Experimental Series 1: Functional loss of *Cntnap2* leads to a larger and delayed auditory-evoked potential in the cortex, but not in brainstem, of adult rats

To determine whether a developmental loss of *Cntnap2* caused differential changes in auditory evoked responses at various levels throughout the central auditory pathway, electrophysiological recordings were made from the auditory brainstem and cortex in wildtype ($n = 7$) and *Cntnap2*^{-/-} rats ($n = 8$) (Fig. 4-1). Brainstem auditory evoked potentials (BAEPs) in response to a click stimulus were recorded using subdermal electrodes positioned on the scalp, whereas cortical auditory evoked potentials (CAEPs) were subsequently recorded in response to a noise burst (50 ms) using a microelectrode array inserted into the auditory cortex. Unlike at the level of the brainstem (Fig. 4-2B), in which there were no genotypic differences in the amplitude of the first or fourth peaks of BAEP (representative of activity in the auditory nerve, and lateral lemniscus/inferior colliculus, respectively), both the N1 and P2 components of the CAEP were significantly larger in *Cntnap2*^{-/-} rats ($p < 0.001$); resulting in a 35% increase in the peak-to-peak N1-P2 potential amplitude compared to the wildtypes ($p < 0.001$; Fig. 4-3B). Furthermore, there was a differential effect observed in the latency of the evoked responses, such that cortical activity in the *Cntnap2*^{-/-} rats had a delayed speed of transmission of the N1 ($p < 0.001$), P1 ($p < 0.001$) potentials and prolonged N1-P1 interpeak latency ($p < 0.001$; Fig. 4-3C), whereas there were no genotypic differences in response latency in the brainstem nuclei (Fig. 4-2C). Taken together, these findings identify that *Cntnap2*-related alterations in auditory evoked potentials manifest as both a larger and delayed response at the level of the auditory cortex, but unlikely at the brainstem, of adult rats.

4.3.3 Experimental Series 1: Multiunit activity in the auditory cortex of *Cntnap2*^{-/-} rats is hyper-responsive to sound and exhibits impaired temporal processing

To investigate the effect of a functional loss of *Cntnap2* on the response dynamics of neurons within the auditory cortex (Fig. 4-1), the spiking activity of multi-unit clusters

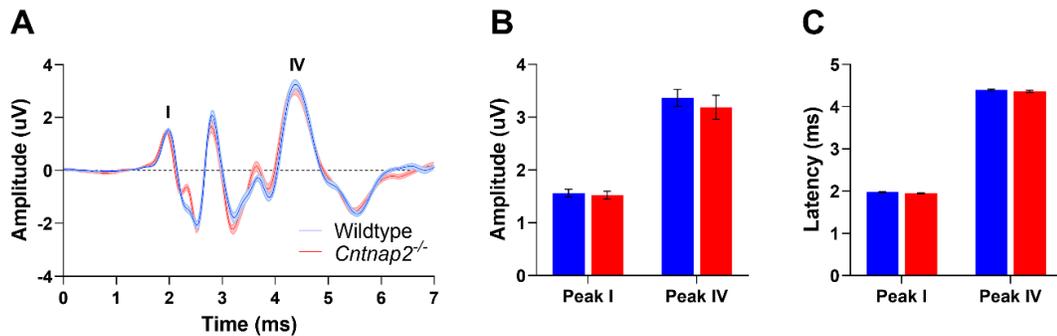


Figure 4-2: Intact auditory brainstem responses in adult *Cntnap2*^{-/-} rats.

(A) Averaged brainstem auditory evoked potential (BAEPs) waveforms from wildtype (n = 7) and *Cntnap2*^{-/-} rats (n = 8), in response to a 90 dB SPL, 0.1 ms click stimulus presented monaurally. Solid line and shaded region denote mean \pm standard error. (B) Amplitude and (C) latencies of peaks I and IV representing activity from the auditory nerve and lateral lemniscus terminating at the inferior colliculus respectively, represented as mean \pm standard error.

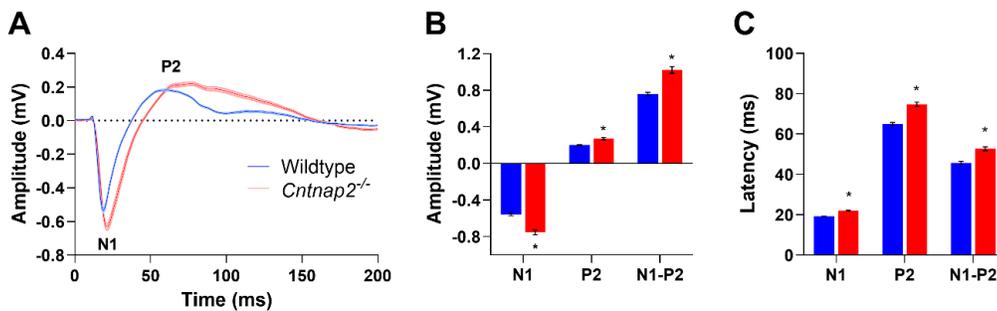


Figure 4-3: *Cntnap2*^{-/-} cortical auditory evoked potentials reflect an immature profile.

(A) Averaged cortical auditory evoked potential (CAEP) waveforms from wildtype (n = 180 waveforms) and *Cntnap2*^{-/-} (n = 161 waveforms) rats in response to a 90 dB SPL noise burst. Solid line and shaded region denote mean \pm standard error. (B) Increased amplitudes and (C) prolonged latencies of the N1 and P2 potentials, reflect an immature response profile. Data represented as mean \pm standard error. * p < 0.05.

(wildtype $n = 180$; *Cntnap2*^{-/-} $n = 161$) were recorded following acoustic stimulation with single noise bursts (50 ms). As seen in the group averaged line peristimulus time histograms derived from the spiking responses to the noise burst stimulation (Fig. 4-4A), there was a genotypic difference in several response features, including a delay in both the response onset ($p < 0.001$; Fig. 4-4B) and peak latency ($p < 0.001$; Fig. 4-4F), as well as a longer response duration ($p < 0.001$; Fig. 4-4C) and increased response magnitude ($p = 0.01$; Fig. 4-4D) in the *Cntnap2*^{-/-} rats compared to the wildtypes. These findings are indicative of a prolonged, hyper-responsiveness to acoustic stimulation of auditory cortex neurons following a functional loss of *Cntnap2*.

To assess whether auditory temporal processing was affected by a loss of *Cntnap2* function, the spiking activity of the multi-unit clusters recorded from auditory cortex of the wildtype ($n = 176$ clusters) and *Cntnap2*^{-/-} rats ($n = 161$ clusters) were compared in response to pulse trains consisting of six noise bursts (25 ms) presented at different repetition rates (0.3, 0.9, 2.8, 5.2, 7.4, and 9.2 pulses per second [pps]; Fig. 4-1). Overall, a differential effect was evident in the spike firing rates (i.e., responsivity) between the genotypes over of the various pulse repetition rates (i.e., significant interaction of repetition rate \times pulse number \times genotype: $p < 0.001$; Fig. 4-5). Most notably, close inspection of the spike firing rates to the 6th pulse of the various trains shows that, while *Cntnap2*^{-/-} multi-unit clusters had a greater spiking activity at the low pulse repetition rates (e.g., 0.3 pps: $p_{Bonf} < 0.001$) than the wildtype multi-unit clusters, this pattern was reversed at the faster pulse repetition rates, with the wildtype multi-unit clusters showing greater spike firing rates to the 6th pulse of the 5.2 pps ($p_{Bonf} < 0.001$), 7.4 pps ($p_{Bonf} < 0.001$) and 9.2 pps trains ($p_{Bonf} < 0.001$).

In an effort to further quantify this genotypic difference in auditory temporal processing, a repetition rate transfer function (RRTF) was calculated, where the average firing rate of noise bursts 2-6 in each six-pulse train was compared to the firing rate evoked by the first noise burst. *Cntnap2*^{-/-} multi-unit clusters responded significantly poorer than wildtype multi-unit clusters to temporally-modulated stimuli at the faster repetition rates (7.4 pps: $p_{Bonf} < 0.001$; 9.2 pps: $p_{Bonf} < 0.001$; Fig. 4-6A). Furthermore, mean temporal modulation-transfer functions (tMTFs) were created by normalizing the RRTF of each multi-unit

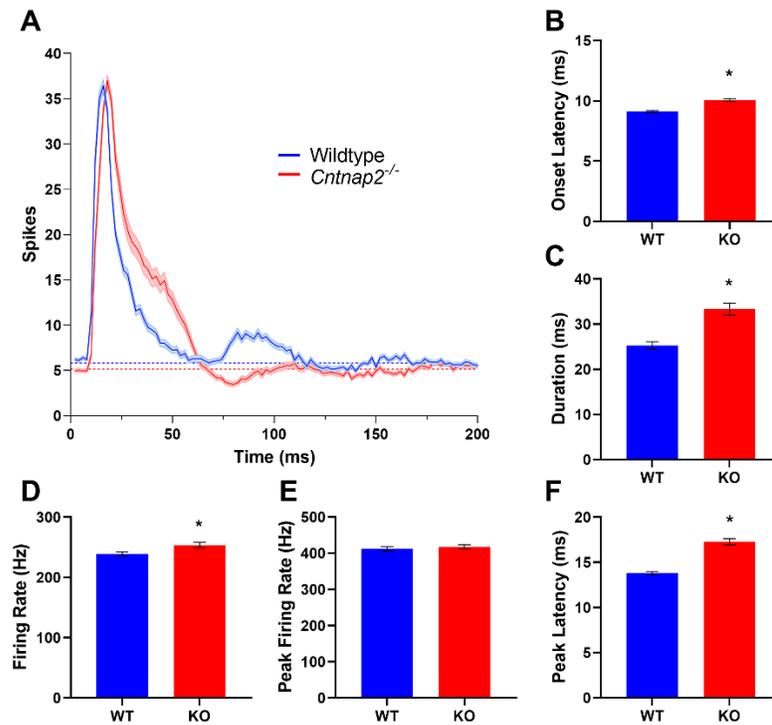


Figure 4-4: Hyper-responsivity in the spiking profile of *Cntnap2*^{-/-} rats' auditory cortex.

(A) Multiunit cortical response dynamics in the primary auditory cortex to a 50 ms noise burst stimulus (monaural, 90 dB SPL), represented as an averaged line peristimulus time histogram (PSTH) from wildtype (n = 180 clusters) and *Cntnap2*^{-/-} (n = 161 clusters) rats. Solid line and shaded region denote mean ± standard error. Horizontal dashed line represents spontaneous activity. (B) Response onset latency, (C) response duration, (D) average response magnitude, (E) peak firing rate, and (F) peak latency represented as mean ± standard error. Longer response duration and increased firing rate are indicative of a hyper-excitable response. * $p < 0.05$.

cluster to the firing rate of the first noise burst in the six-pulse train, such that the magnitude of normalized cortical response was defined as a function of the stimulus repetition rate. The tMTF analysis revealed that for both genotypes the cortical responses to modulated stimuli began to degrade when the rate reached 2.8 pps compared to 0.3 pps (wildtype: $p < 0.001$; *Cntnap2*^{-/-}: $p < 0.001$; Fig. 4-6B). Lastly, to quantify the capacity of cortical neurons to process high-rate stimuli, we calculated the highest temporal rate at which the tMTF of each multi-unit cluster was at half its maximum ($f_{h/2}$), and found it to be significantly lower in the *Cntnap2*^{-/-} multi-unit clusters compared to wildtypes ($p < 0.001$; Fig. 4-6C). Taken together, these established metrics provide evidence that a

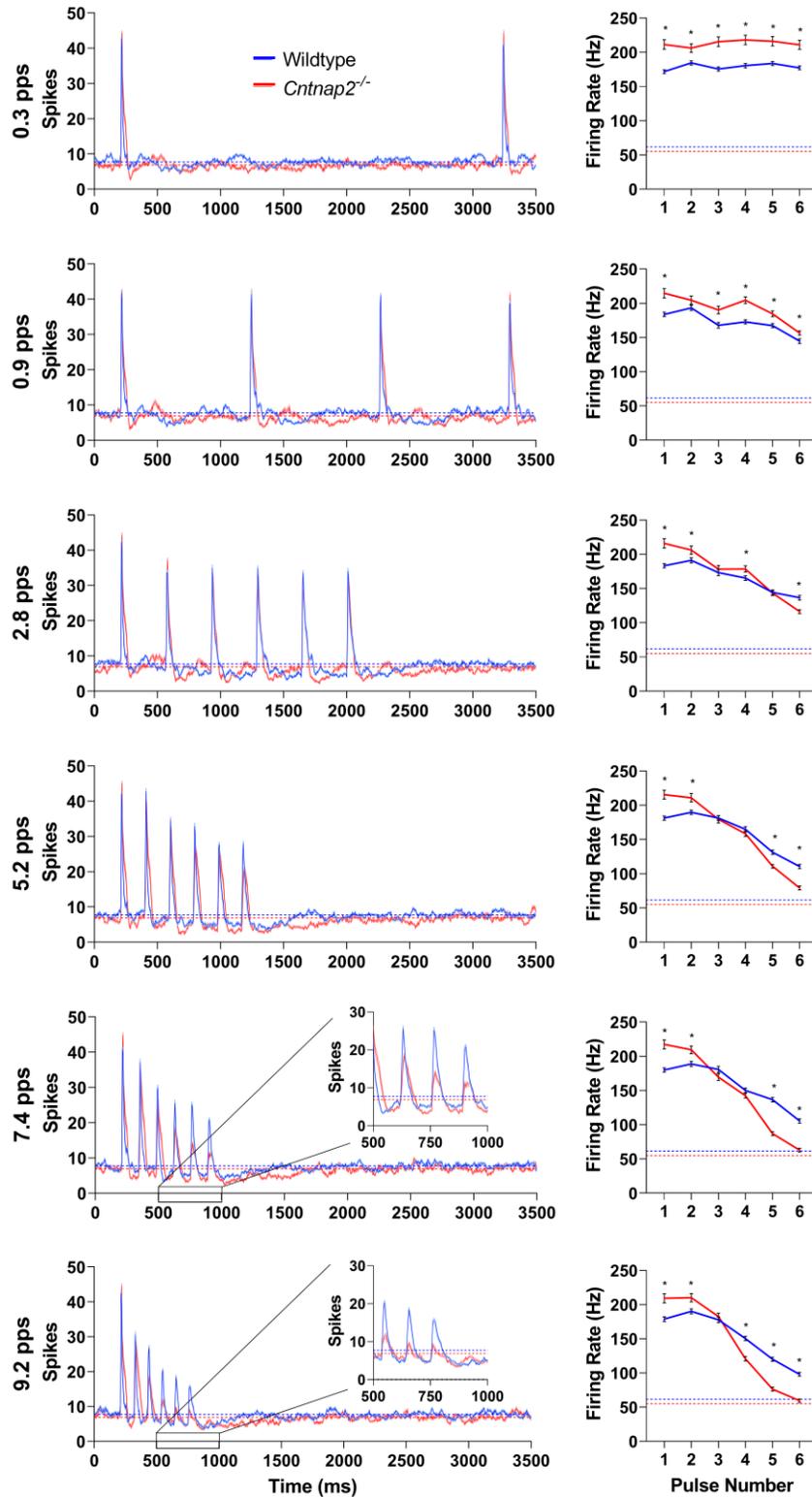


Figure 4-5: *Cntnap2*^{-/-} rats have a reduced ability to consistently respond to a six-pulse noise burst train.

functional loss of *Cntnap2* results in impairments in auditory temporal processing, characterized by an inability of cortical neurons to sustain their spiking activity to rapidly-presented acoustic stimuli.

Finally, the effect of a functional loss of *Cntnap2* on the capacity of cortical neurons to respond in a time-locked fashion to the acoustic stimuli was determined using measures of vector strength and Rayleigh statistics. More specifically, to quantify the temporal fidelity of spikes to the successive noise bursts in the six-pulse train, the vector strength for each multi-unit cluster was calculated. Furthermore, Rayleigh statistics were calculated to estimate the significance of vector strength. In general, both the vector strength (Fig. 4-7A) and Rayleigh statistic (Fig. 4-7B) of *Cntnap2*^{-/-} multi-unit clusters were greater than that of wildtypes at the repetition rates tested in this study, apart from 9.2 pps (vector strength at 0.3 – 7.4 pps: $p_{Bonf} < 0.001$; Rayleigh statistic at 0.3 – 7.4 pps: $p_{Bonf} < 0.01$). Thus, the developmental loss of *Cntnap2* resulted in a temporal fidelity profile shifted in the direction associated with younger age (Chang et al., 2005). However, this outcome can perhaps be associated with the lower spontaneous spiking activity of *Cntnap2*^{-/-} multi-unit clusters ($p < 0.001$), since greater vector strength is associated with lower spontaneous firing rates (0.3 pps: $r = -0.21, p < 0.001$; 9.2 pps: $r = -0.50, p < 0.001$).

Figure 4-5 continued... (Left) Responses of multi-unit clusters in the primary auditory cortex to a six-pulse train of 25 ms noise burst stimuli (monaural, 90 dB SPL), presented at repetition rates of 0.3, 0.9, 2.8, 5.2, 7.4, and 9.2 pulses per second (pps), represented as an averaged line peristimulus time histogram (PSTH) from wildtype (n = 176 clusters) and *Cntnap2*^{-/-} (n = 161 clusters) rats. Solid line and shaded region denote mean \pm standard error. Horizontal dashed line represents spontaneous activity. (Right) Average firing rate in a 35 ms window (5-40 ms after stimulus onset) in response to each noise burst in the six-pulse train for each repetition rate, represented as mean \pm standard error. Horizontal dashed line represents spontaneous activity. As the repetition rate increases, the ability of multi-unit clusters to respond to each noise burst stimulus in the six-pulse train decreases; this effect is more pronounced in the responses of *Cntnap2*^{-/-} rats at rates of 5.2 pps and greater. * $p < 0.05$.

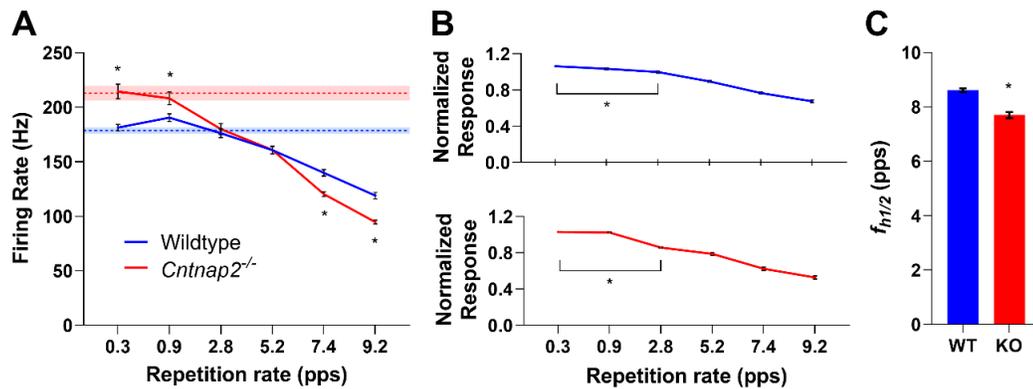


Figure 4-6: Poorer temporal processing is reflected in the decreased rate-following ability in *Cntnap2*^{-/-} rats’.

(A) Repetition rate transfer function (RRTF) reveals the following capacity of *Cntnap2*^{-/-} multi-unit clusters is poorer than wildtypes at repetition rates of 7.4 and 9.2 pps, despite knockout clusters having an increase firing rate to the first noise burst in the six-pulse train. The average firing rate to noise bursts 2 – 6 in the six pulse train are represented by the solid line, with error bars denoting standard error. Dashed line and shaded region represents the mean firing rate \pm standard error to the first noise burst. (B) The temporal modulation-transfer function (tMTF) normalizes the RRTF to the firing rate in response to the first noise burst stimulus, showing that in both wildtype and *Cntnap2*^{-/-} rats, the ability of multi-unit clusters to follow repeated stimuli begins to decrease at a repetition rate of 2.8 pps. (C) The $f_{h/2}$ confirms that *Cntnap2*^{-/-} multi-unit clusters have a poorer capacity for processing high-rate stimuli, since the rate at which the tMTF of each cluster was at half its maximum is lower in *Cntnap2*^{-/-} rats compared to wildtypes. Data represented as mean \pm standard error. * $p < 0.05$.

4.3.4 Experimental Series 2: VGlut1 and GAD67 expression does not differ in the auditory cortex of *Cntnap2*^{-/-} rats

The effect of a developmental loss of *Cntnap2* on cellular markers associated with excitatory and inhibitory neurotransmission were assessed by comparing the expression of VGlut1, a glutamate transporter associated with the synaptic vesical membrane, as well as GAD67, a key synthesizing enzyme for GABA found throughout the cell, in the auditory

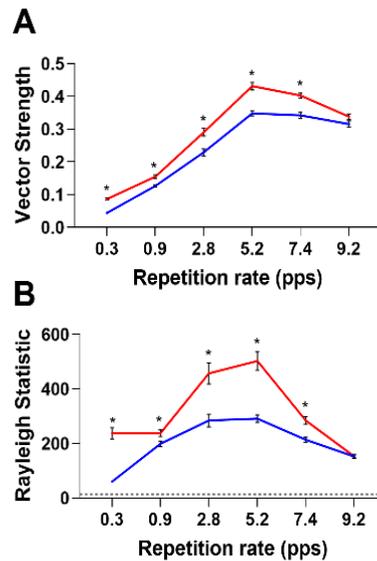


Figure 4-7: No deficit in the cortical phase-locking of multi-unit responses in *Cntnap2*^{-/-} rats.

(A) Cortical spike timing in relation to stimulus phase was determined using vector strength, measured at the various repetition rates for each multi-unit cluster. A higher value signifies more precise spike timing. (B) The significance of the phase locking is measured with the Rayleigh statistic, where > 13.82 indicates $p < 0.001$. Data represented as mean \pm standard error. * $p < 0.05$.

cortex of *Cntnap2*^{-/-} ($n = 7$) and wildtype ($n = 6$) rats. As shown in Figure 4-8, there was no genotypic difference in the percent area coverage of VGluT1 ($p = 0.65$; top panels) or GAD67 staining ($p = 0.69$; bottom panels). Moreover, as GAD67 also stains the cell body, we further compared the number of immune-reactive cell bodies in the auditory cortex of the *Cntnap2*^{-/-} versus wildtype rats, ultimately finding no differences between genotypes ($p = 0.34$).

4.4 Discussion

The present study included a series of experiments to determine if the association between the *CNTNAP2* gene and language impairment arises from its role in the cortical auditory processing. Using adult wildtype and *Cntnap2*^{-/-} rats, we investigated if a loss of *Cntnap2* function would cause poorer temporal processing of sound and altered excitability, similar to what is seen in language related disorders such as ASD and DLD. As predicted, our

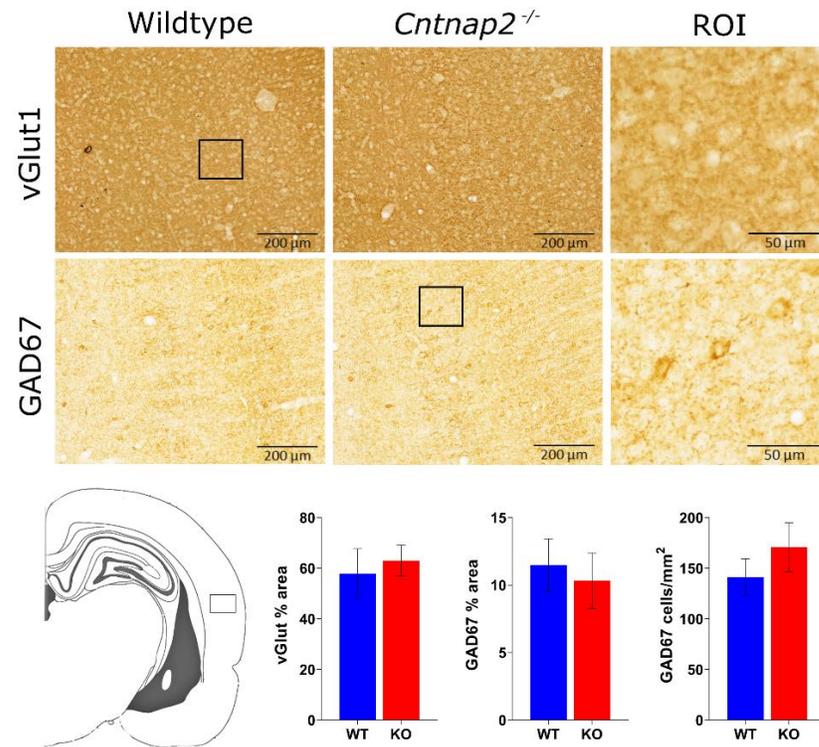


Figure 4-8: *Cntnap2*^{-/-} rats VGlut1 and GAD67 immunoreactivity does not differ from wildtypes.

(Top) Representative images of VGlut1 and GAD67 staining from the auditory cortex (see rectangle on coronal section schematic) of a wildtype and *Cntnap2*^{-/-} rat. Representative ROI images, taken at 20X magnification, are outlined by small black squares. (Bottom) Quantitative analysis of VGlut1 and GAD67 showing the total percentage of area stained, and the number of GAD67 stained cell bodies (wildtype n = 6, *Cntnap2*^{-/-} n = 7). Data expressed as mean ± SEM.

findings recapitulated the immature CAEP profile in ASD, whereby the sound-evoked N1 potential showed a delayed onset and larger amplitude in knockout compared to wildtype rats. Moreover, extracellular electrophysiological recordings of multi-unit activity also showed delayed and prolonged responses to a single noise burst, ultimately resulting in a greater response magnitude. Despite this larger response to simple acoustic stimuli, *Cntnap2*^{-/-} rats had a lower capacity to process high-rate temporally-modulated stimuli compared to wildtype rats, as evidenced by a lower firing rate to rapidly presented stimuli (i.e., RRTF, $f_{h1/2}$). Hyper-excitable pyramidal neurons could underlie the changes in multi-

unit activity observed in the *Cntnap2* rats, as their cells were found to depolarize at a lower voltage, depolarize to a greater extent, and have lower spike thresholds (Appendix A). Although this *Cntnap2*-related alteration led to a greater number of spikes elicited at low levels of stimulation, fewer spikes were generated at higher stimulating rates (Appendix A). Unexpectedly, we did not observe any differences in the expression of VGluT1 or GAD67, immunohistological markers for excitation and inhibition respectively. In the following sections, we discuss our collective findings in the context of cortical maturation, and potential cellular mechanisms.

4.4.1 Immature auditory function

Research in individuals across development and with ASD or DLD has led to the hypothesis that the maturational stage of the auditory cortex, as measured by the CAEP, can be indicative of altered language development, and represents a hallmark of language-related disorders (Berman et al., 2016; Edgar et al., 2015; Kwok, Joanisse, Archibald, & Oram Cardy, 2018; Kwok, Joanisse, Archibald, Stothers, et al., 2018; Oram Cardy, Flagg, Roberts, & Roberts, 2008; Roberts et al., 2010). Similar to the immature CAEP profile in the *Cntnap2*^{-/-} rat, the multi-unit response also had a delayed onset and greater response magnitude. Moreover, the differences in multi-unit activity to temporally-modulated stimuli observed in the adult *Cntnap2* rats in the present study are reflective of the profile commonly observed in young wildtype rats. Specifically, the interaction in the RRTF between firing rate and repetition rate, the reduced rate following ability ($f_{h1/2}$), and the increased temporal fidelity (VS) observed in *Cntnap2*^{-/-} rats, are shifted in the direction that is observed in younger rats compared to old at presentation rates up to 10 pps (Chang et al., 2005). Given that immaturity is reflected in both the N1 potential as well as the multi-unit response profile to temporally-modulated stimuli in the auditory cortex of adult *Cntnap2*^{-/-} rats, *CNTNAP2*-mediated differences in language processing could indeed arise from its role in affecting the cortical response to sound.

The immature profile of responses in the auditory cortex observed in the present study has not been consistently recapitulated in all rodent models for language-related disorders. The

prenatally injected valproic acid (VPA) rat (model for ASD) shows the most similar profile to the *Cntnap2*^{-/-} rats. For example, VPA rats exhibited prolonged CAEP latencies (Engineer, Centanni, Im, Borland, et al., 2014; Gandal et al., 2010), increased N1-P2 amplitudes (Engineer, Centanni, Im, Borland, et al., 2014; cf. Gandal et al., 2010), and delayed multi-unit onset and peak latency in the anterior auditory fields; however, they did not have a greater multi-unit firing rate or altered temporal fidelity (Engineer, Centanni, Im, Borland, et al., 2014). While the *Kiaa0319* knockdown rat (model for dyslexia) also had a prolonged CAEP latency, they had a smaller N1 amplitude, and poorer temporal fidelity (Centanni et al., 2014). Likewise, the N1-P2 amplitude is reduced in *Fmr1* knockout rats (model for fragile X syndrome) compared to wildtypes, with no differences in multi-unit response characteristics in the primary auditory cortex (Engineer, Centanni, Im, Rahebi, et al., 2014). That said, similar to the *Cntnap2*^{-/-} rats, the *Fmr1* knockout mouse model does show an increased unit firing in response to an acoustic stimulus, and a longer response duration, due to a greater variability in spike latency (Rotschafer & Razak, 2013).

Rat models created using developmental perturbations relevant for human development also show immature response profiles to temporally-modulated stimuli. For example, in humans, lead exposure is a risk factor for learning disability, and prenatal lead exposure in rats leads to a decreased cortical capacity for processing the high-rate stimuli (Zhu et al., 2016). Noise exposure during a critical period for auditory development (which is known to disrupt the typical development of the auditory system) similarly leads to poorer processing of temporally-modulated stimuli at rates greater than ~9 pps, a slower maximum rate following ability (i.e., $f_{h1/2}$), as well as increased temporal fidelity at rates up to 9 pps (Zhou & Merzenich, 2008); findings which are similar to those observed in the *Cntnap2*^{-/-} rats.

4.4.2 Excitability

To determine if the altered temporal control over the timing of neural activity in *Cntnap2* knockout rats, resulted from differences in cortical excitability, we assessed the subthreshold synaptic activity and suprathreshold spiking activity of pyramidal neurons in

the auditory cortex using *in vivo* whole-cell patch clamp electrophysiology (Appendix A). While presynaptic function appeared typical in *Cntnap2*^{-/-} cells, post-synaptic pyramidal neurons were hyper-excitable. Cell membrane channels responsible for the rapid inward currents opened at lower voltages and remained open for longer periods of time (i.e., larger half-width), resulting in larger rapid influx current amplitudes. While a reduced cell capacitance and a lower membrane resistance in knockout cells could explain the lower voltage required to elicit a current in *Cntnap2*^{-/-} cells, the larger amplitude and half-widths are not affected by these properties. This hyper-excitability extended to lower spike thresholds in *Cntnap2*^{-/-} cells. The spike release profile of pyramidal neurons was dependant on the extent of stimulation, with cells producing a greater number of spikes at a low current stimulation level, but fewer spikes at higher stimulation levels. Therefore, the increased multi-unit firing rate observed in the *Cntnap2*^{-/-} rats could arise from the acoustic stimulus exciting a greater number of neurons and each neuron firing more action potentials, if a single noise burst is equivalent to a low level of stimulation. Indeed, once the capacity of the auditory cortex to respond to temporally-modulated stimuli was tested by presenting pulse trains consisting of six noise bursts presented at rapid repetition rates (e.g., 9.2 pps), the *Cntnap2*^{-/-} multi-unit firing rate decreased, akin to the reduced ability of knockout pyramidal neurons to spike at high stimulation currents. Therefore, the pyramidal neuron hyper-excitability and spiking profile could explain the differences in multi-unit activity to both temporally simple and complex acoustic stimuli. When we look to other models of *Cntnap2* dysfunction, we find action potential features are varied; spike rate was greater in somatosensory cortex (layer 4) of knockout mice regardless of current injected (Antoine, Langberg, Schnepel, & Feldman, 2019), whereas another study showed no differences in firing threshold, spike amplitude, spike width, or firing rate of the visual cortex (in layers 2/3) of *Cntnap2*^{-/-} mice (Bridi, Park, & Huang, 2017). Future studies could explore the *number of excited cells* hypothesis for the greater multi-unit firing rate of knockout rats using various techniques, such as (1) *in vivo* extracellular single unit recordings to identify the number of cells that respond to an acoustic stimulus in a local area, (2) calcium imaging to globally monitor the activity of hundreds of cells in the

auditory cortex at a given time, or (3) by using a neuronal activity-dependant marker, e.g., c-FOS.

The excitation/inhibition ratio for autism postulates that an imbalance can be caused by a combination of genetic and environmental effects that affect neural development (Rubenstein & Merzenich, 2003). For example, markers of GABAergic inhibition are downregulated in select cortical areas in ASD (Blatt & Fatemi, 2011; Chattopadhyaya & Di Cristo, 2012 *cf.* Kolodny et al., 2017; Pereira, Violante, Mougá, Oliveira, & Castelo-Branco, 2018), while both increases (Brown et al., 2013) and no change in glutamatergic excitation have been documented (Kolodny et al., 2017; Pereira et al., 2018). It is worth noting that rodent studies that used pharmacological manipulations in the auditory cortex to either increase local glutamatergic activity via glutamate application, or reduce GABAergic activity via GABA antagonists (Beh, 2017; Chang et al., 2005; Kurt et al., 2008; Moeller, Kurt, Happel, & Schulze, 2010), have found a pattern of electrophysiological responses akin to what we have observed in the *Cntnap2* knockout rats (i.e., increased N1 CAEP amplitude, increased multi-unit firing rates). Thus, it was surprising that, when we stained for a marker for GABAergic activity, Gad67, and glutamatergic activity, VGluT1, we found no differences in their expression in the auditory cortex of the *Cntnap2* knockout rats compared to wildtypes.

4.4.3 *Cntnap2* and altered development

Here, we have shown that a developmental loss of *Cntnap2* gene function leads to altered pyramidal neuron excitability (Appendix A), poorer temporal control over neural activity, and a profile of immature cortical auditory processing. These findings are largely consistent with past studies that altered the development of the auditory system in wildtype animals. For example, depriving the developing auditory system of normal acoustic experience (by rearing animals in a noisy environment) (Zhou & Merzenich, 2008), caused the same pattern of auditory processing differences in adulthood as observed in the present study. Moreover, gerbils with transient hearing loss during the critical period for auditory development showed an *in vitro* action potential profile similar to what is found in

Cntnap2^{-/-} rats, including smaller action potential amplitudes, increased half-widths, and reduced firing rate (Mowery, Kotak, & Sanes, 2015). In considering the developmental trajectory of auditory processing associated with *Cntnap2*, we have previously shown using our knockout rat model that there is a delayed maturation of the brainstem auditory evoked potential early in life, but this is normalized by adulthood (Scott et al., 2018). Thus, it is reasonable to propose that this *Cntnap2*-related alteration of sensory input during development could cause the persistence of cortical hyper-excitability and profile of immaturity in adulthood. With respect to the possible cellular mechanisms underlying these persistent cortical alterations, it is worth noting that a loss-of-function of the *Cntnap2* protein, Caspr2, has been shown to block experience-dependant homeostatic synaptic plasticity in the visual cortex in response to dark rearing (Fernandes et al., 2019). Ultimately, because homeostatic plasticity is known to be important during the development of sensory systems, *Cntnap2*'s role in long-term cortical auditory dysfunction could be due to differential neuronal homeostatic plasticity during development, brought on by delayed sound processing in the brainstem early in life and prevailing loss of Caspr2 function.

4.4.4 Conclusions

Overall, our findings revealed immaturity and hyper-excitability in the auditory cortex of *Cntnap2*^{-/-} rats. First, the immature CAEP and multi-unit temporal processing profiles further validate the *Cntnap2* knockout rat model for studying auditory system dysfunction with high relevance to language-related disorders, including ASD. Second, the pattern of hyper-excitability and action potential kinematics in pyramidal cell recordings suggest the *Cntnap2* gene is involved in maintaining cortical excitability. Future studies using rodent models with a functional loss of the *Cntnap2* gene could examine the developmental changes in homeostatic plasticity and circuit function in the auditory cortex to ultimately determine the role of *Cntnap2* in central auditory processing across age.

4.5 References

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4.6 Supplemental Statistics Table

Reference to methods section: Data Presentation and Statistics	Figure number	Test	Measure of Interest	Dependent Variable(s)	Independent Variable(s)	Interactions and Main Effects	n: wildtype; Control ²	Degrees of freedom, t-value OR F-value	P value	Levene's Test for Equality of Variances
BAEP	2B	2-way RM ANOVA	Peak Amplitude	Amplitude (mV)	Peak x Genotype	Peak x Genotype, Peak, Genotype	7, 8	F(1,13) = 0.258, F(1,13) = 209.705, F(1,13) = 0.365	0.560, <0.001, 0.556	NA
	2C	2-way RM ANOVA	Peak Latency	Latency (ms)	Peak x Genotype	Peak x Genotype, Peak, Genotype	7, 8	F(1,13) = 0.044, F(1,13) = 31074.416, F(1,13) = 1.698	0.838, <0.001, 0.215	NA
CAEP	3B	Independent samples 2-tailed t-test	N1	Amplitude (uV)	Genotype		180, 161	247.46, 6.55	<0.001	Not assumed
	3B	Independent samples 2-tailed t-test	P2	Amplitude (uV)	Genotype		180, 161	200.56, -5.19	<0.001	Not assumed
	3B	Independent samples 2-tailed t-test	N1-P2	Amplitude (uV)	Genotype		180, 161	231.35, -6.37	<0.001	Not assumed
	3C	Independent samples 2-tailed t-test	N1	Latency (ms)	Genotype		180, 161	216.91, -10.49	<0.001	Not assumed
	3C	Independent samples 2-tailed t-test	P2	Latency (ms)	Genotype		180, 161	289.01, -7.49	<0.001	Not assumed
	3C	Independent samples 2-tailed t-test	N1-P2	Latency (ms)	Genotype		180, 161	315.26, -5.65	<0.001	Not assumed
Multisensory response dynamics to a single noise burst	4A	Independent samples 2-tailed t-test	Response onset	Time (ms)	Genotype		180, 161	339.0, -6.74	<0.001	Assumed
	4B	Independent samples 2-tailed t-test	Response duration	Time (ms)	Genotype		180, 161	269.60, -5.08	<0.001	Not assumed
	4C	Independent samples 2-tailed t-test	Response magnitude	Firing rate (Hz)	Genotype		180, 161	295.21, -2.61	<0.001	Not assumed
	4E	Independent samples 2-tailed t-test	Peak	Firing rate (Hz)	Genotype		180, 161	339.0, -6.63	0.53	Assumed
	4F	Independent samples 2-tailed t-test	Peak	Time (ms)	Genotype		180, 161	255.11, -8.85	<0.001	Not assumed
	NA	Independent samples 2-tailed t-test	Spontaneous	Firing rate (Hz)	Genotype		180, 161	289.0, 3.85	<0.001	Not assumed
	NA	Independent samples 2-tailed t-test	Response offset	Time (ms)	Genotype		180, 161	268.06, -5.72	<0.001	Not assumed
	NA	Independent samples 2-tailed t-test	Responsivity (35 ms window)	Firing rate (Hz)	Genotype		180, 161	235.89, -3.70	<0.001	Not assumed
Multisensory temporal processing	5	3-way RM ANOVA	Response (35 ms window)	Firing rate (Hz)	Repetition Rate x Pulse Number x Genotype	Repetition Rate x Pulse Number, Pulse Number x Genotype, Repetition Rate x Genotype, Pulse Number, Repetition Rate, Genotype	176, 161	F(6,0.2009.35) = 46.48; F(6,0.2009.35) = 327.15; F(1,34, 450.51) = 53.49; F(1, 76, 590.88) = 100.90; F(1,34, 450.51) = 618.14; F(1,76, 590.88) = 452.80; F(1, 335) = 2.0	<0.001, <0.001, <0.001, <0.001, <0.001, <0.001, 0.158	NA
	5	post hoc analysis: Bonferroni corrected t-test			Genotype	+/- v.s. -/-			0.3 pps: pulse 1-6 < 0.001; 0.9 pps: pulse 1 < 0.001, pulse 2 = 0.107, pulse 3 = 0.001, pulse 4 < 0.001, pulse 5 = 0.001, pulse 6 = 0.01; 2.8 pps: pulse 1 < 0.001, pulse 2 = 0.033, pulse 3 = 0.455, pulse 4 = 0.018, pulse 5 = 0.829, pulse 6 < 0.001; 5.2 pps: pulse 1 < 0.001, pulse 2 = 0.002, pulse 3 = 0.700, pulse 4 = 0.265; pulse 5 < 0.001, pulse 6 < 0.001; 7.4 pps: pulse 1 < 0.001, pulse 2 = 0.002, pulse 3 = 0.097, pulse 4 = 0.113, pulse 5 < 0.001, pulse 6 = 0.001; 9.2 pps: pulse 1 < 0.001, pulse 2 = 0.003, pulse 3 = 0.441, pulse 4 < 0.001	NA
Multisensory temporal processing	NA	Independent samples 2-tailed t-test	Spontaneous	Firing rate (Hz)	Genotype		176, 161	269.84, 3.48	0.001	Not assumed
	6A	Independent samples 2-tailed t-test	Mean of all pulse one's	Firing rate (Hz)	Genotype		176, 161	214.72, -4.71	<0.001	Not assumed
	6A	2-way RM ANOVA	RRTF	Firing rate (Hz)	Repetition Rate x Genotype	Repetition Rate x Genotype, Repetition Rate, Genotype	176, 161	F(1,31, 438.6) = 53.60; F(1,31, 438.6) = 632.47; F(1, 335) = 0.146	<0.001, <0.001, 0.703	NA
	6B	1-way RM ANOVA	IMFT-WT	Normalized response	Repetition Rate	Repetition Rate	176	F(5, 965) = 197.03	<0.001	NA
	6B	post hoc analysis: Dunnett's t-test			0.3 v.s. other pps				0.3 pps < 0.001, 0.9 pps = 0.009, 2.8 pps = 0.549, 5.2 pps = 0.956, 7.4 pps < 0.001, 9.2 pps < 0.001	NA
	6B	1-way RM ANOVA	IMFT-XO	Normalized response	Repetition Rate	Repetition Rate	161	F(5, 965) = 249.473	<0.001	NA
Multisensory temporal fidelity	6C	post hoc analysis: Dunnett's t-test			0.3 v.s. other pps			0.9 pps = 0.257, 2.8 pps - 9.2 pps < 0.001	NA	
	6C	Independent samples 2-tailed t-test	fl1/2	Repetition Rate (pps)	Genotype		176, 161	265.76, 7.09	<0.001	Not assumed
	7A	2-way RM ANOVA	Vector strength	Repetition Rate (pps)	Repetition Rate x Genotype	Repetition Rate x Genotype, Repetition Rate, Genotype	176, 161	F(2,77, 928.60) = 6.15; F(2,77, 928.60) = 819.51; F(1, 335) = 41.18	0.001, <0.001, <0.001	NA
	7A	post hoc analysis: Bonferroni corrected t-test			Genotype	+/- v.s. -/-			0.3 pps - 7.4 pps < 0.001, 9.2 pps = 0.074	NA
	7B	2-way RM ANOVA	Rayleigh statistic	Repetition Rate (pps)	Repetition Rate x Genotype	Repetition Rate x Genotype, Repetition Rate, Genotype	176, 161	F(2,1, 703.52) = 16.67; F(2,1, 703.52) = 101.45; F(1, 335) = 38.57	<0.001, <0.001, <0.001	NA
	7B	post hoc analysis: Bonferroni corrected t-test			Genotype	+/- v.s. -/-			0.3 pps < 0.001, 0.9 pps = 0.013, 2.8 pps - 7.4 pps < 0.001, 9.2 pps = 0.924	NA
VGlut1 and GAD67 expression	NA	Pearson Correlation	Vector strength (0.3 pps) and spontaneous firing rate				176, 161	r = -.21	<0.001	NA
	NA	Pearson Correlation	Vector strength (9.2 pps) and spontaneous firing rate				176, 161	r = -.50	<0.001	NA
	8	Independent samples 2-tailed t-test	VGlut1 intensity	% area	Genotype		6, 7	11, -0.46	0.65	Assumed
	8	Independent samples 2-tailed t-test	GAD67 intensity	% area	Genotype		6, 7	11, 0.41	0.69	Assumed
Animal Characteristics	8	Independent samples 2-tailed t-test	GAD67 density	cells/mm ²	Genotype		6, 7	11, -0.99	0.34	Assumed
	NA	Independent samples 2-tailed t-test	In vivo electrophysiology: Age	Age (days)	Genotype		7, 8	10.532, -1.863	0.91	Not assumed
	NA	Independent samples 2-tailed t-test	In vivo electrophysiology: Weight	Weight (g)	Genotype		7, 8	8.318, -0.161	0.876	Not assumed
	NA	Independent samples 2-tailed t-test	In vivo electrophysiology: Hearing Threshold	Threshold (dB SPL)	Genotype		7, 8	13, -1.55	0.15	Assumed

Chapter 5

5 General Discussion

5.1 Establishing a Rodent Model for Neurodevelopmental Disorders

The establishment of valid and reliable animal models for neurodevelopmental disorders is necessary to develop and test treatments for these complex disorders. They also allow researchers to establish the importance of certain risk genes and to analyze the underlying neural mechanisms through which these genes act (Servadio, Vanderschuren, & Trezza, 2015).

Chapter 2 of this thesis aimed to determine if a functional loss of the *Cntnap2* gene is sufficient to cause ASD-related phenotypes. Rats with a homozygous or heterozygous mutation in the *Cntnap2* gene (*SD-Cntnap2^{tm1Sage}*) were compared to wildtype controls across various translational behavioural tasks, including tasks assessing social behaviour and restrictive and repetitive behaviour. Consistent with other *Cntnap2* models, knockout rats displayed reduced social interactions, increased repetitive behaviour, and hyperlocomotion (Brunner et al., 2015; Penagarikano et al., 2011; Thomas, Schwartz, Saxe, & Kilduff, 2016). Given ASD diagnosis is based on behavioural differences in (1) social interaction and communication impairments and (2) restrictive and repetitive patterns of behaviour, interests or activities, the face validity of this model has been established. Unique to my studies, I was able to demonstrate that a loss of *Cntnap2* function also led to both altered pre-attentive (i.e., an increased acoustic startle response and poorer sensorimotor gating), and perceptual behaviour (i.e., increased sound avoidance, unaltered sound intensity categorization, and increased multisensory temporal binding) akin to what occurs in ASD (Bonnell et al., 2010; Foss-Feig, Schauder, Key, Wallace, & Stone, 2017; Khalfa et al., 2004; Kuiper, Verhoeven, & Geurts, 2019; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016; Stevenson et al., 2014; Takahashi, Komatsu, Nakahachi, Ogino, & Kamio, 2016; Takahashi et al., 2014); collective results that strengthen the *SD-Cntnap2^{tm1Sage}* model's validity and utility to explore the mechanisms through which

Cntnap2 acts to alter sensory behaviour. This work was published in *Autism Research* (Scott et al., 2020). In addition to establishing the validity of animal models, it is also critical to use the most translatable phenotypes to close the species gap in research findings, and to establish the validity of the behavioural paradigms used (Kas et al., 2014). The auditory behaviours used in this thesis are explored within this conceptual framework in a commentary article submitted to *Autism Research*, where it has been favourably reviewed and invited for resubmission following minor revisions (Appendix B).

I have also demonstrated reliability in pre-attentive auditory behaviour in the SD-*Cntnap2*^{tm1Sage} rat model. In Chapter 3, the acoustic startle response and sensorimotor gating were tested in a different colony of rats that were bred using a different breeding scheme, again finding an increased acoustic startle response and poorer PPI. This work was published in *The Journal of Neuroscience* (Scott et al., 2018). Of note is the current lack of reliability in the various *Cntnap2* knockout mouse model literature on prepulse inhibition. Three studies have examined PPI in the *Cntnap2*^{-/-} mouse model (B6.129 (Cg)-*Cntnap2*^{tm1Pele/J}), finding no differences in PPI (Penagarikano et al., 2011), poorer PPI (Truong, Rendall, Castelluccio, Eigsti, & Fitch, 2015), or improved PPI (Brunner et al., 2015). Because autism is a spectrum caused by different genetic/environmental impacts, there is heterogeneity in the presence of PPI differences in individuals with autism (Sinclair et al., 2016). However, consistency is needed within a single genetic model to be able to untangle the role that gene plays in the manifestation of a behavioural difference. Therefore, the replication of the increased acoustic startle response and poor PPI found in my two separate studies using the SD-*Cntnap2*^{tm1Sage} model strengthens the rationale for using it to study the mechanisms underlying altered pre-attentive acoustic behaviour, and increases the significance and confidence level of the drawn conclusions.

5.2 Functional Loss of *Cntnap2*: Role in the Auditory System Across Development

In language related neurodevelopmental disorders, including ASD and DLD, auditory information is processed differently in the brainstem and cortex (Baum, Stevenson, & Wallace, 2015; O'Connor, 2012; Sinclair et al., 2016). Previous studies have demonstrated

a delayed neurotransmission throughout the successive relay nuclei of the brainstem, a delayed cortical response reflecting immaturity in the auditory cortex, and temporal processing impairments (Berman et al., 2016; Brandwein et al., 2015; Bruneau, Roux, Adrien, & Barthélémy, 1999; Edgar et al., 2015; Fujikawa-Brooks, Isenberg, Osann, Spence, & Gage, 2010; Gonçalves, Wertzner, Samelli, & Matas, 2011; Høyland et al., 2019; Kwok, Joanisse, Archibald, & Oram Cardy, 2018; Kwon, Kim, Choe, Ko, & Park, 2007; Magliaro, Scheuer, Assumpcao Junior, & Matas, 2010; Matsuzaki et al., 2014; Miron et al., 2016; Oram Cardy et al., 2004; Oram Cardy, Flagg, Roberts, & Roberts, 2008; Port et al., 2016; Roberts et al., 2010, 2019; Rosenhall, Nordin, Brantberg, & Gillberg, 2003; Roth, Muchnik, Shabtai, Hildesheimer, & Henkin, 2012; Samson et al., 2011; Stroganova et al., 2013; Tas et al., 2007; Wong & Wong, 1991). Chapters 3 and 4 of this thesis aimed to determine if the loss of *Cntnap2* function would lead to altered auditory processing as is seen in ASD and DLD. Furthermore, I examined the mechanisms through which *Cntnap2* acts to alter auditory processing by comparing brainstem and cortical neural processing differences between *Cntnap2* knockout rats and wildtype controls. I found a reduction in response amplitudes and a delay in response latency of the ABR (a.k.a. BAEP) in juvenile *Cntnap2* knockout rats compared to age-matched controls that normalized by adulthood, revealing a delayed maturation of auditory processing pathway similar to the findings in autistic people. This work was published in *The Journal of Neuroscience* (Scott et al., 2018). Despite recovery of the ABR in adulthood, cortical auditory function remains altered. Specifically, a functional loss of the *Cntnap2* gene causes (1) immature cortical evoked potentials reflected by delayed latencies and increased amplitudes of the N1 and P2 potentials, (2) delayed multi-unit response latencies, (3) impaired temporal processing, and (4) a pattern of hyper-excitability in both multi-unit and single cell recordings that includes an increased firing rate in multi-unit spiking activity, lower spike release thresholds, and cell membrane channels that opened at lower voltages and remained open for longer periods of time. Ultimately, these results show that a loss of *Cntnap2* function results in a profile reflecting immature cortical auditory processing that parallels differences reported in ASD and DLD. Moreover, the increased cortical excitability caused by the loss-of-function of the *Cntnap2* gene may underlie these alterations. These findings

speak to the excitatory/inhibitory imbalance theoretical model of ASD (Baum et al., 2015; Rubenstein & Merzenich, 2003), which suggests that excitation may be increased, affecting cortical functioning. In line with this theory, Rubenstein and Merzenich (2003) noted that genetic predisposition or an altered environment that leads to hyper-excitability could predispose the cortex to epilepsy or other forms of brain dysfunction like that observed in autism. Indeed, in addition to the ASD-like behavioural phenotype, grand mal seizures were observed in some adult *Cntnap2*^{-/-} male rats.

In Chapter 4, the effect of the *Cntnap2* gene on the inhibitory and excitatory systems was investigated by comparing the expression patterns of a marker for glutamatergic activity, VGluT1, as well as for GABAergic activity, Gad67, in the auditory cortex of *Cntnap2*^{-/-} rats to that of wildtypes. Interestingly, using standard immunohistological procedures and analyses, no genotypic differences were found in either VGluT1 or Gad67 expression. Within the rodent literature on ASD models, there is variability as to whether or not differences exist in the expression of cortical excitatory or inhibitory markers, and it appears that these results largely depend on the region of interest (reviewed in Cellot & Cherubini, 2014). For example, when the cortical region was not discriminated, the level of protein expression as measured by western blot was found to be increased for VGluT1 and decreased for Gad67 in a VPA rat model (Kim et al., 2013). However, when various cortical regions are separated, a different pattern can emerge. For example, in the VPA rat model used by Hou and colleagues (2018), no difference in Gad67 expression was found in the somatosensory cortex, whereas VPA rats exhibited reduced expression in the temporal cortex, and increased expression in the prefrontal cortex as measured by immunohistochemistry. Similarly, neuroligin-3 knockout (model for ASD) mice show no differences in the protein expression of VGluT1 in the somatosensory cortex (Tabuchi et al., 2007). Moreover, findings also differ depending on the model of use. For example, in contrast to findings in the VPA model, the prefrontal cortex of a prenatal immune activation model had reduced levels of Gad67 mRNA in the prefrontal cortex (Labouesse, Dong, Grayson, Guidotti, & Meyer, 2015), and a mouse model lacking *Mecp2* gene function (Rett Syndrome linked gene) from GABA releasing neurons also had a reduction of Gad67 in the forebrain (Chao et al., 2010). See section 5.5 for experimental limitations.

5.3 Separating the Auditory Processing and Acoustic Behaviour Phenotypes

In Chapter 3, I postulated that increased central gain in the auditory brainstem during development may explain poorer PPI in the *Cntnap2*^{-/-} rats, but questioned this relationship in adulthood. I was unable to draw a strong relationship between the delayed maturation of the ABR and the altered pre-attentive acoustic behaviours in *Cntnap2*^{-/-} rats. Preliminary data re-evaluating the ABR, ASR, and PPI in *Cntnap2* knockout rats further calls into question the relationship between brainstem auditory processing in the central auditory pathway and brainstem- and midbrain-mediated acoustic behaviour. While Chapter 3 was conducted using a mixed breeding strategy, using a heterozygous breeding strategy, the preliminary data set does not find any differences in the ABR of *Cntnap2*^{-/-}, *Cntnap2*^{+/-}, or wildtype littermates in juvenile, adolescent or adult animals, but again reveals an increased ASR and poorer PPI in the *Cntnap2* knockouts across age. First, this difference in the results based on breeding strategy highlights the independence of the pre-attentive auditory behaviours from auditory processing in the brainstem. Second, it suggests that the genotype of the dam and littermates a pup is reared with affects the development of the auditory system.

In the mixed breeding strategy (i.e., Chapter 3), three different crossings were used. *Cntnap2*^{-/-} rats were obtained from homozygous knockout crossings (i.e., both the dam and buck were *Cntnap2*^{-/-}), *Cntnap2*^{+/-} rats from crossings of a wildtype dam and *Cntnap2*^{-/-} buck, and wildtype rats from wildtype crossings. Using this strategy, each litter was comprised of only a single genotype, and only the homozygous *Cntnap2* knockout litter was reared by a *Cntnap2*^{-/-} dam. In the heterozygous breeding strategy (i.e., preliminary data), both the dam and buck were heterozygous for the *Cntnap2* gene mutation, and a mixed litter of *Cntnap2*^{-/-}, *Cntnap2*^{+/-}, and wildtype pups were born and reared together by the heterozygous dam. Therefore, the rearing environment differs in the two studies, resulting in two different *Cntnap2*^{-/-} rat profiles of auditory system development. Studies of altered input during development, such as those caused by transient hearing loss, have illustrated the important role of normal sensory experience for typical auditory maturation

(Mowery, Kotak, & Sanes, 2015). To my knowledge, USV's of adult female *Cntnap2*^{-/-} rats have not been recorded to determine if maternal vocalization differences could be the cause of differential sensory input during development; although, differences in the ultrasonic vocalizations of *Cntnap2*^{-/-} and wildtype juvenile mice have been documented (Burkett, Day, Peñagarikano, Geschwind, & White, 2015; Penagarikano et al., 2011). Thus, the question remains if knockout pups bred via homozygous crossings are exposed to fewer vocalizations by their dams or littermates during development which ultimately influences their auditory system maturation. Given the influence of breeding strategy on the electrophysiological phenotype, the *SD-Cntnap2*^{tm1Sage} model could prove to be very useful in future studies exploring gene × environment interactions, as this thesis has shown that, despite a genetic mutation, malleability remains in the *Cntnap2* knockout rat phenotype.

5.4 Exploring Common Mechanisms in ASD

Given that my collective work has shown that a loss of *Cntnap2* function affects auditory processing and behaviour, I contend that future effort to explore the mechanisms through which these alterations might occur could ultimately help to uncover the common molecular pathways involved in auditory dysfunction in ASD. For example, the pattern of auditory processing and behavioural differences observed in the *Cntnap2* knockout rat are most similar to those reported in the VPA model (Engineer et al., 2014; Gandal et al., 2010), a well-studied environmental model for ASD. Indeed, VPA exposure during human pregnancy is associated with autism and poorer language abilities in the child (Roulet, Lai, & Foster, 2013), and rodent studies have identified poorer PPI, a reduced number of vocalizations, and altered cortical auditory processing in VPA exposed pups (Engineer et al., 2014; Gandal et al., 2010). By comparing the downstream effects of a loss of *Cntnap2* function to what is observed in the auditory centers of the VPA model, we might be able to identify common differences that could underlie altered auditory processing in ASD. Moreover, the field could compare and contrast how the functional role of various risk genes (e.g., *Arid1b*, *Chd8*, *Pten*, *Reln*, *Shank3*, *Frm1*, *Syngap1*, *Mecp2*; Möhrle et al., 2020) aligns with the severity or pattern of behavioural phenotypes observed. For example, we could determine if genes involved in neuronal development and axonal guidance (e.g.,

Cntnap2, *Syngap1*, *Shank3*; Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; Pinto et al., 2014) give rise to a behavioural phenotype different from those genes involved in chromatin modification or transcriptional regulation (*Arid1b*, *Chd8*, *Fmr1*, *Mecp2*; Pinto et al., 2014). Alternatively, effort could be made to determine if functionally similar ASD-risk genes alter the molecular/cellular structure of behaviourally relevant brain regions in a comparable manner.

5.5 Experimental Limitations

One limitation of the SD-*Cntnap2*^{tm1Sage} model is that it is a loss-of-function mutation throughout life (i.e., traditional gene knockout). *Cntnap2* is expressed in the developing cortex as early as E14 and broadly throughout the brain during post-natal development because it is involved in both neurodevelopment and myelination (Rodenas-Cuadrado, Ho, & Vernes, 2014). Therefore, we are unable to disentangle the role of the loss-of-function during embryonic development, post-natal development, and adulthood. If a time-dependent, inducible, *Cntnap2* knockout was used, we could determine if a developmental loss of *Cntnap2* is sufficient to cause ASD-like features (e.g., cortical hyper-excitability), or if the continued lack of function is what leads to disorder. Moreover, a tissue-specific conditional *Cntnap2* knockout could help to identify which brain regions are most responsible for an observed deficit. Given that top-down modulation by higher auditory areas via the PPT have been shown to affect both the baseline magnitude of the ASR as well as its inhibition by prepulse stimuli (Fendt, Li, & Yeomans, 2001; Larrauri & Schmajuk, 2006), the selective inhibition of *Cntnap2* in, for example the AC vs. PPT vs. PnC, would help determine where the loss-of-function is most critical.

A second limitation within this thesis (Chapter 4) is the method used to assess the excitatory/inhibitory neurotransmitter systems. It is important to acknowledge that immunohistochemistry, which was used to stain for Gad67 and VGluT1, is a qualitative measure and is not standardized worldwide; methodological issues that, while not restricted to this thesis, nonetheless represent a limitation. Moreover, only a single marker for GABAergic signalling (i.e., Gad67) and glutamatergic signalling (i.e., VGluT1) was used. In the future, this limitation could be overcome by measuring protein expression using

western blot, as this would offer improved quantification. In addition to Gad67 and VGluT1, other molecules important for GABA and glutamate function could also be measured, including synthesis molecules (e.g., Gad67, Gad65), transporters (e.g., VGaT, VGluT1, VGluT2), or various receptor subunits (e.g., receptors: GABA_AR, GABA_BR, AMPAR, NMDAR, mGluR1-8; Kwakowsky et al., 2008; Luján, Shigemoto, & López-Bendito, 2005; Reiner & Levitz, 2018).

Lastly, the behavioural results of the pre-attentive and perceptual acoustic behaviours presented in this thesis were conducted independent from the electrophysiological assessments of brainstem and cortical auditory function. In the discussions of Chapters 3 and 4, I provide rationale for how the observed differences in the neural processing of auditory information may or may not explain the differences in behaviour. However, the direct relationship between the behavioural consequences of altered auditory information processing and neural processing itself in the *Cntnap2* knockout rats has yet to be established. Future studies should conduct behavioural and electrophysiological testing in the same animals, and determine if correlations exist between the degree of differences observed in their neural and behavioural response.

5.6 Future directions

5.6.1 Exploration of Gene × Environment Interactions

Looking forward, one of the exciting possibilities of this rat model is that it provides researchers the opportunity to explore how the environment during development might interact with a loss-of-function of the *Cntnap2* gene. Because I have shown that the auditory brainstem response profile of *Cntnap2*^{-/-} rats is malleable, it calls into question if the auditory phenotype is due to a genetic mutation and/or the altered rearing environment. To untangle this interaction, cross-fostering studies could be conducted so that wildtype, *Cntnap2*^{+/-}, and *Cntnap2*^{-/-} pups are reared by either a wildtype or *Cntnap2*^{-/-} dam. In addition, studies using environmental enrichment could attempt to recover deficits in social behaviour, repetitive behaviour, or auditory behaviour, such as using acoustic enrichment in the form of daily exposures to sounds varied in timing, frequency, and location to

improve auditory processing (Zhu et al., 2014). Finally, the combinatorial effects of an environmental risk factor and a genetic risk factor could be explored by exposing the *Cntnap2*^{+/-} dam to poly(I:C) during pregnancy (using a heterozygous breeding strategy).

5.6.2 Test Predictive Validity

The predictive validity of the *Cntnap2* knockout rat model has yet to be established. Pharmacological manipulations, such as the use of mGluR5 antagonists, R-baclofen (a GABA-B receptor antagonist), risperidone (an atypical antipsychotic that mainly act through serotonin and dopamine antagonism), as well as oxytocin (a neuropeptide), have been shown to correct some behavioural differences in humans and/or other rodent models for neurodevelopmental disorders (Lee et al., 2015; Peñagarikano et al., 2015; Servadio et al., 2015; Silverman et al., 2015). Using such manipulations in *Cntnap2* knockout rats could verify the model's predictive validity if the *Cntnap2*^{-/-} rats mimic the effect seen in humans. These methods could also help to determine the mechanisms through which the *Cntnap2* gene acts.

5.6.3 Discern the Relationship between Altered Auditory Processing and its Behavioural Manifestations

To be able to discern the relationship between altered brain function and behaviour in *Cntnap2* knockout rats, electrophysiology and behaviour must be conducted in the same animals. For example, the neural basis of increased ASR was not elucidated in my studies. The acoustic startle response has a short and conserved pathway, with information passing through the auditory nerve (and cochlear root nucleus in rodents), to the caudal pontine reticular nucleus (PnC), before reaching the spinal cord (Koch, 1999). Given that (1) the neural response arising from the cochlear nucleus is typical in *Cntnap2*^{-/-} knockout rats (i.e., ABR peak I; Chapter 3, 4) and (2) I have shown the presence of Caspr2 in the cochlear root neurons and the giant neurons of the PnC (Chapter 3), the increase in the ASR in *Cntnap2*^{-/-} rats is likely due to differences in PnC function. By testing the ASR in wildtype and *Cntnap2*^{-/-} rats and then recording the activity of the PnC using *in vivo* electrophysiology, we could determine the relationship between PnC neural output and ASR magnitude and latency. Another study could determine how, if at all, hyper-

excitability in the auditory cortex is related to increased sound aversion and intact sound perception. This could be achieved by pharmacologically manipulating the AC to reduce excitability (e.g., with a GABA agonist such as R-baclofen), and monitoring the resulting effects on the perceptual acoustic behaviours.

5.7 Conclusions

Overall, the work in this thesis characterized the role of the autism-linked gene *Cntnap2* in autism-related behaviours and auditory processing in the brainstem and the cerebral cortex. The functional loss of *Cntnap2* is sufficient to cause ASD-related alterations in social behaviour and restricted and repetitive behaviours, thereby validating the SD-*Cntnap2*^{tm1Sage} model for studying ASD. Moreover, differences in pre-attentive and perceptual auditory behaviours in *Cntnap2* knockout rats align with what is reported in autistic people, indicating this model can also be used to study the relationship between altered sensory processing and the more complex diagnostic behaviours for ASD (Chapter 2). In addition, this body of work highlights the importance of the *Cntnap2* gene in sound processing in the brainstem, with a loss of *Cntnap2* function leading to delayed maturation of brainstem auditory processing (Chapter 3). Lastly, the functional loss of *Cntnap2* results in a lasting profile of immaturity and hyper-excitability in the auditory cortex (Chapter 4). Taken together, these studies provide new insight into the role of the *Cntnap2* gene in auditory processing and the behaviours associated ASD.

5.8 References

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Appendices

Appendix A: Immature Neural Population Activity and Hyper-excitability in the Auditory Cortex of Rats with a Functional Loss of the Autism-linked Gene *Cntnap2* - *In-vitro* electrophysiological recordings

Materials and Methods

Slice preparation. Sprague-Dawley wildtype (n = 6) and homozygous knockout (*Cntnap2*^{-/-}; n = 4) rats were anesthetized with isoflurane and their brains quickly removed and transferred into ice-cold slicing solution containing (in mM): 2.5 KCl, 1.25 NaH₂PO₄-H₂O, 24 NaHCO₃, 10 MgSO₄, 11 glucose, 234 sucrose, 2 CaCl₂, 3 Myoinositol, 2 Na-Pyruvate, and 0.4 ascorbate; equilibrated with 95% O₂/5% CO₂. Coronal slices (3.7 and 4.5 mm caudal to bregma) of 300 μm thickness were cut with a vibrating microtome (Compressotome VF-200) in a chamber filled with ice-cold preparation solution, and subsequently transferred into a holding chamber filled with artificial cerebrospinal fluid (ACSF) containing (in mM): 3 KCl, 1.25 NaH₂PO₄-H₂O, 3 MgSO₄, 26 NaHCO₃, 124 NaCl, and 10 glucose; equilibrated with 95% O₂/5% CO₂. CaCl₂ (2mM) was added to the ACSF a few minutes before slices were transferred. ACSF was heated to ~35°C for 30 minutes in order to improve patching success, and the slices were left to rest for an additional 1 hour at room temperature to recover. Slices were kept at room temperature during the experiment.

Whole-cell recordings. Electrophysiological experiments were performed as reported previously (Bosch & Schmid, 2006; Simons-Weidenmaier, Weber, Plappert, Pilz, & Schmid, 2006; Zaman et al., 2017). Pyramidal cells from the auditory cortex layers 2/3 were visualized through an upright microscope (Zeiss Axioskop, Germany), equipped with an EMCCD camera (Evolve 512, Photometric, Tuscon, AZ). Recording electrodes were pulled on a P-97 Puller (Sutter Instrument, Novato, CA) from fabricated borosilicate glass capillaries (1B150F-4, OD: 1.50 mm, ID: 0.84 mm, World Precision Instruments, Sarasota, FL) and had 3 - 7 MΩ resistance when filled with an intracellular solution containing the following (in mM): 140 K-gluconate, 10 KCl, 1 MgCl₂, 10 HEPES, 0.02 EGTA, 3 Mg-

ATP, and 0.5 Na-GTP, pH adjusted to 7.3, 290–300 mosm/L). Signals were sampled at 5 kHz, amplified with Axopatch 200B, digitized with Digidata-1550, and analyzed using pClamp10.4 (all Axon Instruments, Molecular Devices, Sunnydale, CA). Only pyramidal cells with access resistance $< 25 \text{ m}\Omega$ were included in analyses, and parameters were monitored throughout recordings.

Whole-cell voltage clamp electrophysiology of pyramidal neurons in layers 2/3 of A1 (wildtype: $n = 6$ rats, 14 - 16 cells; *Cntnap2*^{-/-}: $n = 4$ rats, 8 - 13 cells) was conducted to assess the spontaneous and evoked EPSC activity, as well as cell capacitance and membrane resistance. The membrane potential was held at -70 mV for all voltage-clamp recordings. sEPSCs were assessed by 5 minute recordings of cell currents. For the evoked EPSCs, layers 5/6 of A1 were stimulated using a bipolar tungsten electrode (Science Products), and paired pulses were generated by a pulse generator (Master-8, AMPI, Israel) in wildtype and *Cntnap2*^{-/-} cells. To determine paired-pulse ratios, interstimulus intervals of 20, 50, 100, 108, 136, 191, 358 ms were used between the first and second stimulation pulses (Fig. A-1). Voltage clamp step recordings were measured by holding the membrane potential (for 300 ms) at -70 mV, hyperpolarizing to -90 mV, and subsequently increasing the holding potential by 10 mV increments until the holding voltage of +40 mV (Fig. A-1).

Current-clamp was used to assess resting membrane potentials in wildtype ($n = 6$ rats, 14 - 17 cells) and *Cntnap2*^{-/-} rats ($n = 4$ rats, 10 cells). Current clamp recordings were made at resting membrane potentials of the cells and involved 1 second long step current injections in 40 pA steps from -120 pA to 480 pA. These recordings were used to assess firing threshold, rheobase, action potential features and kinetics, inter-spike intervals, and firing rates (Fig. A-2).

Offline Analysis. The frequencies and amplitudes of sEPSCs were analyzed in MiniAnalysis (Synaptosoft, Fort Lee, NJ, USA). Voltage clamp step recordings, eEPSCs, and all current clamp recordings were analyzed in pClamp (Molecular Devices). The amplitudes of eEPSCs were taken from the EPSC generated by the first pulse across all the ISIs presented. The paired pulse ratio was calculated as the amplitude of the second eEPSC

divided by the first eEPSC. Action potential half-width was measured as the width (ms) of an action potential at half its amplitude (firing threshold-peak). Action potential after-hyperpolarization was examined for the first action potential. The fast trough was measured as the difference between the AP baseline and trough within 5 ms following the action potential, while slow trough was the difference between the baseline and the lowest trough between two successive action potentials. Inter-spike interval was the time from the baseline of the first action potential to the baseline of the second action potential. Action potential threshold was the baseline voltage of the first action potential, and the rheobase was the accompanying current that elicited the first action potential. For the firing rates, the number of spikes during the 1 s of step current stimulation were counted. Data analyses were performed with pClamp10.4 (Molecular Devices), MiniAnalysis software (Synaptosoft, Fort Lee, NJ, USA), and/or Microsoft Excel 2010 (Microsoft Corp.).

Statistics. Genotypic differences in pyramidal cell function was assessed using a series of independent samples t-tests for cell properties including sEPSC frequency and amplitude, resting membrane potential, cell capacitance, spike firing threshold, rheobase current, and action potential features comprising of peak amplitude, half-width, fast trough, slow trough, and inter-spike interval. The paired-pulse ratios were analysed using a two-way RM-ANOVA for inter-stimulus interval (20, 50, 100, 108, 136, 191, 358 ms) \times genotype (wildtype, *Cntnap2*^{-/-}). A two-way RM-ANOVA for voltage holding level (-90, -80, -70, -60, -50, -40, -30, -20, -10, 0, 10, 20, 30, 40 mV) \times genotype (wildtype, *Cntnap2*^{-/-}) followed by *post-hoc* Bonferroni-corrected t-tests were completed for genotype to assess differences in the rapid influx currents between wildtype and *Cntnap2*^{-/-} cells. Next, to determine the voltage required to elicit a current influx, a one-way ANOVA for voltage holding level (-90, -80, -70, -60, -50, -40, -30, -20, -10, 0, 10, 20, 30, 40 mV) within each genotype was performed followed by *post hoc* Dunnet's t-tests comparing each voltage holding level to -90 mV. The influence of genotype on the relationship between current injection level and number of spikes released was assessed using a two-way RM-ANOVA for current injection level (0.08, 0.12, 0.16, 0.20, 0.24, 0.28, 0.32, 0.36, 0.40, 0.44 nA) \times genotype (wildtype, *Cntnap2*^{-/-}) followed by *post-hoc* Bonferroni-corrected t-test for genotype.

Results

There were also no differences in the age of rats used for *in vitro* electrophysiological slice recordings (wildtype 91 ± 10 days, *Cntnap2*^{-/-} 87 ± 7 days; $p = 0.77$).

Presynaptic neuron function is typical in the auditory cortex of *Cntnap2*^{-/-} rats

In vitro whole-cell patch clamp electrophysiology was used to compare the synaptic properties of neurons in layers 2/3 of the primary auditory cortex (A1) from adult wildtype ($n = 6$) and *Cntnap2*^{-/-} rats ($n = 4$). In voltage-clamp configuration, the frequency (Fig. A-1B) and amplitude (Fig. A-1C) of spontaneous excitatory post-synaptic currents (sEPSCs), reflective of the brief depolarizations of the post-synaptic membrane as a result of spontaneous neurotransmitter release from presynaptic neurons, were found to be unchanged between the genotypes (wildtype: $n = 19$ neurons; *Cntnap2*^{-/-}: $n = 13$ neurons). To further investigate presynaptic function, evoked EPSCs (eEPSCs) were elicited by stimulating layers 5/6 of A1 with a bipolar electrode (Fig. A-1D) using a paired-pulse stimulation protocol, as the relative amplitude evoked by the second pulse can be attributed to altered transmitter release from the presynaptic terminal. Ultimately, assessment of the paired-pulse ratio across the range of inter-stimulus intervals (ISI) that matched those used to assess multi-unit temporal processing (i.e., 108 ms ISI = 9.2 pps, 136 ms ISI = 7.4 pps, 191 ms ISI = 5.2 pps, 358 ms ISI = 2.8 pps; Fig. A-1E) did not reveal differences between the wildtype and *Cntnap2*^{-/-} neurons at any ISI (Fig. A-1F). Overall, the consistent results between the genotypes in the frequency and amplitude of the sEPSC as well as the paired-pulse ratios suggest that a loss of *Cntnap2* did not alter neurotransmitter release or synaptic receptor densities.

Post-synaptic neurons lacking *Cntnap2* require less stimulation for depolarization and spike release

To assess the intrinsic cell properties of pyramidal neurons in layers 2/3 of the primary auditory cortex from adult wildtype and *Cntnap2*^{-/-} rats, a voltage-clamp configuration was used to measure rapid influx currents (normalized to cell capacitance), current half-width,

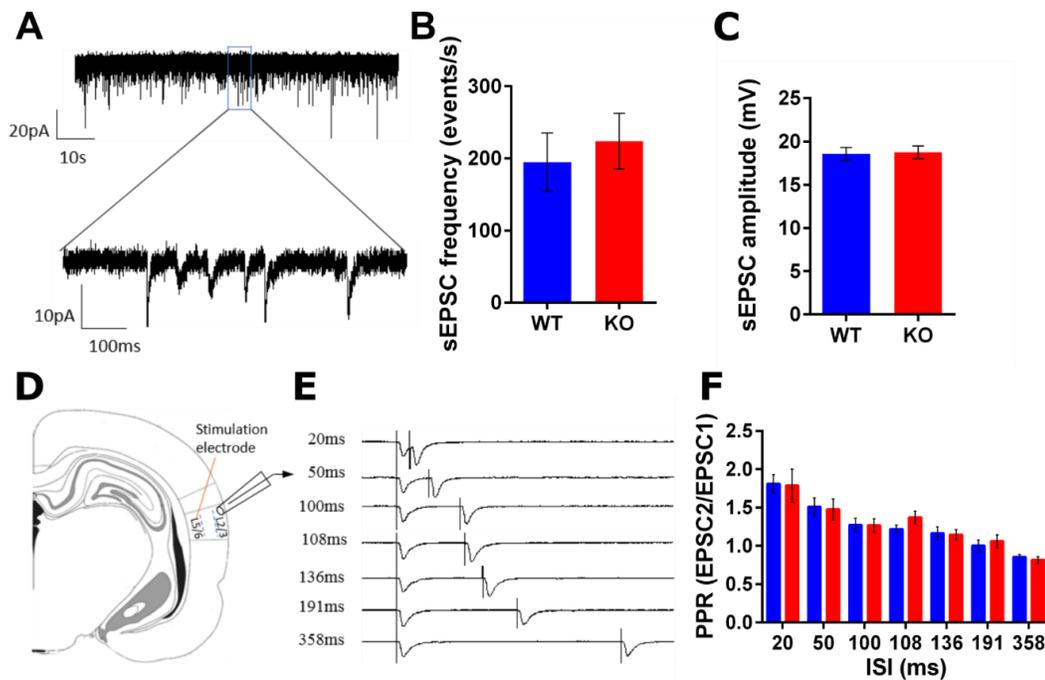


Figure A-1: *Cntnap2*^{-/-} neurons are synaptically comparable to wildtype neurons.

(A) Sample recording trace of spontaneous EPSCs from the auditory cortex of an adult wildtype rat with the (B) mean frequency and (C) mean amplitudes. (D) Representative recording areas including the primary auditory cortex (A1) in the rat, accompanied by the positions of the recording and stimulating electrodes in layers 2/3 and layers 5/6, respectively. (E) Sample traces of paired pulse evoked EPSCs from a wildtype rat at various inter-stimulus intervals, vertical lines represent stimulation pulses, and (F) paired-pulse ratios (amplitude of second EPSC divided by first EPSC). All data are represented as mean \pm standard error (B, C, wildtype $n = 19$ cells, *Cntnap2*^{-/-} $n = 13$ cells; F, wildtype $n = 12$ cells, *Cntnap2*^{-/-} $n = 6$ cells). * $p < 0.05$.

resting membrane potential, cell capacitance, and membrane resistance. Across the full range of voltage levels (-90 to 40 mV), the rapid influx currents were altered in *Cntnap2*^{-/-} neurons compared to wildtypes (genotype \times voltage holding level: $p = 0.002$; Fig. A-2B). For example, in addition to a larger current amplitude (normalized current at -90 – 40 mV: $p_{Bonf} < 0.001$), suggestive of a larger influx of sodium ions during depolarization, the *Cntnap2*^{-/-} neurons showed that current influx could be elicited at a lower voltage (*Cntnap2*^{-/-}: -40 mV vs. wildtype: -30 mV; Fig. A-2B), indicative of a lower threshold for sodium channel opening in neurons lacking *Cntnap2*. Despite this apparent increase in the excitability of the *Cntnap2*^{-/-} neurons, the resting membrane potential was not different

between the genotypes (Fig. A-2C). The *Cntnap2*^{-/-} neurons had smaller membrane capacitances ($p < 0.001$; Fig. A-2D), which could be explained by their lower membrane resistances (wildtype: 44.05 ± 3.75 vs. *Cntnap2*^{-/-}: 28.27 ± 13.22 ; $p = 0.01$). The half-widths of the inward currents were also found to be longer in *Cntnap2*^{-/-} neurons (wildtype: 1.2 ± 0.03 ms vs. *Cntnap2*^{-/-}: 1.65 ± 0.07 ms; $p < 0.001$). With respect to spiking dynamics, the *Cntnap2*^{-/-} neurons exhibited lower spike firing thresholds ($p = 0.01$; Fig. A-2F) and lower rheobase currents ($p = 0.02$; Fig. A-2G); findings which further suggest an increased intrinsic excitability in neurons lacking *Cntnap2*.

Both action potential features and spiking kinematics are altered in cortical neurons with a developmental loss of *Cntnap2*

Considering that genotypic differences were observed in the sound-evoked multi-unit firing rates *in vivo*, patch clamp electrophysiological recordings of auditory cortex neurons were performed to investigate whether a developmental loss of *Cntnap2* altered action potential features and kinetics. Using a current-clamp configuration, it was found that, compared to those from wildtype rats, *Cntnap2*^{-/-} neurons had decreased action potential peak amplitudes ($p = 0.008$; Fig. A-3B) and exhibited longer action potential half-widths ($p < 0.001$; Fig. A-3C). Furthermore, although the fast trough of the after-hyperpolarization was not different between genotypes (Fig. A-3D), the slow trough component was larger in *Cntnap2*^{-/-} neurons ($p = 0.03$; Fig. A-3E). Despite these differences, the inter-spike intervals were not significantly different in *Cntnap2*^{-/-} neurons ($p = 0.07$; Fig. A-3F). Overall, the longer half-width and larger after-hyperpolarization suggest that *Cntnap2*^{-/-} neurons have slower rectifying currents than wildtype neurons. Coupled with the increased excitability (i.e., lower spike threshold), these features of action potentials resulted in an altered firing rate profile (i.e., significant interaction between genotype \times current injection level: $p = 0.001$; Fig. A-3G). More specifically, at low current injection levels, the *Cntnap2*^{-/-} neurons fired a greater number of spikes (e.g., at 0.12 nA: $p_{Bonf} = 0.05$); however, as the current injected was increased, the number of elicited spikes was less in the *Cntnap2*^{-/-} versus wildtype neurons (e.g., at 0.4 nA: $p_{Bonf} = 0.03$; Fig. A-2G).

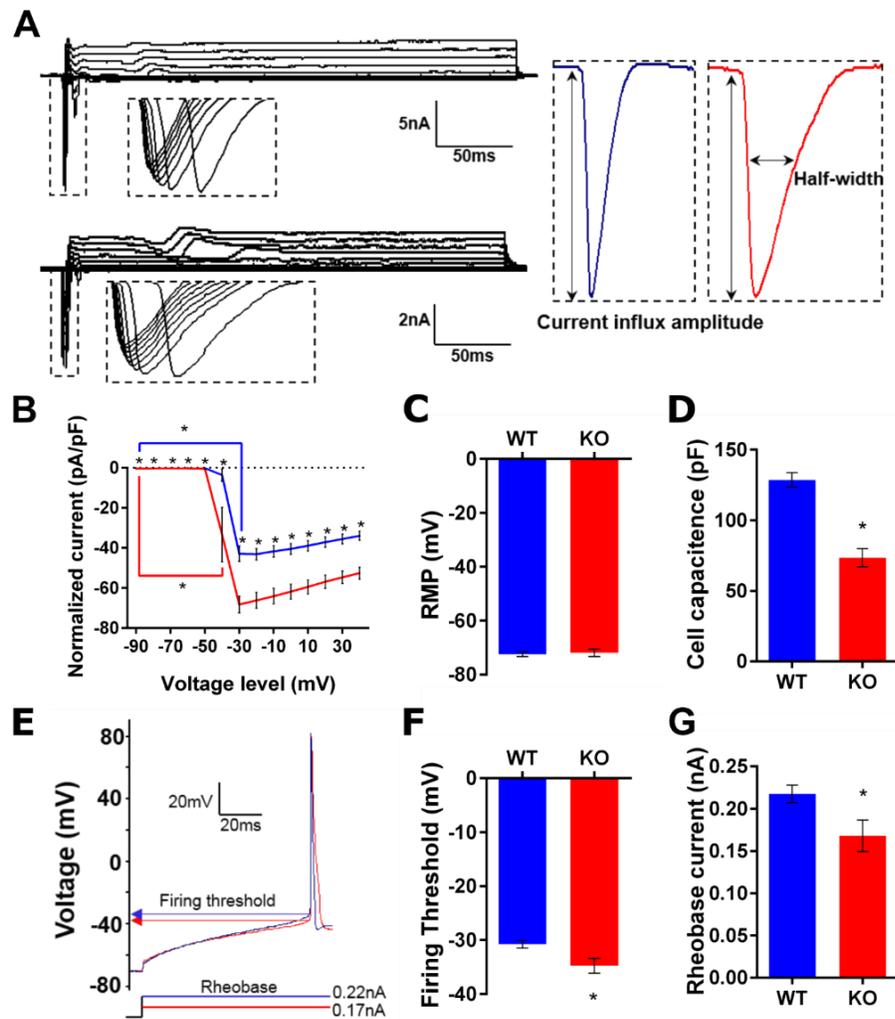


Figure A-2: *Cntnap2*^{-/-} neurons are generally more excitable.

(A) Sample voltage-clamp ramp recordings from wildtype (top left) and *Cntnap2*^{-/-} (bottom left) neurons, and representative magnified first spikes from wildtype (blue) and *Cntnap2*^{-/-} (red) voltage-clamp recordings. (B) Voltage clamp current recordings normalized to cell capacitance reveal *Cntnap2*^{-/-} fire at a lower voltage and have a greater current influx. Intrinsic neuron properties are presented as: (C) resting membrane potential and (D) cell capacitance. (E) Representative current-clamp recording of action potentials from wildtype (blue) and *Cntnap2*^{-/-} (red) neurons with firing threshold and rheobase indicated. The (F) action potential firing threshold and (G) rheobase current at which the neurons fire the first action potential reveal *Cntnap2*^{-/-} neurons are hyperexcitable. All data are represented as mean \pm standard error (B: wildtype n = 16 cells, C, D: wildtype n = 17 cells, F: wildtype n = 15 cells, G: wildtype n = 16 cells; B-G: *Cntnap2*^{-/-} n = 10 cells). * p < 0.05.

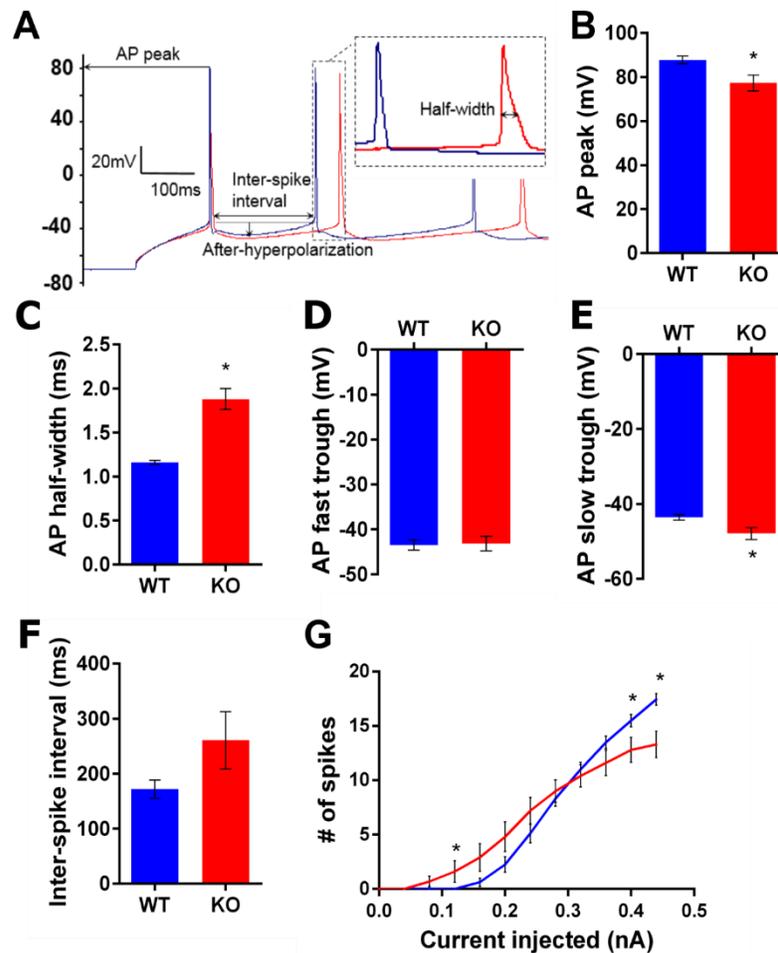


Figure A-3: *Cntnap2*^{-/-} neurons have delayed currents, fire more easily, but elicit fewer maximum spikes.

(A) Sample current clamp action potential recordings of wildtype (blue) and *Cntnap2*^{-/-} (red) neurons with labeled metrics of interest. Action potential features presented are (B) action potential peak voltage, (C) action potential half-width, (D) fast trough of after-hyperpolarization, (E) after-hyperpolarization slow trough, and (F) first inter-spike interval. (G) Firing frequency in response to increasing current injections. All data are represented as mean \pm standard error (B, G: wildtype n = 16 cells, C, F: wildtype n = 15 cells, D: wildtype n = 17 cells, E: wildtype n = 14 cells, B – G *Cntnap2*^{-/-} n = 10 cells). * p < 0.05.

Supplemental statistics table

Reference to methods section: Statistics	Figure number	Test	Measure of Interest	Dependent Variable(s)	Independent Variable(s)	Interactions and Main Effects	n: wildtype, <i>Cntnap2</i> ^{+/+}	Degrees of freedom, t-value OR F-value	P value	Levene's Test for Equality of Variances
Whole cell response properties	1B	Independent samples 2-tailed t-test	sEPSC frequency	events/s	Genotype		19, 13	30, 0.50	0.62	Assumed
	1C	Independent samples 2-tailed t-test	sEPSC amplitude	mV	Genotype		19, 13	30, 0.18	0.86	Assumed
	1F	2-way RM ANOVA	Paired-pulse ratio	EPSC2/EPSC1	Inter-stimulus interval x Genotype	Inter-stimulus interval x Genotype, Inter-stimulus interval, Genotype	12, 6	F(2,208, 35.33) = 0.29, F(2,208, 35.33) = 26.906, F(1, 16) = 0.056	0.77, <0.001, 0.82	NA
	2B	2-way RM ANOVA	Normalized Current	pA/pF	Voltage holding level x Genotype	Voltage holding level x Genotype, Voltage holding level, Genotype	16, 10	F(1,764, 38.798) = 8.114, F(1,764, 38.798) = 161.86, F(1, 22) = 19.08	0.002, <0.001, <0.001	NA
		post hoc analysis: Bonferromi corrected t-test			Genotype	+/- v.s. +/-			-90 mV = 0.013, -80 mV = 0.001, -70 mV = 0.003, -60 mV < 0.0001, -50 mV = 0.012, -40 mV = 0.036, -30 mV - 40 mV < 0.0001	NA
	2B	1-way ANOVA	Normalized Current - WT	pA/pF	Voltage holding level		16	F(13, 223) = 76.45	<0.001	NA
		post hoc analysis: Dunnet's t-test				-90 mV v.s. other mV			-.80 mV to -50 mV = 1.0, -40 mV = 0.94, -30 mV to 40 mV < 0.001	NA
	2B	1-way ANOVA	Normalized Current - WT	pA/pF	Voltage holding level		10	F(13, 139) = 42.63	<0.001	NA
		post hoc analysis: Dunnet's t-test				-90 mV v.s. other mV			-.80 mV to -50 mV = 1.0, -40 mV to 40 mV < 0.001	NA
	2C	Independent samples 2-tailed t-test	Resting membrane potential	mV	Genotype		17, 10	25, 0.363	0.72	Assumed
	2D	Independent samples 2-tailed t-test	Cell capacitance	pF	Genotype		17, 10	25, -6.67	< 0.001	Assumed
	2F	Independent samples 2-tailed t-test	Firing threshold	mV	Genotype		15, 10	23, -2.8	0.01	Assumed
	2G	Independent samples 2-tailed t-test	Rheobase current	nA	Genotype		16, 10	24, -2.52	0.02	Assumed
	NA	Independent samples 2-tailed t-test	Membrane resistance	MΩ	Genotype		17, 10	25, -2.87	0.01	Assumed
	NA	Independent samples 2-tailed t-test	Inward current half-width	ms	Genotype		16, 10	12.34, 6.21	<0.001	Not assumed
Whole cell response properties	3B	Independent samples 2-tailed t-test	Action potential peak	mV	Genotype		16, 10	24, -2.91	0.008	Assumed
	3C	Independent samples 2-tailed t-test	Action potential half-width	ms	Genotype		15, 10	9.637, 5.95	<0.001	Not assumed
	3D	Independent samples 2-tailed t-test	Action potential fast trough	mV	Genotype		17, 10	25, 0.18	0.86	Assumed
	3E	Independent samples 2-tailed t-test	Action potential slow trough	mV	Genotype		14, 10	12.97, -2.43	0.03	Not assumed
	3F	Independent samples 2-tailed t-test	Inter-spike interval	ms	Genotype		15, 10	23, 1.901	0.07	Assumed
	3G	2-way RM ANOVA	Firing frequency	# of spikes	Current injection level x Genotype	Current injection level x Genotype, Current injection level, Genotype	16, 10	F(2,37, 56.95) = 6.83, F(2,37, 56.95) = 269.65, F(1, 24) = 0.003	0.001, <0.001, 0.953	NA
		post hoc analysis: Bonferromi corrected t-test			Genotype	+/- v.s. +/-			0.080 nA = 0.07, 0.12 nA = 0.050, 0.16 nA = 0.051, 0.20 nA = 0.082, 0.24 nA = 0.16, 0.28 nA = 0.57, 0.32 nA = 0.60, 0.36 nA = 0.11, 0.40 nA = 0.03, 0.44 nA = 0.002	NA
Animal Characteristics	NA	Independent samples 2-tailed t-test	In vitro electrophysiology : Age	Age (days)	Genotype		6, 4	8, 0.31	0.77	Assumed

Appendix B: Closing the Species Gap: Translational Approaches to Studying Sensory Processing Differences Relevant for Autism Spectrum Disorder⁵

Abstract

The study of sensory phenotypes has great potential for increasing research translation between species, a necessity to decipher the neural mechanisms that contribute to higher-order differences in autism spectrum disorder (ASD). Over the past decade, despite separate advances in our understanding of the structural and functional differences within the brain of autistic and non-autistic individuals and in rodent models for ASD, researchers have been unable to directly translate the findings in murine species to humans, mostly due to incompatibility in experimental methodologies used to screen for ASD phenotypes. Focusing on sensory phenotypes offers an avenue to close the species gap because sensory pathways are highly conserved across species and are affected by the same genetic and environmental risk-factors as the diagnostic criteria for ASD. By first reviewing how sensory processing has been studied to date, we are able to direct our focus to electrophysiological and behavioural techniques that can be used to study and assess sensory phenotypes consistently across species. Using auditory sensory phenotypes as a template, we seek to improve the accessibility of translational methods by providing a framework for collecting cohesive data in both rodents and humans. Specifically, evoked-potentials, acoustic startle paradigms, and psychophysical detection/discrimination paradigms can be created and implemented in a coordinated and systematic fashion across species. Through careful protocol design and collaboration, sensory processing phenotypes can be harnessed to bridge the gap that exists between preclinical animal studies and human testing, so that mutually held questions in autism research can be answered.

⁵ A version of this appendix has been accepted with revisions in *Autism Research* Scott, K.E., Allman, B. L., Oram Cardy, J., Stevenson R. A., Schmid, S. (Submitted-AUR-20-0503), Closing the Species Gap: Translational Approaches to Studying Sensory Processing Differences Relevant for Autism Spectrum Disorder.

1. Sensory processing in ASD

The study of sensory processing in autism spectrum disorder (ASD) has rapidly gained traction in the last 20 years. Altered sensory processing is thought to be related to difficulty with adaptive behaviour and/or contribute to maladaptive behaviour, making it important to study in ASD (DuBois, Lymer, Gibson, Desarkar, & Nalder, 2017). Since the inclusion of sensory symptoms in the DSM-5, research has taken two approaches to studying sensory processing: the first focuses on identifying sensory symptoms in daily interactions, and the second on basic sensory detection and/or discrimination in research settings (reviewed in Burns, Dixon, Novack, & Granpeesheh, 2017; DuBois et al., 2017; Schauder & Bennetto, 2016). The first approach commonly uses assessment measures such as behavioural questionnaires, e.g. The Sensory Profile, and behavioural observation, e.g. Sensory Processing Assessment (Burns et al., 2017). Research using these metrics have identified an estimated 75% - 90% of children with ASD show sensory processing differences (Crane, Goddard, & Pring, 2009; Robertson & Baron-Cohen, 2017; Schoen, Miller, Brett-Green, & Nielsen¹, 2009; Talay-Ongan & Wood, 2000; Tomchek & Dunn, 2007). Using questionnaires, observations, and qualitative interviews, researchers have been able to identify sensory interactions that can be broken down to their simplest forms for further study. The second approach to studying sensory processing in ASD addresses basic sensory abilities that are thought to underlie complex behaviours (Baum, Stevenson, & Wallace, 2015; Schauder & Bennetto, 2016). Psychophysical behavioural assessments and neuroimaging are used to objectively evaluate sensory responses and the neural mechanisms underlying sensory processing (Baum, Stevenson, & Wallace, 2015; Robertson & Baron-Cohen, 2017; Schauder & Bennetto, 2016). Psychophysical behavioural studies are generally conducted in a lab setting, use objective stimuli, are tested on a continuum (i.e., non-binary), and have specific measurement procedures making them easily repeatable (DuBois et al., 2017); however, variability in protocols, criterion, task design, and stimulus type reduces the consistency of findings across studies and can make comparison difficult. Notably, neurophysiological responses to sensory stimuli can provide information regarding internal sensory experience and sensory information processing in the brain before a behaviour is generated. Although these neurophysiological responses

could prove complementary to traditional clinical assessments, to date, psychophysical testing methods have mostly been focused toward improving our understanding of the sensory differences in ASD.

2. Identifying translational potential

While perhaps less relevant for the daily experiences individuals have with their world, psychophysical behavioural and electrophysiological methods do find overlapping similarities with symptoms identified via questionnaires (DuBois et al., 2017; Schauder & Bennetto, 2016) and importantly they hold the highest translational potential between species. Sensory processing pathways are conserved across species and affected by the same genetic and environmental risk-factors as the diagnostic criteria of ASD, meaning they can be modelled in animals, unlike some of the phenotypes associated with autism that are intrinsically human in nature (e.g., speech development). Therefore, studying sensory processing using psychophysical methods across species offers a unique opportunity to improve the translation from pre-clinical animal studies to human testing.

One of the largest impediments to cross-species translation is the incompatibility of experimental protocols used to screen for the various behavioural or electrophysiological phenotypes in humans and rodents. As a consequence of this experimental incompatibility, over the past decade, research in humans and murine species examining the neural underpinnings of ASD have occurred mostly in parallel. In humans, neuroimaging studies have characterized the structural and functional differences within the brain of autistic and non-autistic individuals in an attempt to understand the neural differences underlying altered behavioural profiles associated with the disorder. At the same time, a variety of rodent models have been developed to uncover the molecular, cellular, and circuit-based mechanisms that drive autism-like phenotypes in rodents. Despite these separate advances, very little progress has been made in the direct translation between such mechanistic studies in rodents and the complex behavioural profiles observed in autistic individuals. Consequently, we are far from understanding the cellular mechanisms that contribute to the higher-order differences in ASD.

Box 1. Case example - Lack of coordination between species could lead to incorrect conclusions

Audiovisual temporal binding has been extensively studied in autistic and non-autistic individuals using both complex speech stimuli (e.g. /ga/; both sensory and semantic processing required) and simple flash-beep stimuli (Siemann, Veenstra-VanderWeele, & Wallace, 2020; Stevenson & Wallace, 2013; de Boer-Schellekens, Eussen, & Vroomen, 2013) finding that the presence of behavioural dysfunction in ASD may depend both on stimulus complexity and task design (Stevenson et al., 2014; Stevenson et al., 2014; de Boer-Schellekens, Eussen, & Vroomen, 2013). In psychophysical tasks where the synchrony of an auditory and visual stimulus must be judged, dysfunction is identified in ASD when speech stimuli are used but this is not always the case when simple stimuli are used. Since rats do not have speech, a team of preclinical researchers were able to design an analogous task for rats based on simple stimuli (Schormans et al., 2017), and conduct assessments of audiovisual temporal processing in a genetic rodent model for ASD (*Cntnap2* knockout), revealing a similarly intact integration profile to autistic individuals when simple flash-beep stimuli are used (Scott et al., 2020; Stevenson et al., 2014). These studies show the necessity of task similarity when comparing results across species – had the preclinical researchers only had a task using speech stimuli (added complexity of semantics can pose a cross-species translation hurdle) to compare to the human literature, they may have incorrectly concluded the behaviour of the rat model did not resemble audiovisual temporal processing in ASD.

This significant gap in translation exists largely because researchers have been unable to directly relate the findings in rodents with those in humans (and vice-versa). Put simply, humans and rodents have rarely been systematically tested using the same paradigms, and as such, it has not been possible to translate the relevant findings across species. While sensory research is becoming more prevalent in the literature, at this time more coordinated efforts in methodologies between human and animal research is needed.

3. Cross species translational framework to study sensory processing in ASD

The systematic testing of sensory phenotypes across species using psychophysical behavioural and electrophysiological methods will allow us to overcome the cross-species translational issue in ASD. Audition, vision, olfaction, and touch can be assessed with similar electrophysiological signatures, startle-based behaviours, and behavioural detection/discrimination paradigms in humans and rodents. But currently, few accessible, translatable paradigms exist that can provide valid and reliable metrics across species, and reduce the heterogeneity among protocols between and within human and animal research.

Here we use auditory sensory phenotypes to provide a template to develop paradigms with translational potential (Fig. 1). We emphasize interdisciplinary collaboration to develop and utilize cross-species assays in a targeted fashion to answer questions specific to sensory processing in ASD. Simplifying task design by stripping stimuli down to their basic characteristics (e.g., for acoustic stimuli, modulating intensity or temporal structure or frequency) and using as few instructions as possible in tasks of detection or discrimination, i.e. using implicit or very simple behaviours, one can remove potentially confounding cognitive variables such as motivation, distractibility, or the understanding of instructions. Implementing these changes to behavioural and electrophysiological assays will allow for

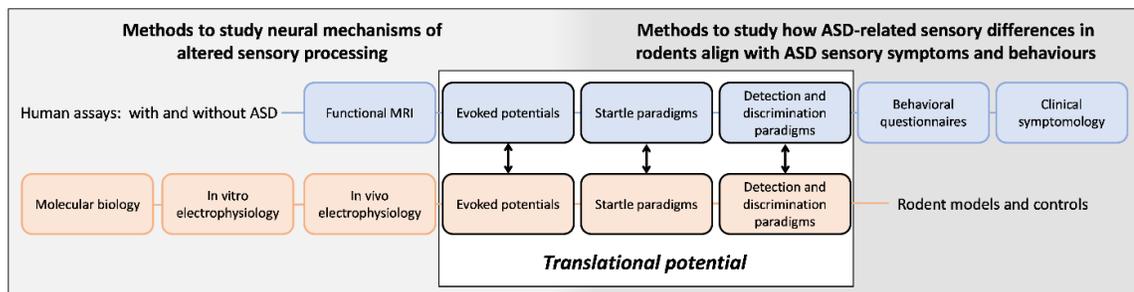


Figure 1: Translational framework for studying auditory processing differences relevant for ASD.

It is important to note, that multiple techniques should be used from the template framework and optimized for the respective species. All the proposed framework methods quantitatively measure the relationship between a controlled sensory stimulus and a subject's behavioural or electrophysiological response (e.g., detection, discrimination, and/or comfort thresholds).

clearer translation between humans and animal models. Only then can we determine the mechanisms contributing to ASD, untangle gene/environment interactions, or assess intervention (e.g., drug) efficacy.

a. Evoked potentials

Sensory-evoked potentials (i.e., event-related potentials) are the most accessible cross-species platform for studying the neural activity related to stimulus processing (utility for social stimuli processing reviewed in Modi & Sahin, 2017) (Fig. 2). In humans, electroencephalography (EEG) allows for the collection of the averaged electrical field generated by extracellular currents from large populations of neurons. Using surface electrodes, by varying the electrode placement/analysis, stimulus characteristics, and filtering frequency, the dynamics of the brain's response to an acoustic stimulus can be determined at the level of the brainstem (brainstem auditory evoked potentials [BAEP], a.k.a. auditory brainstem response [ABR]; (Ankmal-Veeranna, Allan, & Allen, 2019), midbrain (middle latency response [MLR]), and cortex (cortical auditory evoked potential [CAEP]; Kwok, Joanisse, Archibald, & Oram Cardy, 2018; Kwok et al., 2018). Similarly, in rodents, subdermal electrodes, bone screws, or in-vivo extracellular recordings allow for the collection of BAEPs (Scott et al., 2018), MLRs, and CAEPs (Wieczerzak et al, 2020). To date, evoked potentials have been utilized to assess the validity of animal models, and this approach has helped to uncover the neural mechanisms of stimulus processing, with a consistent finding of delayed BAEPs and CAEPs in a variety of human and rodent literature on altered sound processing in various neurodevelopmental disorders, including ASD (Ankmal-Veeranna et al., 2019; Kwok et al., 2018; O'Connor, 2012; Scott et al., 2018; Sinclair, Oranje, Razak, Siegel, & Schmid, 2016).

b. Acoustic startle paradigms

Behavioural paradigms relying on the acoustic startle response (ASR), that is the motoric reaction to an acoustic stimulus, have gained traction in the last 20 years for assessing acoustic reactivity in ASD (reviewed in Sinclair et al., 2016). Importantly, the acoustic startle pathway is short, well defined, and conserved across species (Koch, 1999), making

the study of the ASR a preserved and accessible behavioural readout of pre-attentive auditory function (Fig. 3). The acoustic startle response is elicited in humans and rodents by presenting a sudden, loud acoustic stimulus and assessed by measuring the contraction of the musculus orbicularis oculi using electromyography in humans (Takahashi et al., 2014), and the whole-body contraction using a movement-transducing platform in rodents (Valsamis & Schmid, 2011). Dependent on the symptom severity, autistic individuals and rodent models for ASD show increases in startle amplitudes and latencies (Scott et al., 2020, 2018; Sinclair et al., 2016; Takahashi et al., 2014; Takahashi, Nakahachi, Stickley, Ishitobi, & Kamio, 2017), and the ASR response is correlated with sensory behavioural questionnaires (Takahashi, Nakahachi, Stickley, Ishitobi, & Kamio, 2018). The ASR can be modulated by both internal and external stimuli, and has a non-zero baseline, meaning it can show both reduction and enhancement. Sensorimotor gating, a process that limits some sensory information from reaching higher cognitive centers, can be operationally measured using prepulse inhibition (PPI). PPI is the modification (i.e., reduction) of the ASR by a sensory event preceding the startling stimulus, called a prepulse stimulus, by 30 – 500 msec (Hoffman & Ison, 1980). Typically, when a non-startling acoustic prepulse precedes a startling stimulus, there is a reduction in the ASR; however this process is disrupted in ASD and other neurodevelopmental disorders, and the models thereof (Scott et al., 2020, 2018; Sinclair et al., 2016; Takahashi et al., 2014).

When designing ASR paradigms, it is important to remember that numerous factors, including features of the stimulus (e.g., its intensity, length, and rise time), the sensory environment, the subject's internal state (e.g., attention, anxiety), genetics, and medication/drugs, will all influence the response. Therefore, it can be difficult to compare between studies that have used different test protocols (Koch, 1999). This again highlights the need for collaboration between research groups to enable consistent testing parameters.

c. Psychophysical paradigms: detection and discrimination

Compared to the use of acoustic startle paradigms, psychophysical testing can assess sensory processing at a higher-cognitive level. This is possible because psychophysical testing can be designed so that subjects are essentially asked to report their perception of a

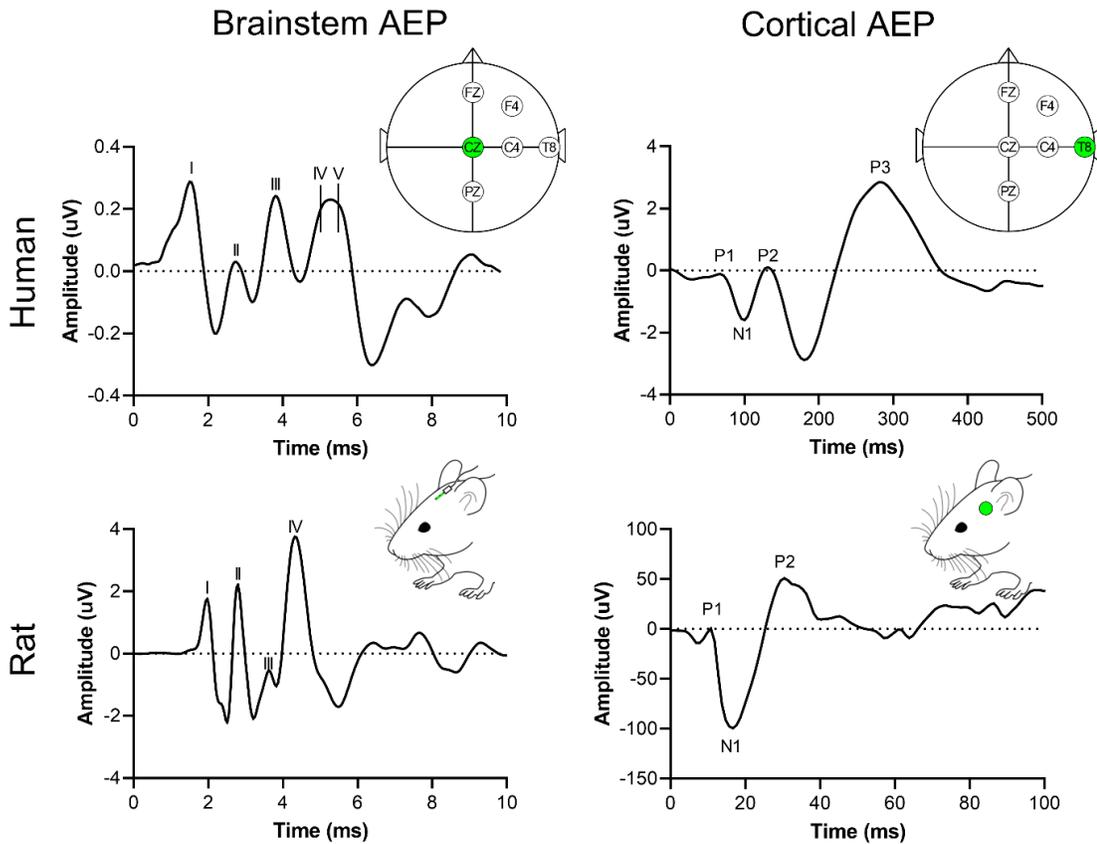


Figure 2: Mean auditory evoked potentials from the brainstem and cortex of children with no developmental concerns and adult wildtype rats.

Top Left BAEP from children aged $\sim 4 - 16$ ($n = 22$) recorded from position CZ. Adapted from Ankmnal-Veeranna et al., 2019 (Figure 7) with permission from © Georg Thieme Verlag KG. Bottom Left BAEP from rats ($n = 17$) recorded with a subdermal electrode positioned at the vertex of the scalp, adapted from data presented in Scott et al., 2018. In both humans and rats BAEPs, activity from the auditory nerve is best represented by peak I. While the cochlear nucleus is represented by peak III in humans, peak II best represents this activity in rats. Lastly, the activity from the lateral lemniscus is seen in peak V in humans and peak IV in rats. Top Right CAEP from children aged $\sim 7 - 10$ ($n = 67$) recorded from position T8. Adapted from Kwok et al., 2018 (Figure 1) with permission from John Wiley and Sons. Bottom Right CAEP from rats ($n = 10$) recorded with an epidural screw electrode positioned over the auditory cortex. Adapted from Wiczerzak et al., 2020 (Figure 2A) with permission from Elsevier. Green denotes recording position.

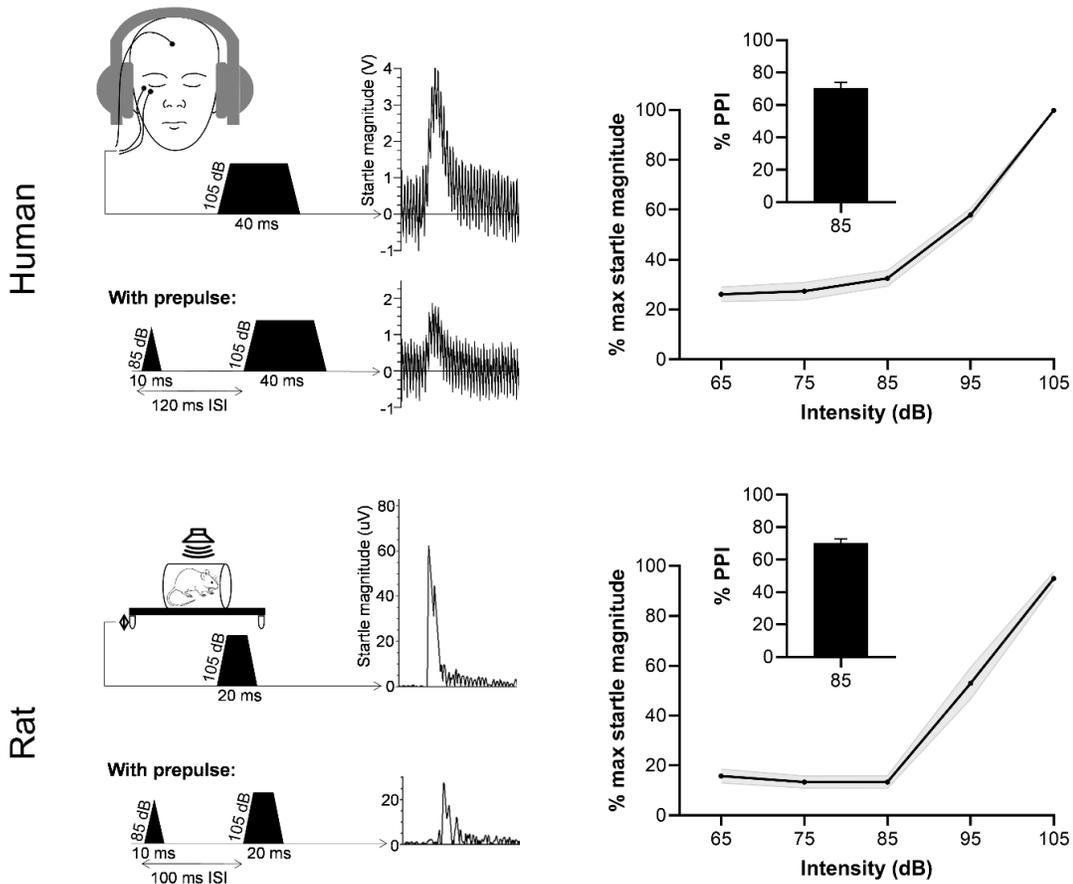


Figure 3: Average acoustic startle responses (ASRs) and prepulse inhibition (PPI) of neurotypical humans and wildtype rats.

Top Left Schematic of the eyeblink reflex electromyography recording system (SR-HLAB™ EMG, San Diego Instruments, San Diego, California) and stimulus characteristics used to elicit the human ASR, shown by the representative trace. Top Right ASR to various acoustic stimuli, represented as the percentage of maximum recorded startle, and percent PPI to an 85 dB acoustic stimulus, presented binaurally through noise-cancelling headphones to individuals aged ~ 17 – 20 ($n = 35$), mean \pm SEM; unpublished data. Bottom Left Schematic of task set-up and stimulus characteristics used to elicit the rat ASR, shown by the representative trace. The rat is placed in a movement-limiting tube set on a motion sensitive platform (StartFear System, Panlab, Barcelona, Spain). Bottom Right ASR to various acoustic stimuli, represented as the percentage of maximum recorded startle, and percent PPI to an 85 dB acoustic stimulus, presented free-field in background noise (60 dB) to adult rats ($n = 22$), mean \pm SEM; adapted from data presented in Scott et al., 2018.

given stimulus; a request that ultimately requires them to make a sensory-based decision. From there, researchers can assess at what level of stimulus complexity dysfunction occurs in ASD by making minor alterations to the characteristics of the stimuli used. Through both clinical and personal reports, two aspects of acoustic processing that have been identified as difficult for autistic individuals are acoustic temporal processing and loudness sensitivity (O'Connor, 2012).

Temporal Processing

In addition to the audiovisual temporal discrimination task described above (see Box 1), through collaboration our research team has developed an auditory temporal rate discrimination task (RD) to assess the ability of humans and rats to perceive small changes in the presentation rate of a train of acoustic noise bursts. Using a two-alternative forced choice task modifiable for both humans and rats, subjects were instructed/trained to categorize a train of acoustic noise bursts presented at various rates as more similar to a slow template noise burst train or a fast template noise burst train (Fig. 4). Because of the simplicity of this task, humans did not require extensive instruction, and rats only required ~2-3 weeks of training to learn the task, ultimately allowing for a similar psychophysical curve to emerge from testing both species.

Auditory temporal processing can also be assessed by determining the extent that a subject can detect a brief silent gap in an otherwise continuous background noise. In humans, this gap detection ability has been assessed using a psychophysical task (Stone et al., 2017), whereas testing in rodents has used a modified acoustic startle paradigm (see section 2), in which a silent gap (rather than a prepulse) was presented just prior to the startle-eliciting stimulus (Fitch et al., 2015); the degree of startle attenuation was indicative of gap detection ability. Despite both studies finding reduced gap detection in ASD or the model thereof, because the tasks rely on different cortical or midbrain circuits (detection vs. sensory filtering), the results cannot be directly compared between species. Fortunately, because both the perceptual and pre-attentive tasks offer translational potential, coordinated efforts between human and rodent research groups could allow for stronger conclusions to be drawn about the validity of the rodent model, and the mechanisms

underlying gap temporal processing dysfunction when the same task is used across species.

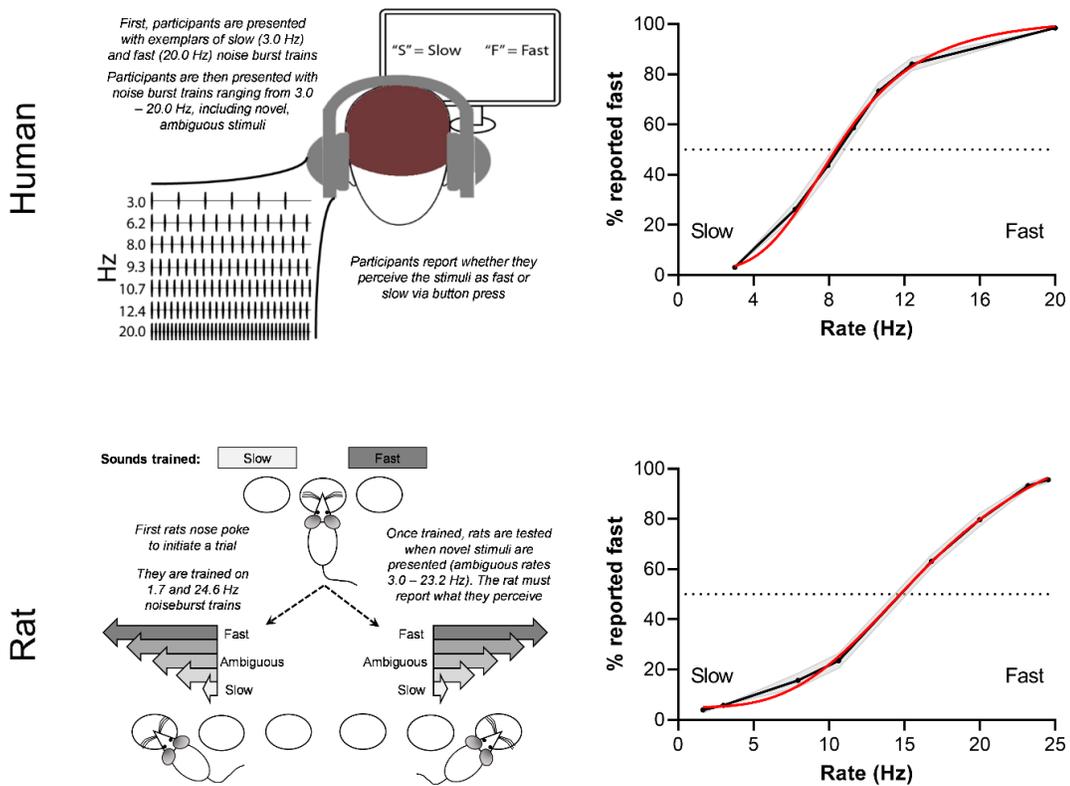


Figure 4: Rate discrimination ability of neurotypical humans and wildtype rats

Top Left: Schematic of stimulus types and trial format used for the RD task in humans. All stimuli were presented using Matlab with PsychToolBox extensions. Acoustic stimuli were presented binaurally through noise-cancelling headphones. Top Right Average rate discrimination performance in individuals aged $\sim 17 - 20$ ($n = 53$), mean \pm SEM, reported as the percentage of trials reported fast; unpublished data. Bottom Left: Schematic of stimulus types and trial format used for the RD task in rats, acoustic stimuli presented free-field; see Scott et al., 2020 for similar methodological procedure. Bottom Right Average rate discrimination performance in adult rats ($n = 8$), mean \pm SEM, reported as the percentage of trials reported fast; unpublished data. Psychometric curves are fit with a nonlinear regression (red).

Loudness detection and perception

Currently, our research group is in the process of refining paradigms to assess loudness detection and perception in both human and rodents. Three tasks that will allow for behavioural translation between species are described. First, Schulz and Stevenson 2020

have developed an acoustic detection task to ascertain the lowest sound intensity that can be heard by individuals (i.e., their acoustic threshold; a.k.a. quiet threshold), which is similar to a sound detection task that has been created for rats (Möhrle et al., 2019; Rüttiger, Ciuffani, Zenner, & Knipper, 2003). Modelled after a categorical loudness scaling task in humans (Khalifa et al., 2004), a second task developed by Scott et al., 2020 for rats determines at what intensity a sound is perceived as too loud (a.k.a. loudness discomfort level) by assessing their sound avoidance behaviour. This is an example of a paradigm originally designed for humans that was too complex to directly transition to a lower-order species. Since rats are unable to verbalize when a sound becomes too loud, researchers instead modified the task to take advantage of rats' natural preference for dark enclosed spaces, and gradually made this area aversive by presenting incrementally louder sounds, so as to ultimately determine at what sound level a quieter + bright space is preferred over a louder + dark space. Third, the ability to discriminate sound intensities can be studied using methods akin to those described in the temporal rate discrimination task, except now the stimulus characteristic that is varied is sound intensity, as opposed to presentation rate (Scott et al., 2020).

4. Conclusions

Translational paradigms hold great potential for shaping the way in which ASD researchers across disciplines approach mutually held questions. Sensory processing differences are thought to contribute to higher-order symptoms such as impaired language development, restricted behaviour, fixated interests, and compulsive behaviours (Baum et al., 2015; Cascio, Woynaroski, Baranek, & Wallace, 2016; Schauder & Bennetto, 2016; Schulz & Stevenson, 2019; Sinclair et al., 2016). By identifying the mechanisms through which they manifest, we can inform autistic individuals and their caregivers about how to tend to the sensory environment with the goal of minimizing sensory distress and improving downstream behavioural responses. Since auditory processing is well studied and highly conserved between species, objective, quantifiable, and comparable measures used in both animal models and humans provide an avenue to translate results from animal research to clinical trials by reducing the dependence of clinical trials on non-translatable metrics (e.g.,

parental reports). Moreover, electrophysiological and startle paradigms are accessible across a range of intellectual abilities and in both youth and adults; important considerations in ASD research. Using our framework as a guide, it is our hope that other researchers can develop translational platforms to study ASD. If the phenotypes of ASD can be studied systematically across species, collectively we can advance our understanding of the mechanisms contributing to ASD and enhance the translation of putative treatments from animal research to clinical trials.

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Appendix D: Animal Use Protocols



2013-020::1:

AUP Number: 2013-020

AUP Title: Neurophysiological Basis of Multisensory Processing

Yearly Renewal Date: 08/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2013-020 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee



AUP Number: 2017-162
PI Name: Allman, Brian
AUP Title: Neurophysiological Basis of Multisensory Processing
Approval Date: 12/01/2017

Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2017-162:1, entitled "Neurophysiological Basis of Multisensory Processing" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

- 1) Animals used in this research project will be cared for in alignment with:
 - a) Western's Senate MAPPs 7.12, 7.10, and 7.15
http://www.uwo.ca/univsec/policies_procedures/research.html
 - b) University Council on Animal Care Policies and related Animal Care Committee procedures
http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
- 2) As per UCAC's Animal Use Protocols Policy,
 - a) this AUP accurately represents intended animal use;
 - b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
 - c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
 - d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined
http://uwo.ca/research/services/animalethics/animal_use_protocols.html
- 3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
 - a) be made familiar with and have direct access to this AUP;
 - b) complete all required CCAC mandatory training (training@uwo.ca); and
 - c) be overseen by me to ensure appropriate care and use of animals.
- 4) As per MAPP 7.15,
 - a) Practice will align with approved AUP elements;
 - b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
 - c) UCAC policies and related ACC procedures will be followed, including but not limited to:
 - i) Research Animal Procurement
 - ii) Animal Care and Use Records
 - iii) Sick Animal Response
 - iv) Continuing Care Visits
- 5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets
<http://www.uwo.ca/hr/learning/required/index.html>

Submitted by: Copeman, Laura
 on behalf of the Animal Care Committee
 University Council on Animal Care

Curriculum Vitae

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CIHR Silver Prize Oral Presentation
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2018

Province of Ontario Graduate Scholarship
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Autism Scholars Award, Council of Ontario Universities
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Natural Sciences and Engineering Research Council (NSERC)
Masters Award
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Related Work Experience Virtual Social Events Team
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