Oxytocin and epigenetic mechanisms in mouse models of empathy and autism

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Dedication

This dissertation is dedicated to:

My family – to whom I owe more than I know.

The late Irv Gottesman, PhD – a friend and mentor.

Abstract

Empathy, the capacity to infer the emotional state of another, represents a normal process of social cognition that is impaired in several psychiatric diseases. This dissertation research investigates (1) behavioral indices and features of empathy in mice, (2) the role of the neuropeptide oxytocin in mouse empathy, and (3) empathic and other endophenotypes in an epigenetic mouse model of autism spectrum disorders (ASDs). Empathy was modeled using a novel set of behavioral paradigms that measure fear transmitted from a distressed "demonstrator" mouse to an "observer" conspecific. Socially transmitted fear was influenced by the sex of the observer, familiarity to the demonstrator, and distress vocalizations emitted from the demonstrator. Repeated observation of a distressed conspecific elicited a switch from freezing to escape fear behaviors that was specific to familiar conspecifics. Oxytocin – whether exogenously applied or released via chemogenetic stimulation – enhanced socially transmitted fear in unfamiliar mice, whereas oxytocin receptor antagonism reduced this empathic behavior in familiar mice. Genetically-modified mice lacking chromodomain helicase DNA-binding 5 (Chd5), a chromatin remodeler that regulates neurodevelopmental processes, exhibited specific impairments in these empathic behaviors. $Chd5^{-/-}$ mice further displayed characteristic socio-communicative and neophobic behaviors reminiscent of ASD symptomology. Cortical tissue from $Chd5^{-/-}$ mice revealed altered transcriptional expression and neuron-level morphological variations characteristic of ASD etiology and pathophysiology. Thus, this research furthers our understanding of neural and epigenetic contributions to neurodevelopment and social cognition, and provides clues to understanding empathy and treating psychiatric disease.

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Chapter 1: Introduction

Social Cognition & Empathy

Social relations are vital for individual and group well-being. Normal social interactions are intrinsically rewarding, whereas impairments in social functioning epitomize numerous psychiatric disorders.(Kennedy & Adolphs, 2012) The genetic and neural aspects underlying normal and psychopathological social behaviors remain some of the most complex and enigmatic in neuroscientific research. Social behaviors are multifaceted, and the processes that mediate such behaviors are more broadly classified as "social cognition".(Adolphs, 2003; Frith, 2008) One long-standing hypothesis posits that social cognition in mammals co-evolved with the expansion of the neocortex, thus allowing for adaptive behaviors in the context of more dynamic social hierarchies.(Dunbar, 1998) Increased brain size and complexity has also been proposed to underlie the pathogenesis of psychiatric disease, and several examples, including autism spectrum disorders (ASDs) and schizophrenia, are conceptualized as deviations of the "social brain".(Crespi & Badcock, 2008)

Social cognition is intimately linked to emotion.(Olsson & Ochsner, 2008) Empathy, the capacity to infer emotional states of others, represents a core process of social cognition.(de Waal, 2008) Empathy is thought to have originated as a parental capacity to infer emotional states of offspring and consequently to increase the likelihood of their survival.(Decety, 2011; Decety, Norman, Berntson, & Cacioppo, 2012) Empathy draws upon various neural systems. Accordingly, components of empathy can be mapped onto these neural systems. “Affective” empathy is sub-served by the evolutionarily more primitive sub-cortical and brainstem regions that in-part encompass the limbic system. This system is homologous in mammalian species and mediates basic forms of empathy.(J. Panksepp & Panksepp, 2013) These forms of affective empathy include emotional “contagion” and “state-matching”, such as infectious crying among babies, as well as yawning and vicarious pain responses among adults.(J. Panksepp, 2011a) Interestingly, brain responses to vicarious pain mirror those during direct nociception, indicating overlap between basic emotional systems and those involved with this form of empathy.(Lamm, Decety, & Singer, 2011) “Cognitive” empathy extends to cortical regions that mediate higher-order capacities, including “theory of mind”, defined as the capacity to infer intentional or belief states of others. Mirror neurons represent a key substrate of the cognitive empathy system. These specialized neurons and their corresponding networks mediate motor mimicry and facets of communication.(Rizzolatti & Craighero, 2004) Cognitive empathy ultimately gives rise to sympathetic concern, targeted helping, and altruism.(de Waal, 2008)

Affective and cognitive empathy involve highly integrated neural systems. One theory contends that affective empathy systems are nested within higher-order cognitive systems, and represent proximate mechanisms underlying directed, altruistic behavior.(de Waal, 2008) In mammalian species, empathy has thus evolved from basic and instinctive emotional capacities to more cognitively-based processes. For example, vicarious pain responses, a form of affective empathy, can be modulated by relationship to the conspecific in pain or prior experience with the painful stimulus.(Cheng et al., 2007)

Empathy has thus expanded from a parental role to more dynamic social relationships. In humans, empathy is a fundamental socio-emotional capacity and “glue” of our social world.(Simon Baron-Cohen & Wheelwright, 2004) The importance of empathy is highlighted by its disruption in numerous psychiatric diseases, including ASDs, schizophrenia, antisocial personality disorder, etc.(S Baron-Cohen, Leslie, & Frith, 1985; Decety, Chen, Harenski, & Kiehl, 2013; Derntl et al., 2009) Empathic impairments in these diseases are strong predictors of social functioning and treatment outcomes.(Couture, Penn, & Roberts, 2006; Eapen, Crnčec, & Walter, 2013) Thus, uncovering the neural substrates that underlie empathy may enhance our understanding of this form of social cognition, and further both the identification and treatment of relevant deficits in psychiatric disease.

The Oxytocin Empathogen

Oxytocin represents a putative neural substrate of social cognition. This nine amino acid neuropeptide is synthesized within magnocellular and parvocellular neurons of the paraventricular, supraoptic, and accessory sub-nuclei of the mammalian hypothalamus. Magnocellular neurons project via the hypophyseal portal system to the posterior pituitary gland where the neuropeptide is released into circulation. Systemic circulation of oxytocin has been classically implicated in child birth/rearing functions, including uterine contraction during parturition and milk production following childbirth, and sexual behavior, including orgasm.(Gimpl & Fahrenholz, 2001; Rilling & Young, 2014)

A second population of parvocellular oxytocinergic neurons project to local targets throughout the mammalian brain.(Knobloch et al., 2012) Oxytocin released at these targets binds to the oxytocin receptor (OXTR), a G protein-coupled receptor containing seven transmembrane domains and enriched throughout limbic and cortical regions of the rodent brain.(Gimpl & Fahrenholz, 2001; Grinevich, Knobloch-bollmann, Eliava, Busnelli, & Chini, 2016) OXTRs are coupled to several G proteins - G_q, G_i, G_o – that signal via disparate molecular cascades depending on the cell and tissue type.(Stoop, 2012; Zingg & Laporte, 2003) The primary G protein coupled to OXTRs within the brain is G_q, which signals the increase of intracellular levels of calcium and leads to neuronal excitability.(Sanborn et al., 1998)

In addition to its canonical roles in parturition, milk production, and sexual behavior, oxytocin has also garnered considerable attention for its involvement in social cognition.(Lim & Young, 2006; Meyer-Lindenberg, Domes, Kirsch, & Heinrichs, 2011) As such, many brain regions linked with social cognition processes are enriched with oxytocin receptors(Mitre et al., 2016) and receive projections from parvocellular oxytocinergic neurons of the hypothalamus.(Knobloch et al., 2012) Roles for oxytocin in social cognition build upon canonical roles to include parent-infant attachment(F. S. Chen, Barth, Johnson, Gotlib, & Johnson, 2011; Gordon, Zagoory-Sharon, Leckman, & Feldman, 2010), social recognition(Ferguson et al., 2000; Ferguson, Aldag, Insel, & Young, 2001), pair bonding(Gonzaga, Turner, Keltner, Campos, & Altemus, 2006), and empathy.(Bartz et al., 2010; Domes et al., 2007; Singer et al., 2008)

Recent years have borne witness to a surge of research that link oxytocin to social cognition, and – more specifically - empathy. Indeed, several hypotheses have been formulated to account for the various experimental evidence. One leading hypothesis attributes the apparent pro-social effects of oxytocin to the engagement of core emotional systems influencing fear, anxiety, and stress.(Neumann & Slattery, 2016) Under this hypothesis, oxytocin signaling modulates neural activity within various limbic regions.(Lahoud & Maroun, 2013; Viviani et al., 2011) Supporting this hypothesis, intranasal oxytocin in humans attenuates amygdalar reactivity to fearful faces(Domes et al., 2007) and reduces anxiety.(Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003) Systemically administered oxytocin also enhances fear extinction, and has been proposed as a treatment for post-traumatic stress disorder.(Eckstein et al., 2014) This hypothesis, however, is not without contradictory findings.(Lahoud & Maroun, 2013; Toth, Neumann, & Slattery, 2012) For example, oxytocin can either reduce(Domes et al., 2007; Missig, Ayers, Schulkin, & Rosen, 2010) or enhance(Eckstein et al., 2015; Grillon et al., 2012) measures of fear and anxiety.

Accounting for these contradictory findings, another leading hypothesis proposes that oxytocin instead modulates the emotional salience of social cues.(Shamay-Tsoory & Abu-Akel, 2015) For instance, oxytocin increases attention to social cues, thereby enhancing emotional recognition(Bartz et al., 2010; Shahrestani, Kemp, & Guastella, 2013) and socially-mediated learning.(Choe et al., 2015; Hurlemann et al., 2010) This “social salience” hypothesis of oxytocin agrees with a growing number of studies pointing to a direct role of oxytocin in empathy.(Bartz et al., 2010; Domes et al., 2007;

Singer et al., 2008) Consistent with its traditionally ascribed role in parturition, milk production and sexual behavior, oxytocin could more generally serve to enhance recognition of emotional cues from offspring or otherwise. Social behavior is intimately linked with underlying genetics(Ebstein, Israel, Chew, Zhong, & Knafo, 2010), and numerous single nucleotide polymorphisms (SNPs) in the oxytocin receptor (OXTR) gene have been associated with reduced empathy in otherwise neurotypical individuals. OXTR SNPs modulate levels of autonomic arousal and empathic scores in response to others in pain.(Smith & Wang, 2013) Several SNPs have also been linked to social recognition(Skuse et al., 2013) and cooperation(Haas, Anderson, & Smith, 2013), and mediate brain responses to emotional cues.(Loth et al., 2014) One replicated finding across individuals with SNPs in the OXTR gene is reduced performance on the "Reading the Mind in the Eyes" test, which assays identification of emotional expressions.(Domes et al., 2007; Rodrigues, Saslow, Garcia, John, & Keltner, 2009) Thus, these findings implicate the oxytocin system in social cognition, particularly emotional processing and empathy.

Oxytocin & Autism Spectrum Disorders (ASDs)

Given its various pro-social effects, a dysregulated oxytocin system may have significant psychosocial relevance to psychiatric conditions in which social relations are impaired. Most prevalent among these is ASD, a set of neurodevelopmental conditions characterized by impairments in socio-communicative interaction and repetitive/stereotypic patterns of behavior.(Association, 2013) Nearly 1 in 68 children in the United States is diagnosed with an ASD(Report, 2012), with a disproportionate number of diagnosed individuals being male. Individuals with ASDs exhibit impairments in social cognition, including diminished facial processing(Dalton et al., 2005), emotional recognition(Sucksmith, Allison, Baron-Cohen, Chakrabarti, & Hoekstra, 2013), and "theory of mind".(S Baron-Cohen et al., 1985) These features represent endophenotypes, or intermediary components of disease that functionally link underlying genetics and overt behavioral symptoms.(Gottesman & Gould, 2003) Supporting the contention that these features are genetically linked, undiagnosed individuals with a familial risk of psychiatric disease show patterns of social dysfunction, including decrements across domains of social cognition.(Bailey, Palferman, Heavey, & Le Couteur, 1998; Goussé et al., 2002; Oerlemans et al., 2014; Sucksmith et al., 2013; Tarbox & Pogue-Geile, 2011) These decrements have been reported in unaffected co-twins(Bailey et al., 1995; Le Couteur et al., 1996), immediate and distant relatives(de Achával et al., 2010; F. Irani et al., 2006; Pickles et al., 2000; Piven et al., 1997), and generalize across the normal population.(Losh et al., 2009; Losh & Piven, 2007; Sasson, Nowlin, & Pinkham, 2013) Interestingly, these deficits in social cognition have also been proposed to underlie nosological socio-communicative impairments.(Hughes, Soares-Boucaud, Hochmann, & Frith, 1997)

Several forms of evidence suggest a dysregulated oxytocin system in ASDs.(Guastella & Hickie, 2015) Numerous studies have identified SNPs in the OXTR gene among individuals with ASDs.(Gregory et al., 2009; Gurrieri & Neri, 2009; Jacob et al., 2007; Loparo & Waldman, 2014; S. Wu et al., 2005) Additionally, clinical cohorts have exhibited reduced oxytocin plasma levels.(Green et al., 2001; Modahl et al., 1998) Oxytocin has also been proposed as a promising treatment, particularly for remediating impairments in social cognition.(Bakermans-Kranenburg & van IJzendoorn, 2013; Young & Barrett, 2015) For subjects with ASDs, oxytocin administration increases eye contact(Auyeung et al., 2015; Guastella, Mitchell, & Dadds, 2008), social cooperativity(Andari et al., 2010), and recognition of emotional cues.(Hollander et al., 2007) These behavioral outcomes correlate with modulation of underlying neural systems. Intranasal oxytocin in adults with ASDs increases activity within various regions of the limbic system.(Y Aoki et al., 2014; Yuta Aoki et al., 2014) Furthermore, oxytocin increases amygdala, fusiform gyrus, and inferior occipital gyrus activity during presentation of facial stimuli(Domes et al., 2013; Gordon et al., 2013), an effect opposite to neurotypical individuals.(Domes et al., 2007) Numerous other studies have also demonstrated promising improvements in social behaviors and neural correlates(Andari et al., 2010; Guastella et al., 2010; Hollander et al., 2007), and clinical trials are currently underway.

The Epigenetic Hypothesis of ASDs

ASDs comprise one of the most heritable psychiatric diseases(Sullivan, Daly, & O'Donovan, 2012), and are therefore thought to involve a significant genetic component to their etiology.(Bailey et al., 1995) Mirroring the heterogeneous presentation and range of symptom severity of ASDs, genetic sources of disease are complex and incompletely understood.(de la Torre-Ubieta, Won, Stein, & Geschwind, 2016; Sullivan et al., 2012)

Only a small fraction of individuals with ASDs have an identifiable genetic variant – either a single, *de novo* or inherited mutation or multiple-gene copy number variation – and thus conform to a major-gene model of disease.(Lee et al., 2012; Pinto et al., 2010; Purcell et al., 2009; Šestan, State, & Sestan, 2013) In fact, nearly half (41%) of cases involve a yet to be identified genetic cause.(de la Torre-Ubieta et al., 2016) Thus, the largest predicted component of genetic risk derives from multiple common genetic variants, each with a small effect that – in combination with environmental factors – result in the disease state.(Gaugler et al., 2014; Klei et al., 2012) Genetic loading of these common variants contribute to convergent underlying mechanisms that ultimately produce the disease phenotype. This polygenic risk model adheres closely to the endophenotype concept – convergent mechanisms are genetically linked and features of disease are evident in undiagnosed relatives.(Lowe, Werling, Constantino, Cantor, & Geschwind, 2015; Lyall et al., 2014; Virkud, Todd, Abbacchi, Zhang, & Constantino, 2010) Genome-wide association studies (GWAS), albeit presently limited by their statistical power, have identified numerous variants of interest and are moving the field towards a more complete picture of relevant biological pathway.(Anney 2012; Geschwind 2015)

In light of the enigmatic genetic architecture of ASDs, a recent theory has postulated that pathogenesis occurs through epigenetic dysregulation.(Feinberg, 2007; Gräff & Mansuy, 2009; Grafodatskaya, Chung, Szatmari, & Weksberg, 2010; Petronis, 2010; Tsankova, Renthal, Kumar, & Nestler, 2007; Urdinguio, Sanchez-Mut, & Esteller, 2009) Epigenetic processes comprise those mechanisms that regulate gene expression independent of changes in the DNA code sequence. Conceptually, dysregulated epigenetic mechanisms could alter the expression of one or more highly penetrant risk genes (independent of any nucleotide-specific variation) that then contribute to a disease state. Epigenetic processes also span complex gene-environment interactions, which are known to influence disease progression.(Sutherland & Costa, 2006)

Various epigenetic mechanisms have been characterized. The most ubiquitous of these, transcription factors, are nuclear proteins that form complexes to directly mediate gene expression. Aside from transcription factors, there exist three secondary forms of epigenetic regulation: methylation, nucleosome modifications, and chromatin remodeling (**Fig 1**). Direct methylation of nucleotides at cytosine residues, a process mediated by DNA methyltransferases, physically blocks transcription factors from reading genetic code. Nucleosome modifications comprise post-translational alterations of histone tails (e.g., acetylation, methylation, phosphorylation, etc.) that alter the chromatin structure and accessibility of transcriptional machinery. These modifications can be acquired during mitosis and may even be transmitted trans-generationally, thereby producing long-term epigenetic effects.(Petronis, 2010)

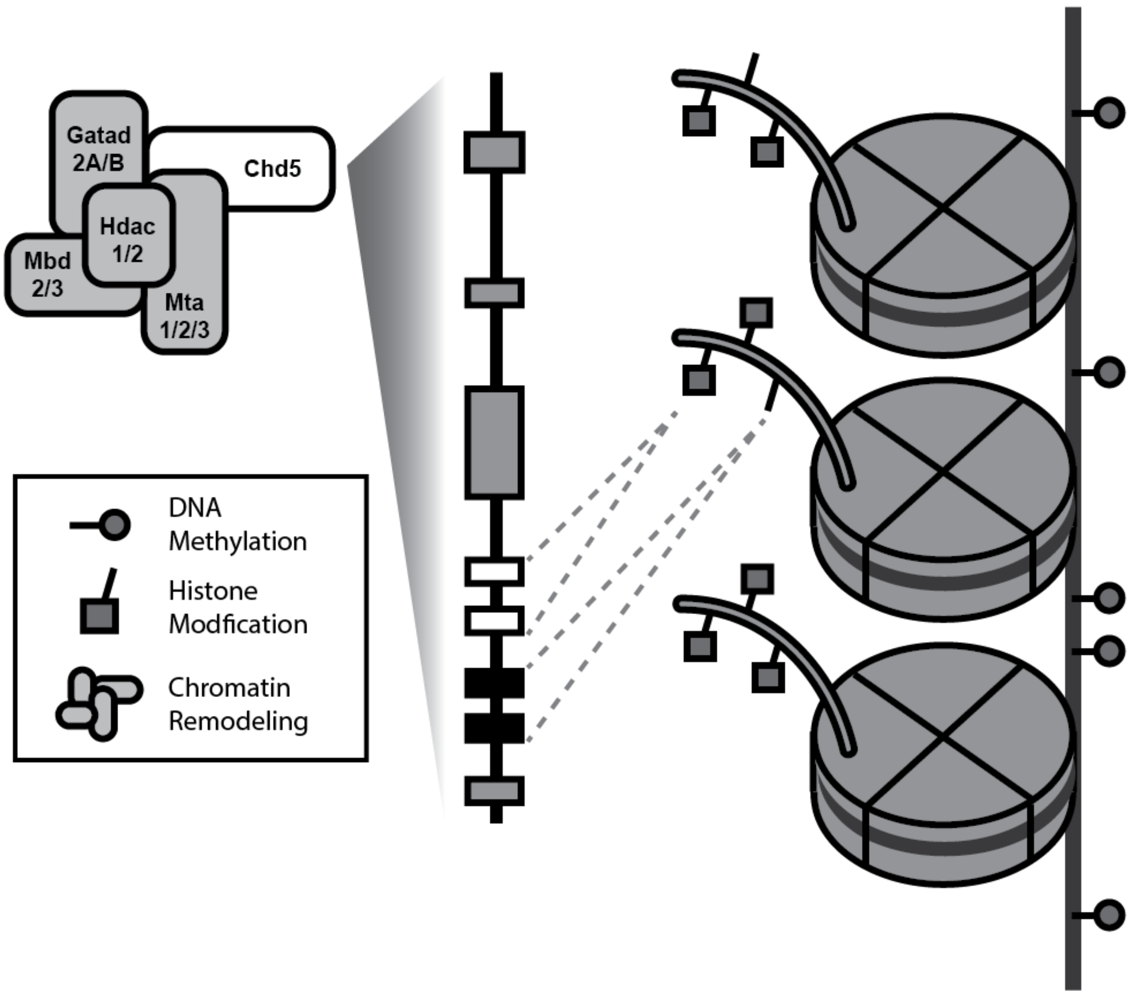

Figure 1. Illustration of epigenetic mechanisms & Chd5 chromatin remodeler. *(**left**) The Chd5 chromatin remodeling complex, (**middle**) functional domains of the Chd5 protein, including tandem chromodomains (white) and plant homeodomains (black), and (**right**) chromatin, composed of DNA (black line) bound to nucleosomes (gray discs) and histone tail modifications (squares). Hdac1/2, histone deacetylase 1 or 2; Mbd, methyl-CpG-binding domain 2 or 3; Mta, metastasis tumor antigen 1, 2 or 3; Gatad, GATA zinc finger domain 2A or B.*

Chromatin remodeling in neurodevelopment and ASDs

One remaining epigenetic mechanism is chromatin remodeling. Chromatin remodeling proteins form large complexes that influence accessibility of histone-associated DNA (nucleosomes) to transcriptional machinery. This dynamic process is commonly mediated by ATP hydrolysis, which causes restructuring of the chromatin complex and either opening or closing of nucleosomes.(Kouzarides, 2007; Marfella & Imbalzano, 2007) Chromatin remodeling has recently emerged as a key epigenetic mechanism relevant to neurodevelopment and psychiatric disease. In fact, variants of genes encoding chromatin remodelers have been repeatedly associated with ASDs via GWAS, making it one of the most common biological pathways implicated in the psychiatric disease.(De Rubeis et al., 2014; Huguet, Ey, & Bourgeron, 2013; Krumm, O'Roak, Shendure, & Eichler, 2013; Pinto et al., 2014)

Chromatin remodeling is accomplished via several classes of remodelers. Of these, chromodomain helicase DNA-binding (CHD) chromatin remodelers have received particular attention. Several genes of the CHD family have been associated with ASDs, namely CHD1, CHD2, CHD7 and CHD8.(Iossifov et al., 2012, 2014) In fact, mutations of CHD8 are the most commonly identified across GWAS(Neale et al., 2012; O'Roak et al., 2012), making it the *de novo* variant that explains the highest percentage of liability in ASDs.(de la Torre-Ubieta et al., 2016) More detailed examinations further demonstrate that reduced Chd8 protein function produces pathophysiological features of ASDs.(Cotney et al., 2015; Willsey et al., 2013) Mutations in CHD7 cause CHARGE syndrome, a set of deficiencies that include intellectual disability and other ASD-related features.(Janssen et al., 2012; Vissers et al., 2004) Interestingly, Chd8 has also been shown to associate with Chd7 and could therefore contribute to the underlying etiology of CHARGE syndrome.(Batsukh et al., 2010) While the aforementioned members of the

CHD family have received considerable attention via GWAS, several others remain largely unexplored.(Hall & Georgel, 2007; Marfella & Imbalzano, 2007)

CHD5 represents one outstanding member of this family of chromatin remodelers. The Chd5 protein has ~70% amino acid homology to Chd3 and Chd4, and shares several functionally similar domains.(Stanley, Moore, & Goodarzi, 2013) Much like Chd3/4, Chd5 associates with a nucleosome remodeling and histone deacetylase (NuRD)-like complex that includes histone deacetylase (Hdac) 1/2, methyl-CpG-binding domain (Mbd) 2/3, metastasis tumor antigen (Mta) 1/2/3, and GATA zinc finger domain (Gatad) 2A/B **(Fig 1, left)**.(Eberl, Spruijt, Kelstrup, Vermeulen, & Mann, 2013) As a component of this complex, Chd5 binds with lysine residues on histone protein tails, thus influencing accessibility of nucleosome-associated DNA. Recognition and binding to histone tails is governed by tandem plant homeodomains (at unmodified H3K4 residues) and chromodomains (at tri-methylated H3K27 residues) within the Chd5 residue sequence **(Fig 1, middle and right)**.(Egan et al., 2013; Oliver et al., 2012; Paul et al., 2013) CHD5 gene mutations have also been identified in large genome-wide association studies of ASDs(Bucan et al., 2009), including the most recent and largest whole-exome sequencing study to date.(De Rubeis et al., 2014), and the Chd5 protein has been mapped to specific gene co-expression networks associated with ASDs.(Parikshak et al., 2013)

ASDs have long been classified as neurodevelopmental disorders, and – not surprisingly – epigenetic mechanisms assert strong influences on neurodevelopment. Indeed, most epigenetically-driven events overlap with sensitive periods of early embryonic development, a time during which the “epigenome” is highly responsive to environmental influences and transcriptional variability.(Dolinoy, Weidman, & Jirtle, 2006; Rutten & Mill, 2009) Thus, temporally and functionally relevant epigenetic processes influence neurodevelopment, as well as the initiation and progression of psychiatric disease. Human GWAS have also revealed functional gene networks in ASDs that relate to neuronal development.(Pinto et al., 2014) Neuropathological studies in humans and non-human models of ASDs have pointed to a range of features stemming from errors in cortical development – decreased neuron size, aberrant neuronal migration and orientation, reductions in white matter integrity, and dendritic abnormalities.(J. A. Chen, Peñagarikano, Belgard, Swarup, & Geschwind, 2015; de la Torre-Ubieta et al., 2016)

Chromatin remodeling has a primary role in neurodevelopmental processes (Ho & Crabtree, 2010; Yoo & Crabtree, 2009). Chd3 and Chd4, relatively well-characterized members of the CHD family, associate within NuRD complexes that mediate myelination processes.(Eberl et al., 2013; Hung, Kohnken, & Svaren, 2012) Chd4 has also been shown to inhibit glial differentiation in the developing cortex.(Sparmann et al., 2013) Chd2 is required for normal organismal development(Marfella & Imbalzano, 2007) and embryonic neurogenesis in the developing cerebral cortex.(Shen, Ji, Yuan, & Jiao, 2015) Unlike other Chd chromatin remodelers, Chd5 is expressed almost exclusively in neural tissue(Thompson, Gotoh, Kok, White, & Brodeur, 2003) where it binds to nucleosomes to influence expression of various developmentally-relevant genes, including several associated with ASDs and intellectual disability.(Potts et al., 2011) Recently, Chd5 was also been reported to regulate neuronal differentiation in the mammalian cortex.(Egan et al., 2013) These convergent lines of evidence suggest that Chd5 may have a crucial epigenetic role in regulating neurodevelopment and therefore the pathogenesis of ASDs.

Studying endophenotypes in mouse models

Mouse models are vital tools to understanding genetic bases and neural systems underlying behavior and psychiatric disease, as well as informing the development of therapeutics to be used in humans.(McCammon & Sive, 2015) Although recent advances in mammalian cell culture systems promise to extend our understanding of molecular mechanisms in health and disease, whole organism models provide a wider perspective spanning genes to behavior. In particular, the common mouse (*mus musculus*) possesses the unique advantages of sharing high gene synteny (>90%) with humans(Waterston et al., 2002), amenability to genetic and genomic manipulation, and availability of a wide array of established behavioral assays. The majority of genetic mouse models of ASDs have focused on a single gene – for example, fragile X mental retardation 1 (FMR1), tuberous sclerosis 1 and 2 (TSC1/2), and contactin associated protein-like 2 (CNTNAP2). These transgenic mouse lines have provided valuable knowledge of underlying pathophysiology. Conversely, modeling a polygenic model of ASDs is intractable given current means of engineering the mouse genome. The closest alternative to modeling common variants in mice is to identify and disrupt an epigenetic source that selectively dysregulates expression of risk genes.

While a widely utilized animal system, mouse models of ASDs are fraught with obvious issues of validity: mice cannot express quintessentially human cognitive capacities, nor manifest the complete diagnostic symptomology of disease. Even so, the classic approach to characterizing mouse models of disease involves assigning behavioral phenotypes based on human symptomology.(Buxbaum et al., 2012; Kazdoba, Leach, & Crawley, 2016) This approach, albeit a standard practice in the field, is not based on etiology or pathophysiology, nor does it necessarily recognize the advantage of studying endophenotypic features of disease. The endophenotype approach instead aims to reduce genetically-based complex behaviors into more defined and quantifiable components.(Gould & Gottesman, 2006) Animal models characterized via the endophenotype approach are more congruent with the human disease. Indeed, many endophenotypes observed in humans have tangible and quantifiable antecedents in mice. The brain regions and circuits thought to be affected by psychiatric diseases are also evolutionarily conserved and homologous between mouse and man. Subcortical and brainstem structures have similar connectivity, and these neural circuits give rise to analogous behavioral output. Therefore, mouse models that exhibit particular endophenotypes offer powerful tools for elucidating pathophysiological mechanisms underlying psychiatric disease.

One ASD-associated endophenotype that may prove to be particularly useful in characterizing mouse models is impaired social cognition. Long acknowledged in primates, recent findings have begun to identify capacities for social cognition processes in rodents. For example, several studies have now documented fear behavior in rodents observing the distress of a conspecific.(Q. Chen, Panksepp, & Lahvis, 2009; Gonzalez-Liencres, Juckel, Tas, Friebe, & Brüne, 2014; Jeon et al., 2010; Kim, Covey, & Kim, 2010; Sanders, Mayford, & Jeste, 2013) These studies highlight the conserved nature of emotional “state-matching”, a form of affective empathy.(J. Panksepp, 2011a) However, very few investigations of empathy utilize the mouse model system, including its rich repertoire of genetic and genomic techniques and resources. Consequently, the underlying neurobiological substrates of empathy remain largely undefined, and little evidence exists to suggest that mouse models of ASDs exhibit empathic impairments. Coupling the use of mouse models that target epigenetic mechanisms with the adoption of reliable endophenotypic measures of social cognition promises to shed light on the etiology and pathophysiology of ASDs.

Outline of experiments

This dissertation work aims to characterize the source of normal empathic behavior and endophenotypic impairments in a mouse model of psychiatric disease. The first set of experiments utilizes both clinically-relevant (intranasal drug administration) and new-generation forms of neuromodulation (chemogenetics) to identify a basic neurochemical mechanism underlying empathy. The second set of experiments detail a novel epigenetic mouse model of disease that recapitulates transcriptional, neuron-level morphological, and behavioral endophenotypes of ASDs. The specific experiments described within this dissertation work are outlined below.

In the first set of experiments, we describe socially transmitted fear behavioral paradigms used to measure empathic behavior in mice. These experiments reveal several characteristics mediating empathic behavior, including sex, familiarity, early-life environment, and vocal communication. We then investigate the contribution of oxytocin in normal empathic behavior. These experiments utilize intranasal and systemic delivery of the neuropeptide or an antagonist to its receptor, respectively, as well as chemogenetic/DREADD stimulation of oxytocinergic neurons.

In the second set of experiments, we describe a novel mouse model of ASDs. This model, resulting from deletion of the neuron-specific Chd5 chromatin remodeler, represents a source of epigenetic and neurodevelopmental dysregulation. We first outline the brain-restricted genetic deletion of the Chd5 chromatin remodeler in mice, then highlight transcriptional and cellular alterations within the mouse cortex. We further characterize socio-communicative and neophobic behaviors that mirror those observed in ASDs. Finally, we detail impairments in empathic behaviors of the Chd5 mouse model of ASDs using the socially transmitted fear paradigms validated within the first set of experiments.

Chapter 2: Behavioral paradigms for measuring empathy in mice

Introduction

Affective empathy is a socio-emotional facility having evolved from sub-cortical structures conserved across mammalian evolution. Thus, lower mammalian species, including rodents, have the neural foundations for affective empathy. Although an analysis of this type of empathy in rodents is relatively nascent, several reports have documented evidence supporting its existence. For instance, pain responses in mice are modulated by the observed pain of a conspecific,(Langford et al., 2006) and rats even exhibit directed helping (“altruistic-like”) behavior, a form of higher-level cognitive empathy.(Ben-Ami Bartal, Rodgers, Bernardez Sarria, Decety, & Mason, 2014) More commonly, several groups have employed modified Pavlovian conditioning assays that examine fear expression in an observing rodent in order to study affective empathy.(Q. Chen et al., 2009; Jeon et al., 2010; Kim et al., 2010) These studies have aided in revealing several features of empathy analogous to those observed in humans.(Preston & de Waal, 2002) One such feature, emotional “state-matching”, has commonly been observed, and involves the fear experienced by one animal to be mirrored in another. Another feature, “familiarity bias”, describes the way in which empathic behavior scales with social relatedness. This particular feature has been confirmed by manipulating the relationship or prior social experience between conspecifics.(Gonzalez-Liencres et al., 2014; Jeon et al., 2010) Lastly, “self-other distinction”, relies on the notion that an observer animal recognizes its autonomy from another. Evidence supporting this particular feature in rodents has been restricted to rats(Ben-Ami Bartal et al., 2014) and prairie voles.(Burkett et al., 2016) Collectively, evidence of these features contribute to the notion that rodent species indeed possess the capacity for affective empathy.

These studies have set precedents for examining empathic behavior in rodents. Even so, the study of this socio-emotional capacity is incomplete, and further examination is needed. Few studies have utilized the mouse model system, including its diverse set of genetic and genomic resources. Moreover, several of these aforementioned features of empathy (in addition to others – e.g., sex and environment) have also yet to be shown in mice. In this first set of experiments, we develop behavioral paradigms to examine empathic behavior in mice. Importantly, we identify influences of familiarity, vocal communication, sex, and early-life environment on this emotional "state-matching" behavior. We also introduce a novel paradigm, social conditioned place aversion (sCPA) to quantify a unique switch in fear behaviors – from freezing to escape. These experiments identify features and expand upon our understanding of affective empathy in mice.

Materials and Methods

Animals

Mice were bred and maintained according to US National Institutes of Health guidelines for animal care and use and Institutional Animal Care and Use Committee of the University of Minnesota – Twin Cities. Mice were provided food and water ad libitum except during experimental testing, and housing lights were maintained on a 12:12hr light/dark cycle.

Socially Transmitted Fear Behaviors

Male or female mice were pair-housed with littermates from the time of weaning and tested at 8-12 weeks of age. Observer mice were acclimated to handling by an experimenter for five consecutive days (5mins/day), followed by habituation to the testing context and acoustic startle over three consecutive days. All sessions commenced with a 5min acclimation period following placement of the observer-demonstrator pair into adjacent cages. Activity measurements were recorded from the observer animal (confined to the cage on the load cell transducer) every 5s. The US was a 0.8mA, 1.5s scrambled foot-shock delivered through the bars of the cage to the demonstrator. This shock intensity was piloted to elicit reliable auditory and visual signs of distress (i.e., vocalizations and freezing behavior, respectively) from demonstrators. The conditioning protocol was composed of 15 trials, each containing 12 activity measurements preceding the US (60sec inter-trial interval). Prior to demonstrator conditioning days, observer mice underwent two consecutive days of control conditioning experiments: the first without inclusion of the demonstrator and with the US (noDem); the second with the demonstrator and without the US (noShock). Non-specific freezing during these experiments was assessed over “conditioning” trials (i.e., excluding acclimation period). Observer mice did not have experience with foot-shock (i.e, “priming”) prior to demonstrator conditioning (cf.(Q. Chen et al., 2009; Kim et al., 2010; Sanders et al., 2013)). Freezing behavior was calculated as the number of measurements per trial (12 total) below a 0.3AU activity threshold (see Fig 2, and (Gewirtz, Falls, & Davis, 1997)). A subset of videos of observer mice was also recorded during conditioning sessions using infrared web cameras. These videos were scored for freezing behavior by a treatment-naïve experimenter using Button Box 5.0 (Behavioral Research Solutions, LLC).

During conditioning sessions, vocalizations were collected using a high-frequency microphone, data acquisition hardware (UltraSoundGate 416H; Avisoft Bioacoustics, Inc.), and recording software (Recorder USGH; Avisoft Bioacoustics). For analysis, recordings were processed using a custom-built R script. In brief, spectrograms were generated from raw WAV files and band pass filtered (10-110kHz). A time-varying parameter, spectral purity, was computed as the fraction of total power within a single frequency bin, and filtered over 8ms. Identification of a vocalization bout was based on three parameters: minimal length (>5ms), spectral purity (>0.15), and holding time (<10ms), defined as the threshold time in which separate bouts were merged. Total duration was computed by summing the vocalizations identified across the entire conditioning session. Vocalizations were presumed to be elicited from demonstrators based on coincidence with foot-shock onset/offset and previously identified characteristics of distress vocalizations elicited by foot-shock.(Q. Chen et al., 2009) An experienced experimenter confirmed the accuracy of automated call detection using the generated spectrogram. Vocalization recordings were not collected for one familiar male, one unfamiliar female, and two unfamiliar males with acute intranasal saline due to technical error.

The social conditioned place aversion (sCPA) paradigm represents a novel method for studying escape-specific social fear behavior in mice. All experimental days entailed a 20min testing session within a conditioned place preference arena (20x25x45cm) containing both tactile (bars vs grids) and visual (vertical lines vs none) cues at 50 lux. The demonstrator mouse was confined to a small cage placed within a window on one side of the arena (counter-balanced). On two consecutive days, observer mice were habituated to the arena without inclusion of the demonstrator. The following day (pre-test), a demonstrator mouse was introduced into the adjacent conditioning cage and the observer was allowed to freely explore. On the first day of demonstrator conditioning (Cond (open)), the observer mouse was confined to the same side as the demonstrator. After 5mins acclimation, the demonstrator was exposed to foot shocks (1.5sec, 1mA scrambled current per 1min; 15 total) using the Advanced Startle software (Med Associates, Inc.). On the second day of demonstrator conditioning (Cond (closed), the observer mouse was allowed to explore the entire arena while the demonstrator was conditioned using the same protocol. On the final day of testing (post-test), observer mice were tested using an identical procedure as the pre-test. A sCPA score was calculated as the change in percentage time spent on the demonstrator-containing chamber between pre- and post-test: [post time - pre time) / (pre time)]. Video was collected during all sessions using infrared web cameras and scored over two-minute bins by trained, group-naïve experimenters using Button Box 5.0 (Behavioral Research Solutions, LLC).

Results

Sex, familiarity, and litter size effects on socially transmitted fear

To investigate empathic behavior in mice, we devised a social fear paradigm in which Pavlovian fear conditioning of a demonstrator mouse produces freezing in an observer mouse (**Fig 2a, top**). We used a load cell transducer system to quantify decreases in force-generated locomotor activity of observers across several intra-trial measurements. Quantifying measurements below threshold produced a reliable proxy for freezing *(average counts (<0.3AU) vs manual freezing scores: Pearson* $R^2 = 0.65$, $p < 0.0001$). Non-specific freezing by observer mice was first measured during control experiments conducted two days prior to conditioning (**Fig 2a, bottom**). In the first control experiment electrical current was passed through an empty demonstrator cage (noDem); in the second, the demonstrator was present but not conditioned (noShock).

We first investigated effects of sex and familiarity on socially transmitted fear behavior. While social fear behaviors in mice are known to be influenced by familiarity ("familiarity bias"),(Gonzalez-Liencres et al., 2014; Jeon et al., 2010) sex-specific effects have been less studied (see(Sanders et al., 2013)). In female mice, we discerned no effect of familiarity – that is, sibling (henceforth, "familiar") and non-sibling/non-cagemate (henceforth, "unfamiliar") observers exhibited high and comparable levels of freezing during demonstrator conditioning (**Fig 2b, right**). We also detected no difference in non-specific freezing behavior of females during control experiments (*noDem:* $t(19) = 0.36$, $p = 0.72$; *noShock:* $t(19) = 0.21$, $p = 0.83$). In contrast, unfamiliar male observers exhibited a significant deficit in freezing compared to familiar observers – both when quantified across conditioning trials (**Fig 2b, left**) and when averaged (**Fig 2b, right**). We detected no differences in non-specific freezing of males during control experiments (*noDem:* $t(25) = 1.1$, $p = 0.27$; *noShock:* $t(25) = 1.39$, $p = 0.18$)

This familiarity effect in males was not attributable to the duration or characteristics of individual vocalizations recorded from demonstrator mice. However, compared to males, we noted a significantly elevated duration of vocalizations from females, implying either enhanced nociceptive sensitivity(Mogil, 1999) or heightened communication of distress ($F_{1,43} = 6.37$, $p = 0.016$). Vocalizations contribute to socially acquired fear in rats(Kim et al., 2010); therefore we also analyzed the relationship between demonstrator vocalizations and observer freezing across conditioning trials by individual pairs of mice. Familiar males showed a significantly higher average correlation compared to unfamiliar males (**Fig 2c**). This finding suggests that vocalizations contribute to social fear exclusively between familiar conspecifics, even though there were no discernable qualitative differences between familiar and unfamiliar mice.

Early life environment contributes strongly to the development of emotional processes and social behavior. Therefore, we next investigated the relationship between litter size and socially transmitted fear. Familiar observers raised in smaller litters (2-6 pups) exhibited higher freezing levels compared to those raised in larger litters (7-12 pups), whereas unfamiliar mice showed the reverse relationship (**Fig 2d**). Importantly, average litter size did not differ between any of these groups (**Table 1**).

We further analyzed locomotor activity measurements obtained immediately after demonstrator foot-shock (measurement (M)1) or across the remainder of each trial (M2-12) (see **Fig 1a, top**). Initial locomotor activity (M1) was equivalent across groups, indicating similar orientation responses for all observers; however, unfamiliar male mice failed to maintain freezing behavior throughout each conditioning trial (M2-12) (**Fig 1e**). This finding suggests that processing of sensory signals emanating from demonstrator mice (e.g., vocalizations) remained intact in all observer mice, yet only produced sustained fear responses in familiar males. We noted lower locomotor activity in familiar observers prior to conditioning, indicating non-specific freezing in response to a confined demonstrator conspecific (**Fig 1f**). We thus tested whether observer mice would exhibit conditioned freezing during acclimation on a subsequent demonstrator conditioning day (Cond2; see **Fig 1a, bottom**). Not only was freezing absent in both familiar and unfamiliar male mice, but we noted a graded increase in locomotor activity across three conditioning days in familiar males only (**Fig 1f**).

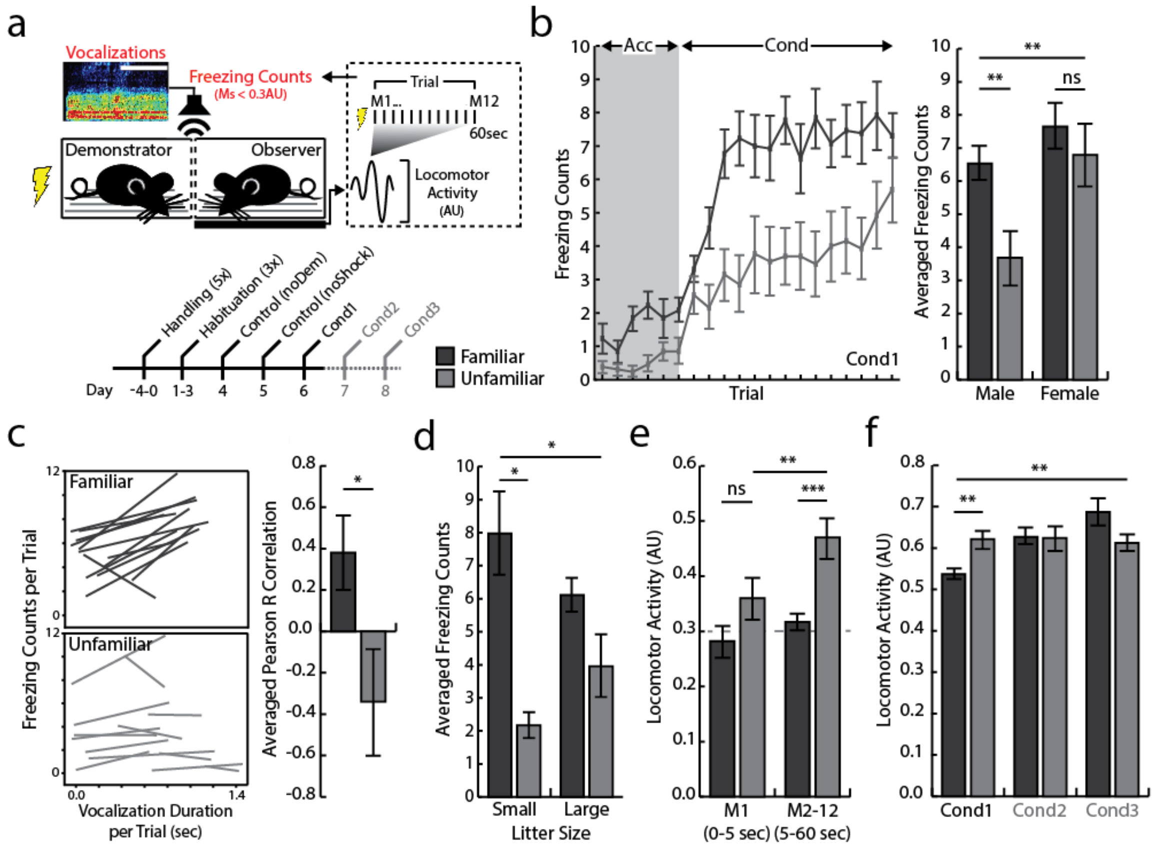

Figure 2. Empathic behavior: the socially transmitted fear paradigm. *(**a**) (**top**) Schematic of the socially transmitted fear paradigm (AU, arbitrary unit; M, measurement; scale bar, 100msec), and (**bottom**) schedule (Acc, acclimation; Cond, demonstrator conditioning). (**b**) Unfamiliar male observers (gray, n = 13) exhibited significantly less freezing compared to familiar male observers (black, n=13) (**left**) across demonstrator conditioning trials (repeated measure ANOVA: $F_{1,390}$ = 6.00, p < 0.0001), and (**right**) when averaged (two-tailed student's t test: t(25) = 2.96, p = 0.008). Familiar (black, n = 10) and unfamiliar (gray, n = 10) female mice did not exhibit significant differences in levels of freezing (two-tailed student's t test: t(19) = 0.728, p = 0.48) (ANOVA: $F_{3,45}$ = 5.55, p = 0.003; effect of familiarity $F_{1,45}$ = 6.06, p = 0.018; effect of sex $F_{1,45}$ = 7.83, p = 0.008; familiarity x sex interaction $F_{1,45}$ = 1.76, p = 0.192). The gray box (on **left**) indicates the acclimation period prior to demonstrator conditioning. (**c**) (**left**) The relationship between observer freezing counts and demonstrator vocalization durations across conditioning trials in (**top**) familiar and (**bottom**) unfamiliar mice. (**right**) Average correlations were significantly different (Fisher transformation, two-way student's t test: t(24) = 2.25, p = 0.035). (**d**) Socially transmitted fear differed between familiar (black; small: n = 3, large: n = 10) and unfamiliar (gray; small: n = 2, large: n = 11) male mice when the observer was reared within a relatively small litter (small: 2-6 pups; large: 7-12 pups) (ANOVA: $F_{3,25}$ = 3.69, p = 0.027; effect of familiarity, $F_{1,25}$ = 10.28, p = 0.004; effect of size $F_{1,25}$ = 0.001, p = 0.970; familiarity x size interaction, $F_{3,25}$ = 2.16, p = 0.156; two-tailed student's t test: small litters only, t(4) = 4.38, p = 0.035). (**e**) Locomotor activity measurements recorded over the first 5 seconds (M1) or subsequently (M2-12) after demonstrator foot-shock were significantly different in unfamiliar*

*observers (two-tailed student's t test: effect of measurement t(25) = 3.70, p = 0.002), and in unfamiliar compared to familiar observers at M2-12 (two-tailed student's t test: effect of familiarity t(25) = 3.96, p = 0.001), indicating less sustained freezing within trials. The dashed line indicates locomotor activity threshold used for freezing counts, as in (b). (**f**) Averaged locomotor activity during acclimation of Cond1 was significantly lower in familiar observers (two-tailed student's t test: t(25) = 3.08, p = 0.008) compared to unfamiliar observers and increased significantly across subsequent conditioning days (repeated measure ANOVA: $F_{5,77}$ = 3.86, p = 0.004; effect of familiarity $F_{1,77}$ = 5.96, p = 0.017; effect of day $F_{2,77}$ = 4.28, p = 0.018; familiarity x day interaction $F_{2,77}$ = 5.36, p = 0.007). Error bars represent s.e.m. * p < 0.05; ** p < 0.01; *** p < 0.001; ns = not significant.*

Repeated observation of demonstrator conditioning elicits escape

Increased locomotor activity of familiar observer mice suggested a transition from freezing to escape behavior. Escape is an ethologically-relevant social fear behavior elicited by the distress of conspecifics intending to signal environmental threats.(Sherman, 1977) To test the hypothesis that familiar observer mice exhibit escape behavior following extended exposure to demonstrator distress, we designed a novel socially conditioned place aversion (sCPA) paradigm (**Fig 3a**). In this paradigm, observers experienced demonstrator conditioning on two subsequent days: on the first (Cond (closed)), observers were confined to the demonstrator-containing side; on the second (Cond (open)), observers were allowed to escape to the opposite side. During acclimation on this second conditioning day, both familiar and unfamiliar observer mice exhibited significantly more time on the non-demonstrator side compared to familiar non-conditioned controls. However, only familiar observers maintained this behavior during conditioning trials (**Fig 3b**). Although side preferences were equivalent for all groups at pre-test (measured prior to conditioning), only familiar male observers exhibited a significant preference for the non-demonstrator side at the post-test, measured on the day following the last conditioning day (**Fig 3c**). Lastly, a normalized score of escape behavior, sCPA, indicated that only familiar observer mice exhibited a conditioned avoidance of the demonstrator-containing side (**Fig 3d**).

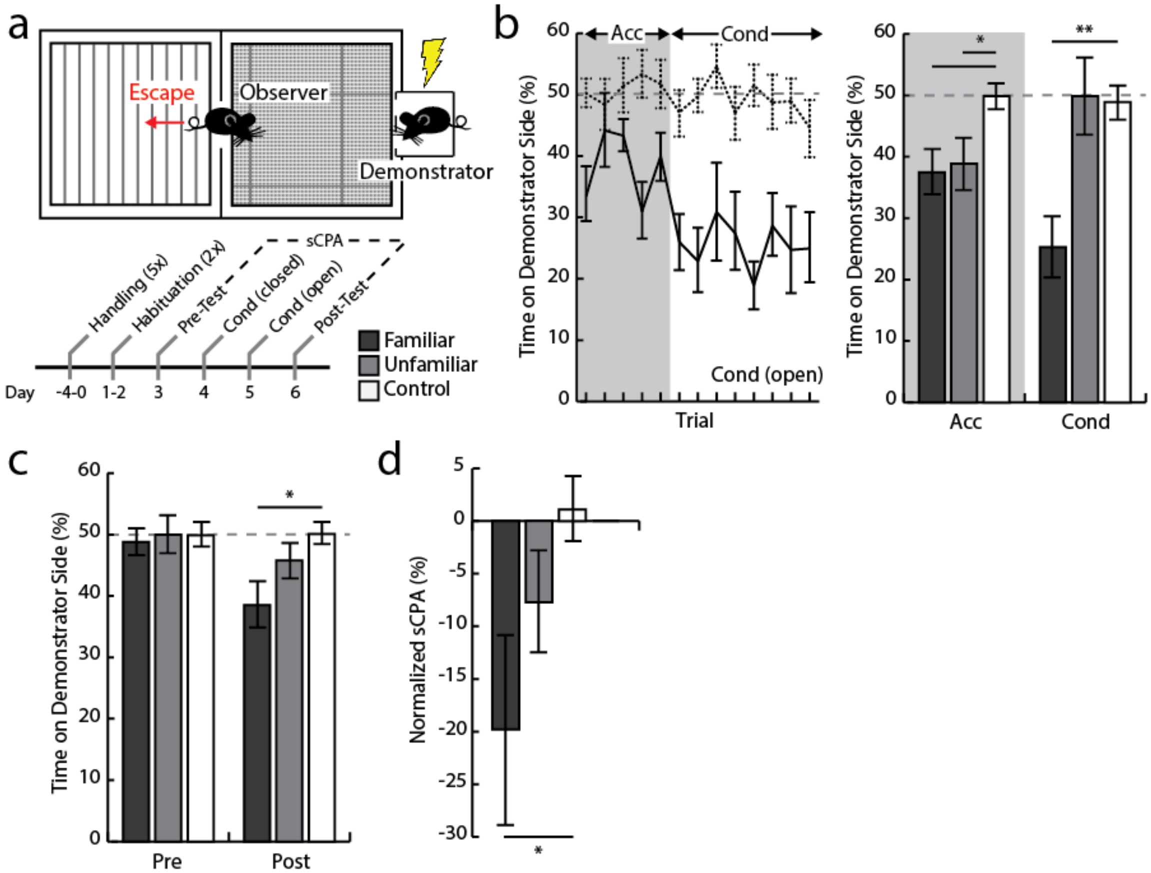

Figure 3. Empathic behavior: the socially conditioned place aversion paradigm. *(**a**) (**top**) Schematic of the sCPA paradigm, and (**bottom**) schedule (Acc, acclimation; Cond, demonstrator conditioning; Control, familiar, non-conditioned). (**b**) (**left**) On the second conditioning day (Cond (open)), familiar mice (black line, n = 10) but not non-conditioned controls (dashed line, n = 12) escaped the demonstrator-paired context during acclimation and conditioning. The gray box indicates acclimation period prior to demonstrator conditioning. (**right**) Compared to non-conditioned controls (white, n = 12), both familiar (black, n = 10; two-tailed student's t test: t(21) = 2.90, Dunnett's post-hoc, p = 0.017) and unfamiliar (gray, n = 8; t(19) = 2.32, Dunnett's post-hoc, p = 0.049) observer mice exhibited initial escape behavior during acclimation, whereas only familiar mice maintained escape during demonstrator conditioning (familiar: two-tailed student's t-test: t(21) = 4.09, Dunnett's post-hoc, p = 0.001; unfamiliar: t(19) = 0.14, Dunnett's post-hoc, p = 0.981). (**c**) Compared to controls, familiar observers exhibited a significant escape behavior at post-test (two-tailed student's t test: t(21) = 2.73, Dunnett's post-hoc, p = 0.012), whereas unfamiliar mice did not (two-tailed student's t test: t(19) = 1.27, Dunnett's post-hoc, p = 0.488). (**d**) Familiar observer mice exhibited a significant difference in normalized sCPA scores compared to controls (two-tailed student's t test: t(21) = 2.17, Dunnett's post-hoc, p = 0.029). Error bars represent s.e.m. * p < 0.05; ** p < 0.01.*

Discussion

In the preceding experiments we successfully demonstrated a model of empathy using mice. The form of empathy we studied was the tendency of one mouse to adopt the emotional state of another. This phenomenon, referred to as emotional "state-matching" or "affect sharing"(de Waal, 2008; Preston & de Waal, 2002), was measured here as the social transmission of fear, analogous to "fear contagion" in humans.(J. Panksepp & Panksepp, 2013) This form of affective empathy was manifested initially as expression of freezing by the observer mouse. Freezing both coincided with, and extended beyond, the foot-shock of the demonstrator mouse, and correlated with the demonstrator's emission of audible distress vocalizations. When this procedure was repeated over several days the freezing response was replaced by increased activity and efforts at escape. Freezing and escape are incompatible behaviors (involving a cessation versus initiation of activity, respectively) and are prototypical expressions of fear across mammalian species.(Blanchard & Blanchard, 1989; Gelder, Snyder, Greve, Gerard, & Hadjikhani, 2004; Gozzi et al., 2010) Within naturalistic contexts, rats show a similar transition from "passive" freezing to "active" escape, depending on the proximity of the threat.(Blanchard & Blanchard, 1989) Moreover, observational or "vicarious" fear conditioning in rats is dependent on the vocalizations of the demonstrator.(Kim et al., 2010) Taken together, the exhibition and timing of these responses in mice strongly suggest that fear is transmitted to the observer via the vocalizations of the demonstrator.

Supporting the contention that affective empathy in mice and humans are analogous phenomena, a similar directionality of gender differences is seen in both species. Females are more empathic than males across several domains of human social interaction,(Chakrabarti & Baron-cohen, 2006; Rueckert & Naybar, 2008; Schulte-Rüther, Markowitsch, Shah, Fink, & Piefke, 2008) and here we found stronger emotional "state-matching" in females than in male mice. The female superiority in empathy has been reported only rarely in rats(Ben-Ami Bartal et al., 2014) and mice(Jules B Panksepp, Lahvis, Panksepp, & Lahvis, 2016). This is hardly surprising since female subjects have rarely been included in rodent studies of socially transmitted or vicarious fear.(Q. Chen et al., 2009; Gonzalez-Liencres et al., 2014; Jeon et al., 2010; Kim et al., 2010; Martin et al., 2015; Sanders et al., 2013) This gender difference may be a result of higher receptivity of female mice to the vocalizations of other mice, or of greater vocal expressiveness among female demonstrator mice. Consistent with the latter possibility, female mice produce more high frequency calls than do male mice(Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2012), and we found a similar sex difference in the frequency of audible vocalizations during foot-shock.

We traced the origins of the lower level of socially transmitted fear in males to two factors: one related to their social experience in adulthood and a second to their social milieu during development. First, responsiveness of male mice was positively related to their familiarity with the conspecific in distress. This "familiarity bias" has been widely demonstrated in mice(Gonzalez-Liencres et al., 2014; Jeon et al., 2010; Martin et al., 2015) and rats(Ben-Ami Bartal et al., 2014) within experimental settings and ground squirrels within their natural environment,(Sherman, 1977) suggesting an evolutionarily conserved function. Second, social transmission of fear between unfamiliar males depended on the size of the litter in which the observer mouse had been raised. That is, male mice raised in large litters were more responsive to an unfamiliar male than were those raised in small litters. This effect could result from several epigenetically-mediated factors, including perinatal nutritional or hormonal environment, the quality of maternal care, and/or degree of sibling interaction.(Curley, Jensen, Mashoodh, & Champagne, 2016) The latter factor may be important in providing the experience of interacting with – and especially communicating with – a wider circle of conspecifics. Interestingly, 24hrs of social isolation during adolescence was sufficient to reduce social transmission of fear in male mice.(Jules B Panksepp et al., 2016) Alternatively, it may be more stressful for mice to be raised with few siblings,(Champagne et al., 2008; Dimitsantos, Escorihuela, Fuentes, Armario, & Nadal, 2007) and heightened stress has been proposed to underlie the reduced emotional "state-matching" among unfamiliar conspecifics.(Martin et al., 2015) Regardless of its underlying cause, our litter-size effect comports with the human literature showing that the number of siblings can influence measures of social cognition.(McAlister & Peterson, 2007; Perner, Ruffman, & Leekam, 1994)

Chapter 3: Oxytocin modulation of mouse empathy

Introduction

The underlying neurobiological substrates of empathy remain largely undefined. One putative substrate of empathy is oxytocin, a neuropeptide classically implicated in parturition and mother-infant care. In neurotypical humans, intranasally administered oxytocin enhances emotional recognition(Domes et al., 2007) and empathy(Bartz et al., 2010) by modulating amygdala activity(Hurlemann et al., 2010) and amygdala-cortical connectivity.(Kovács & Kéri, 2015; Meyer-Lindenberg et al., 2011) Oxytocin is conserved across mammalian species, and evidence in rodents suggests its involvement in various social behaviors. For instance, oxytocin signaling plays a role in social approach(Crawley et al., 2007; Peñagarikano et al., 2015; Teng et al., 2013), recognition(Dumais, Alonso, Immormino, Bredewold, & Veenema, 2016; Ferguson et al., 2001), and learning(Choe et al., 2015; Zoicas, Slattery, & Neumann, 2014), as well as in consolation behavior.(Burkett et al., 2016)

While such data demonstrate the role of oxytocin in social cognition processes, no studies to date have investigated the direct role of oxytocin using a mouse model of empathy. Therefore, we sought to investigate oxytocin using our social fear paradigms – validated indices of affective empathy. To test whether alterations in oxytocin neurotransmission are sufficient to induce empathic behavior, we administered oxytocin intranasally to unfamiliar male mice using both acute and chronic regimens, and activated endogenous oxytocinergic neurons using chemogenetics/DREADDs. To further test the necessity of oxytocin, we administered a selective oxytocin receptor antagonist to male mice that were familiar to one another.

Materials and Methods

Animals & Genotyping

All experimental mice were pair-housed with same sex littermates. Oxt-IRES-Cre/+ transgenic mice (Jackson Laboratories #024234) were genotyped using the following primers: 5′-TTTGCAGCTCAGAACACTGAC-3′ (F); 5′-ACACCGGCCTTATTCCAAG-3′ (Mut-R), 5′-AGCCTGCTGGACTGTTTTTG-3′ (WT-R).

Drug Administration

Oxytocin (Sigma-Aldrich #O3251) was dissolved in sterile saline and aliquots were frozen at -80C. The oxytocin antagonist (OXTA, L-368,899 hydrochloride, Tocris #160312-62-9) was dissolved in sterile saline to make a 0.625mg/mL working solution, and aliquots were frozen at -20°C. For intranasal oxytocin, mice were briefly anesthetized with isoflurane and quickly administered oxytocin (20ug/kg) or saline at 5uL per nostril as described previously.(K L Bales et al., 2014) Chronic oxytocin was administered at approximately the same time for five consecutive days. For systemic OXTA, mice were anesthetized with isoflurane for approximately 30sec and administered the OXTA (5 or 10mg/kg, I.P.). Systemically administered L-368,899 hydrochloride is known to reach the brain, where it preferentially binds with oxytocin receptors.(Boccia, Goursaud, Bachevalier, Anderson, & Pedersen, 2007)

CSF Collection and Oxytocin Measurements

Cerebrospinal fluid (CSF) was obtained according the techniques described previously.(Fleming, Ting, Stohlman, & Weiner, 1983) Mice were deeply anaesthetized using Beuthenasia (200mg/kg, I.P.) at 30mins following intranasal oxytocin or saline administration. Mice were then placed into a stereotaxic frame and a longitudinal cut was made along the posterior neck. Subcutaneous tissue and muscles were exposed, sectioned along the rim of the occipital bone, and then removed laterally to reveal the clear arachnoid membrane overlying the cisterna magna. A glass capillary was pulled to ~5mm diameter and inserted horizontally into the cisterna magna. CSF samples were obtained between 1-3pm, and typically 2-3 µl of total CSF was collected. Samples contaminated with blood were discarded. Samples were immediately frozen at -80°C. Oxytocin quantification was completed using standard ELISA techniques (Enzo Life Sciences, Inc.). Briefly, CSF samples were added to an equivalent volume of 0.1% trifluoroacetic acid (TFA) in water, then applied to a C18 spin column (Thermo Scientific, Inc.) and washed with 0.1% TFA. The extracted oxytocin sample was eluted using a 95% acetonitrile/5% of 0.1% TFA solution and evaporated at 4°C. Samples were then reconstituted in assay buffer and run in duplicate on a spectrophotometer (Multiskan EX, Thermo Scientific, Inc.) at 405nm. Optical density values were converted to concentration using the manufacturer’s standard curve.

Quantitative RT-PCR

Total RNA was obtained from littermate-matched, experimentally-naïve male mice on the day following (18-24hrs) the last chronic intranasal treatment (oxytocin or saline) (see Fig 4a). Mice were deeply anaesthetized using Beuthenasia (200mg/kg, I.P.) and tissue was dissected bilaterally from the whole amygdala and anterior cingulate cortex (ACC) using a mouse brain atlas reference, then quickly submerged in RNA Later (Qiagen) and frozen at -80C. Isolated RNA was processed for DNAase digestion and reverse-transcribed using the GoScript reverse transcriptase (Promega) according to the manufacture's manuals. The cDNA was diluted 1:7 before preparing the qRT-PCR reaction, which was then run using the GoTaq qPCR master mix (Promega). Oxytocin receptor (Oxtr) mRNA primers were chosen from previous work(Zheng et al., 2014) and purchased through the University of Minnesota's Genomics Center. Primers were as follows: 5′-CCGCACAGTGAAGATGACCT-3′ (forward) and 5′-AGCATGGCAATGATGAAGGCAG-3′ (reverse). Mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as the endogenous control. For each sample, a duplex PCR reaction was set-up containing a target gene primer set and a Gapdh primer set. We conducted three replicate PCR reactions for each sample. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 62°C for 1 min on the CFX96 Real-Time System (BioRad, Inc.). The relative levels of gene expression were determined according to the standard curve methods described in the BioRad company manual. The expression value of the target gene in each well was first normalized by the expression value of the Gapdh. The median of three repeated reactions was used to represent the relative quantity of the target gene.

Chemogenetics/DREADDs

Standard aseptic stereotaxic surgical procedures were utilized as described previously.(Athos & Storm, 2001) The pAAV-hSyn-DIO- rM3D(Gs)-mCherry virus (~10^{12} virus particles/mL) was produced by the University of North Carolina Vector Core Facilities (Addgene plasmid #50458). Male Oxt-IRES-Cre/+ mice (7-8 weeks old) were sedated with a ketamine:xylazine cocktail (100:10mg/kg) and bilateral burr holes were created above the paraventricular nuclei (PVN) of the hypothalamus. A Hamilton syringe was loaded with virus and slowly lowered to the PVN (AP: -0.85mm; ML: +/-1.1mm; DV: -5.4mm; 10° angle). A total of 0.5 microliters was injected bilaterally at 0.1uL/min, and the needle was left at the injection site for 10 minutes to allow for virus absorption. Animals were provided 500uL saline (S.C.) post-operatively and 5mg/kg Ketofen (S.C.) daily for three days, then allowed a minimum of three weeks recovery before behavioral experiments. Mice were then either injected with CNO (3mg/kg, I.P.) or saline at thirty minutes prior to social fear conditioning or Pavlovian fear conditioning. For c-Fos immunohistochemistry, mice were administered CNO (3mg/kg, I.P.) ninety minutes prior to sacrifice.

Histology & c-Fos Immunohistochemistry

All animals used for chemogenetics experiments had their brains removed and processed to confirm viral injection location. Mice without Pavlovian fear conditioning experience were used for quantification of c-Fos+ neurons. Mice were deeply anaesthetized using Beuthenasia (200mg/kg, I.P.) and transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde in PBS. Brains were fixed overnight in 4% paraformaldehyde and 30% sucrose in PBS then sliced at 40 micron thickness using a vibratome (Leica VT1000S). Free-floating coronal sections containing the PVN hypothalamus were incubated for 3hrs in blocking solution containing 0.2% Triton-X, 2% normal horse serum, and 0.05% Tween20. Sections were then incubated 72hrs with primary antibodies (rabbit anti c-Fos 1:1000, Santa Cruz; mouse anti-mCherry 1:1000, Abcam), washed six times, then incubated overnight with secondary antibodies (donkey anti-rabbit Alexa Fluor 488 IgG 1:1000, Life Technologies; goat anti-mouse Alexa Fluor 647 IgG 1:1000, Abcam). Slides were washed and counter-stained for twenty minutes with 4',6-diamidino-2-phenylindole (DAPI) (1:50,000, Life Technologies), and mounted. Fluorescent images were acquired on a Zeiss LSM 710 scanning confocal microscope using a 20x objective. Quantification of c-Fos+ neurons was performed across eight slices containing the PVN by a treatment-naïve experimenter.

Results

Acute intranasal oxytocin enhances socially transmitted fear

One putative substrate of empathy is oxytocin, a neuropeptide conserved through mammalian evolution. In humans, intranasal oxytocin bolsters emotional recognition(Shahrestani et al., 2013) and empathy.(Hurlemann et al., 2010) We therefore sought to improve socially transmitted fear behavior in unfamiliar male mice using intranasal oxytocin administration (**Fig 4a**). To assay oxytocin levels within the brain, we collected cerebrospinal fluid (CSF) samples thirty minutes following intranasal administration (a sufficient period of time for oxytocin to reach the brain(Neumann, Maloumby, Beiderbeck, Lukas, & Landgraf, 2013)) and quantified oxytocin concentration using the manufacturer's ELISA procedure (Enzo Life Sciences, Inc.). Intranasal oxytocin (20ug/kg) successfully elevated CSF levels in behaviorally naïve mice, thereby confirming the entry of oxytocin into brain CSF (**Fig 4b**). Using an acute regimen, we then administered a single intranasal dose (20ug/kg) thirty minutes prior to demonstrator conditioning. For unfamiliar male mice, this regimen produced a significant enhancement of freezing compared to saline-treated controls (**Fig 4c**). Conversely, for familiar male mice, intranasal oxytocin did not produce effects on freezing compared to saline-treated controls (*Fam/OXT: 6.81 +/- 0.91; Fam/SAL: 7.95 +/- 0.90; t(9) = 8.6, p = 0.41*).

Oxytocin has been shown to influence measures of explicit fear(Campbell-smith, Holmes, Lingawi, Panayi, & Westbrook, 2015; Lahoud & Maroun, 2013; Modi et al., 2016; Viviani et al., 2011) and anxiety(Ring et al., 2006; Waldherr & Neumann, 2007; Yoshida et al., 2009) in rodent models. Therefore, we also assayed the effect of single-dose intranasal oxytocin on classic measures of fear, including acquisition (freezing), expression (cue- and context-specific fear-potentiated startle (FPS)), and extinction. We did not note any differences in these measures when intranasal oxytocin was given acutely prior to conditioning (*Acquisition:* $t(22) = 0.179$, $p = 0.859$; *Contextual FPS:* $t(22) = 0.859$, $p = 0.401$; *Cued FPS:* $t(22) = 0.03$, $p = 0.975$). Therefore, the effects of oxytocin on socially acquired fear were not secondary to alterations in the capacity for fear conditioning in general.

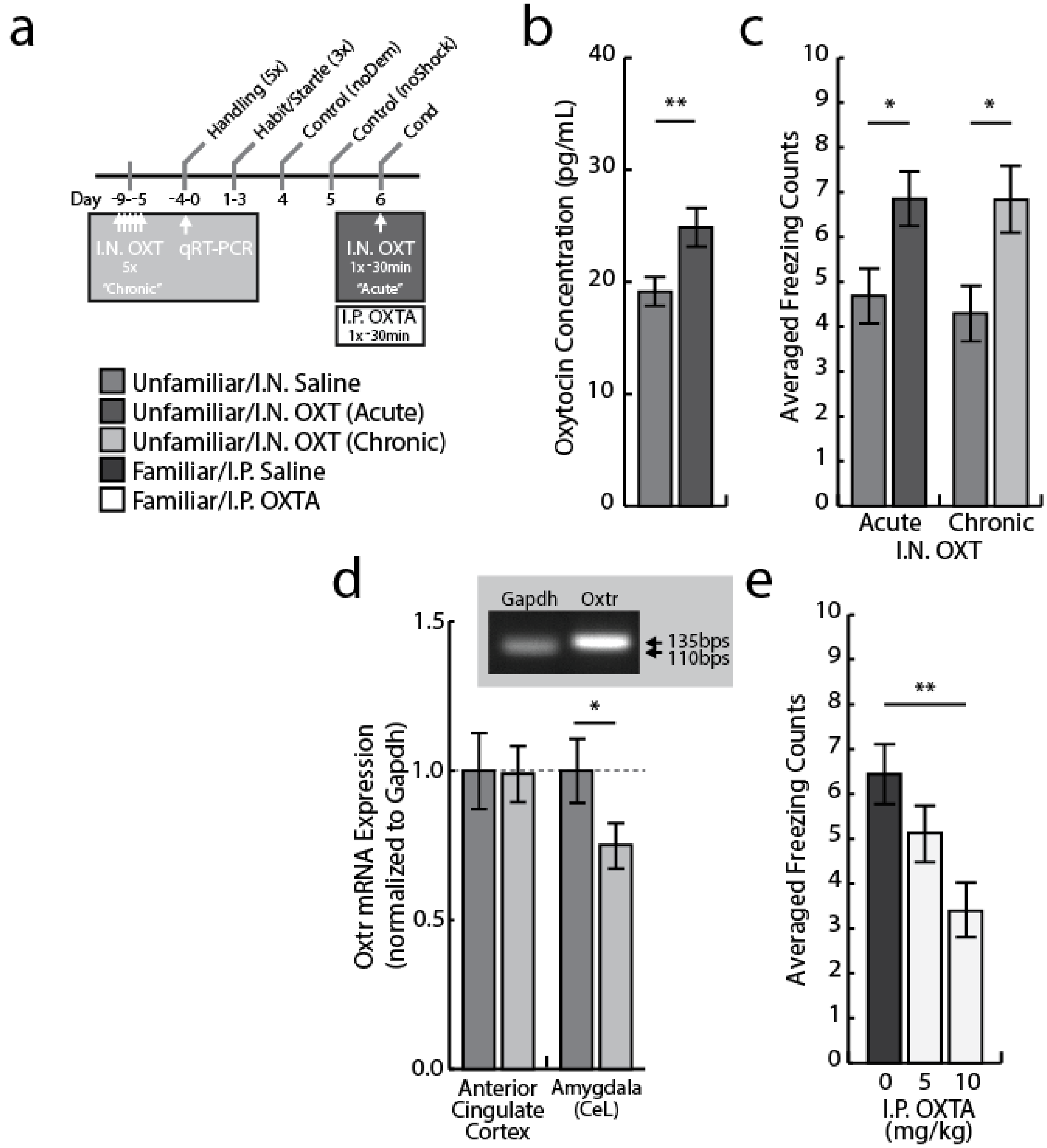

Figure 4. Oxytocin modulates empathic behavior. *(**a**) Schematic of oxytocin drug regimens and time of sample collection for qRT-PCR (habit, habituation; cond, demonstrator conditioning; OXT, oxytocin; OXTA, oxytocin receptor antagonist). (**b**) In behaviorally naïve mice administered acute intranasal oxytocin (dark gray, n = 10), concentrations of the neuropeptide were significantly elevated compared to saline-treated controls (gray, n = 10) (two-tailed student's t test: t(19) = 2.73, p = 0.015). (**c**) Unfamiliar male observers given acute (single dose, thirty minutes prior; dark gray, n = 12; two-tailed student's t test: t(23) = 2.49, p = 0.020) or chronic (five daily doses; light gray, n = 11; two-tailed student's t test: t(21) = 2.64, p = 0.016) intranasal oxytocin exhibited a significant enhancement of freezing compared to saline-treated controls (gray, n = 12/11, respectively). (**d**) Chronic intranasal oxytocin significantly reduced*

*oxytocin receptor (Oxtr) transcript expression in the central lateral nucleus of the amygdala (CeL) (two-tailed student's t test: $t(19) = 2.08$, $p = 0.05$), but not the anterior cingulate cortex (ACC) (two-tailed student's t test, $t(19) = 0.06$, $p = 0.95$), assayed one day after completion of oxytocin treatment. (**inset**) qRT-PCR amplicons of the Gapdh (110bps) and Oxtr (135bps) transcripts at expected sizes. (**e**) Familiar male observers systemically administered the oxytocin receptor antagonist L-368,899 hydrochloride (OXTA; single dose, thirty minutes prior; white, n = 12/group) showed dose-dependent reductions in socially transmitted fear at 5mg/kg and 10mg/kg, compared to saline-treated controls (black, n = 12) (ANOVA: $F_{2,35} = 5.73$, $p = 0.007$). Error bars represent s.e.m. * $p < 0.05$; ** $p < 0.01$.*

Chronic intranasal oxytocin enhances socially transmitted fear and reduces receptor expression in the amygdala

Duration of oxytocin exposure has been proposed to influence behavioral outcomes in both humans(Yatawara, Einfeld, Hickie, Davenport, & Guastella, 2015) and animal models.(Karen L. Bales et al., 2013; Huang et al., 2014) Given that repeated administration of low doses of oxytocin can produce lasting effects on social interaction behavior(Teng et al., 2013, 2015) we employed a chronic regimen in which intranasal oxytocin was administered on five consecutive days, followed by a ten day period without drug exposure (see **Fig 4a**). Similar to acute oxytocin, this regimen produced a significant enhancement of freezing in oxytocin-treated unfamiliar observers, compared to saline-treated controls (**Fig 4c**). These oxytocin effects could not be attributed to differences in the duration of vocalizations of the demonstrator mice ($F_{3,68} = 0.013$, $p = 0.908$) or acquisition of Pavlovian fear in general following chronic intranasal oxytocin administration to the observer mice ($t(12) = 0.134$, $p = 0.896$).

Oxytocin stimulates a distinct population of neurons within the central lateral nucleus (CeL) of the amygdala that express the G protein-coupled oxytocin receptor (OXTR).(Huber, Veinante, & Stoop, 2005) In light of the established role of the amygdala in fear behaviors, alterations in OXTR expression in the CeL may be involved in the behavioral effects of chronic intranasal oxytocin. We therefore collected brain tissue from mice treated with chronic oxytocin and measured levels of the Oxtr mRNA transcript in the amygdala and anterior cingulate cortex (ACC), an area implicated in empathic behaviors in rodents.(Burkett et al., 2016; Jeon et al., 2010) Compared to saline-treated controls, amygdala tissue recovered from OXT-treated mice showed a significant downregulation of the Oxtr transcript, whereas there was no difference in the ACC (**Fig 4d**).

Systemic oxytocin receptor antagonism reduces socially transmitted fear

To investigate the contribution of endogenous oxytocin signaling in socially transmitted fear, we systemically administered the nonpeptidergic oxytocin receptor antagonist (OXTA) L-368,899 hydrochloride to familiar male mice. Administration of the OXTA (5mg/kg and 10mg/kg, I.P.) thirty minutes prior to demonstrator conditioning (see **Fig 4a**) produced a dose-dependent reduction in freezing compared to saline-treated controls (**Fig 4e**), but did not produce differences in direct acquisition of Pavlovian fear in these same observer mice ($F_{2,30} = 0.506, p = 0.608$).

We further investigated whether levels of freezing measured as a result of socially transmitted fear correlated with directly acquired fear resulting from contextual Pavlovian conditioning. We predicted that these measures of fear, whether acquired socially or directly within the same mice, would involve common neural systems but would only be associated for mice with intact oxytocin signaling. Indeed, saline-treated familiar male mice exhibited a significant correlation between socially and directly acquired freezing levels (*Pearson* R^2 *= 0.434, p = 0.038)*, a relationship that was not seen in OXTA-treated mice (*5mg/kg: Pearson* R^2 *= 0.035, p = 0.606; 10mg/kg: Pearson* R^2 *= 0.160, p = 0.194*). Collectively, these findings support the idea that socially and directly acquired fear are subject to individual differences along a common trait of fearfulness(Vaidyanathan, Patrick, & Bernat, 2009) and that oxytocin neurotransmission contributes exclusively to the former.

Chemogenetic activation of oxytocinergic neurons enhances socially transmitted fear

Circulating oxytocin levels appear to contribute to elevated socially transmitted fear in familiar male mice. To investigate the remediating effects of enhanced endogenous oxytocin signaling on socially transmitted fear behavior in unfamiliar mice, we utilized designer receptors exclusively activated by designer drugs (DREADDs) to chemogenetically activate oxytocinergic neurons. We injected a conditional rM3D(Gs)-encoding adeno-associated virus (**Fig 5a**) bilaterally into the hypothalamic paraventricular nuclei (PVN) of Oxt-IRES-Cre/+ mice(B. G. Irani et al., 2010), for which Cre expression is restricted to oxytocin-producing neurons (**Fig 5b**). In mice administered CNO (3mg/kg, I.P.) ninety minutes prior to sacrifice, immunofluorescent imaging revealed co-localization of rM3D(Gs) receptors and c-Fos+ neurons within the PVN (**Fig 5c**), and the number of c-Fos+ cells was significantly elevated compared to mice administered saline (**Fig 5d**). In unfamiliar male mice expressing rM3D(Gs) within oxytocinergic neurons, activation of DREADDs via systemic CNO (3mg/kg, I.P.) administered thirty minutes prior to demonstrator conditioning produced a significant enhancement of freezing compared to saline-treated mice (**Fig 5e**). This enhancement reached a level commensurate with that seen in familiar mice (see Fig 1). There were no significant differences in demonstrator vocalizations ($t(18) = 0.48, p = 0.64$), nor did CNO-treated mice exhibit differences in acquisition of directly acquired fear ($t(14) = 1.380, p = 0.190$).

We next tested the necessity of endogenous oxytocin signaling in empathic behavior by chemogenetically activating oxytocinergic neurons concurrent with either systemic or region-specific receptor antagonism. Using the same DREADD strategy as above, we bilaterally infected the PVN of Oxt-IRES-Cre/+ mice with the rM3D(Gs)-encoding adeno-associated virus and systemically injected CNO at thirty minutes prior to unfamiliar conspecific conditioning. Systemically injecting the OXTA L-368,899 hydrochloride (10mg/kg, I.P.) at forty-five minutes prior to demonstrator conditioning did not attenuate the enhanced socially transmitted fear behavior compared to saline-treated controls (**Fig 5f**). Freezing scores, however, were comparable to CNO-treated mice (see **Fig 5e**), indicating that oxytocinergic neurons were successfully activated. We then tested whether bilateral local infusion of OXTA into the ACC, a region implicated in empathic behavior(Burkett et al., 2016; Jeon et al., 2010), would reduce DREADD-induced behavioral enhancement. Similar to systemic administration, we did not document reductions in socially transmitted fear when compared to saline-treated controls (**Fig 5f**). These data left open the possibility that the pharmacokinetics of the OXTA were ineffective in blocking receptors following chemogenetic activation and release of hypothalamic oxytocin. Therefore, we lastly tested whether direct infusion of

CNO (3uM, 0.5uL) into the ACC would enhance socially transmitted fear via activation of DREADDs at hypothalamic terminals. Compared to saline, local CNO infusion successfully enhanced freezing in unfamiliar observers. Thus, although additional experimentation is needed, these data suggest that endogenous oxytocin acts within the ACC to enhance socially transmitted fear.

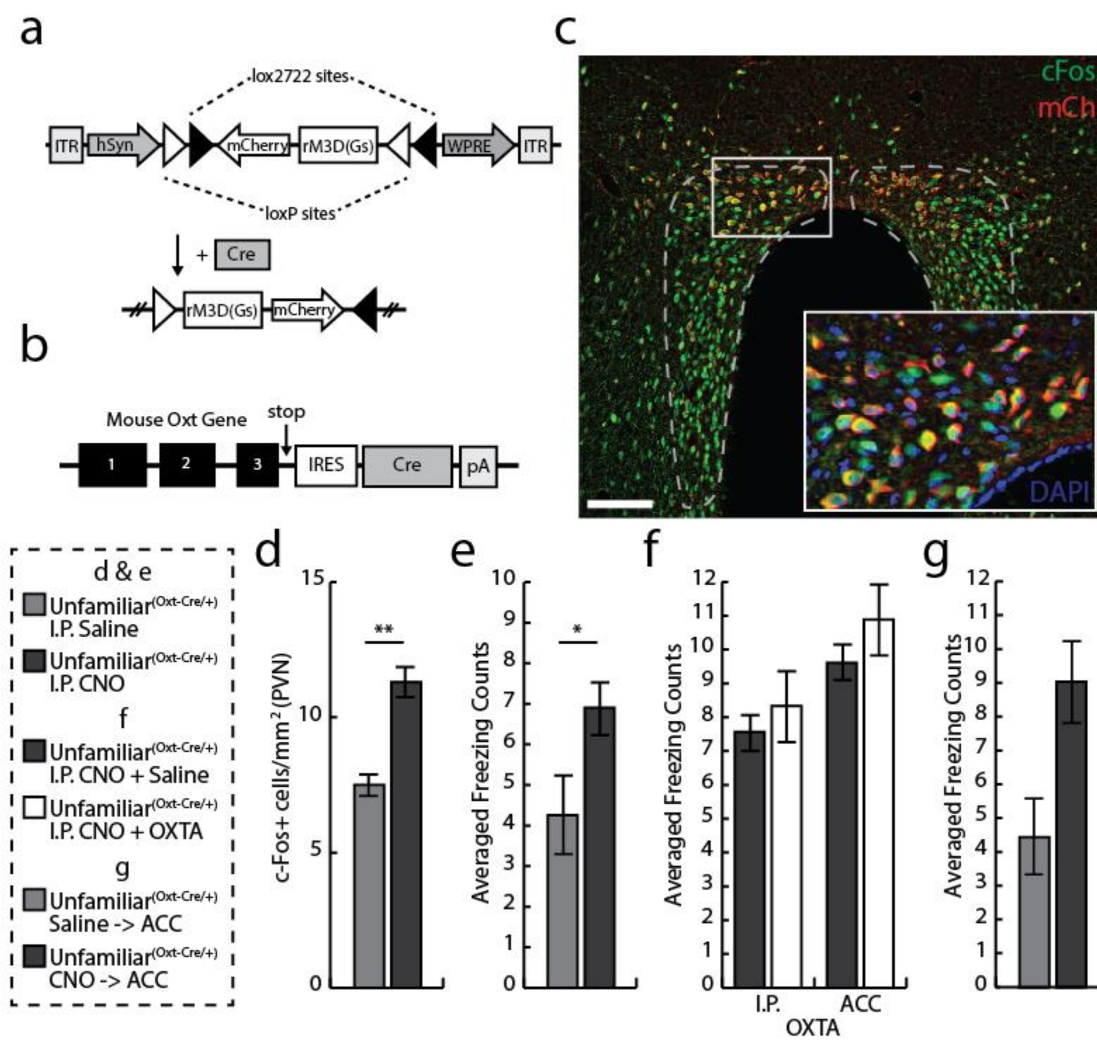

Figure 5. Chemogenetic enhancement of empathic behavior. *(**a**) Schematic of construct used for viral incorporation of the rM3D(Gs) receptor before (**top**) and after (**bottom**) Cre-dependent recombination. (**b**) Schematic of Cre recombinase transgene insertion at the mouse oxytocin gene. (**c**) Representative c-fos and mCherry immunoreactivity within the paraventricular nuclei (PVN; dashed outline) expressing rM3D(Gs) receptors (scale bar, 100um) and (**inset**) magnified view of co-labeled c-fos+/mCherry-expressing neurons. (**d**) Quantification indicating significantly elevated labeling of bilateral c-fos+ neurons in the PVN of CNO-treated mice (3mg/kg, I.P.; black, n = 4 mice, n = 8 slices/mouse) compared to saline-treated controls (gray, n = 4 mice, n = 8 slices/mouse) (two-tailed student's t test: t(7) = 4.9, p = 0.003). (**e**) Administration of CNO (3mg/kg, I.P.) thirty minutes prior to demonstrator conditioning enhanced freezing in unfamiliar male mice (black, n = 10) compared to saline-treated controls (gray, n = 9) when averaged over conditioning trials (two-tailed student's t test: t(19) = 2.25, p = 0.041). (**f**) Administration of the OXTA (white; 3mg/kg I.P., n = 5, two-tailed student's t test: t(9) = 6.5, p = 0.54; 3uM ACC infusion, n = 2, two-tailed student's t test: t(7) = 3.15, p = 0.09) did not reduce freezing compared to saline-treated controls (black, n = 5 (I.P.), 2 (ACC)). (**g**) Local infusion of CNO into the ACC (black, n = 2) non-significantly enhanced freezing compared to saline-treated controls (gray, n = 2; two-tailed student's t test: t(7) = 2.3, p = 0.15). Error bars represent s.e.m. * p < 0.05; ** p < 0.01.*

Discussion

Having established reliable emotional “state-matching” in mice as a form of empathy (Chapter 2), in these experiments we set about investigating its neural substrates. Recent evidence has demonstrated a critical role for oxytocin in forms of empathic behavior: maternal retrieval of mouse pups(Jin et al., 2007), as well as grooming of a stressed conspecific (“consolation”) and social transmission of fear in prairie voles.(Burkett et al., 2016) We demonstrated the necessity of oxytocin signaling for social transmission of fear in mice by administering an oxytocin receptor antagonist and thereby reducing freezing in familiar observers. To demonstrate the role of the central oxytocin system in social transmission of fear, we then conditionally induced DREADD expression in oxytocinergic neurons within the paraventricular nuclei (PVN) of the hypothalamus. This approach allowed us to stimulate oxytocinergic neurons in mice during an episode of observing the distress of an unfamiliar conspecific. Activation of DREADDs produced social transmission of fear in observer mice, showing that stimulation of PVN oxytocin neurons can lead to the elicitation of observational fear in circumstances where it would not otherwise be seen. Together, these findings indicate that the oxytocin system is both necessary and sufficient for the social transmission of fear.

The involvement of the oxytocin system in emotional “state-matching” is consistent with evidence in human subjects of the prosocial effects of oxytocin administered acutely, including increased trust(Kosfeld, Heinrichs, Zak, Fischbacher, & Fehr, 2005), emotional recognition(Domes et al., 2007), and empathy.(Bartz et al., 2010)

Similarly, we found that administration of oxytocin, comparable in route of delivery (i.e., intranasal) and dose(Anagnostou et al., 2014; Bartz et al., 2010), fully rescued social transmission of fear in unfamiliar male mice. Of greater clinical relevance(Pedersen et al., 2011; Tachibana et al., 2013), we saw similar facilitation after an extended oxytocin dosing protocol that has been found to enhance social behavior in mice.(Teng et al., 2013, 2015) Since the last dose of oxytocin had been administered a full ten days prior to behavioral testing, the facilitation of social transmission of fear must have occurred through persistent neuroadaptive changes rather than through the direct effects of the drug. These changes most likely occurred in the hypothalamic-cingulate-amygdalar circuitry that mediates affective empathy.(Meyer-Lindenberg et al., 2011) The anterior cingulate cortex (ACC) and central nucleus of the amygdala (CeA) are two primary targets of hypothalamic oxytocinergic neurons.(Gimpl & Fahrenholz, 2001; Knobloch et al., 2012) Activity in the ACC has been implicated in several empathy-related processes across mammalian species(Jeon et al., 2010; Singer et al., 2004), the CeA is a critical locus for the expression of fear conditioning(Phelps & LeDoux, 2005), and oxytocin enhances the functional connectivity between these regions.(Meyer-Lindenberg et al., 2011; Sripada et al., 2013) Hence, oxytocinergic interplay between these structures likely plays a critical role in the social transmission of fear.

Indeed, we found that the same chronic oxytocin treatment that enhanced social transmission of fear down-regulated transcription of the oxytocin receptor in the CeA. This is consistent with the internalization of the receptor in the CeA seen *in vitro* following extended oxytocin overstimulation.(Terenzi & Ingram, 2005) Hence, the persistent facilitative effect of repeated oxytocin administration appears to involve adaptation within the hypothalamic-amygdala oxytocin pathway. In contrast, the prosocial effects of acute intranasal oxytocin and stimulation of oxytocinergic neurons are likely mediated by the hypothalamic-cingulate oxytocin pathway, since local infusion of an oxytocin receptor antagonist into this structure blocks social transmission of fear.(Burkett et al., 2016) We found no long-term change in oxytocin receptor transcription in the ACC, suggesting a dissociation between the loci of acute and chronic oxytocin effects on social transmission of fear. Along with chemogenetic activation of oxytocinergic neurons of the hypothalamus, we propose a brain circuitry model that accounts for the enhanced empathic effect of oxytocin (**Fig 6**).

It is notable that in several experiments we found no effects of either increasing or decreasing oxytocin activity acutely on Pavlovian fear conditioning. This is an important control in order to show that oxytocin affects the social transmission of fear rather than the acquisition or expression of fear itself. We also found no effect of our chronic oxytocin regimen on fear conditioning. This suggests that downregulation of oxytocin receptors in the CeA (specifically, CeL) is restricted to neurons that are also activated by socio-emotional cues, perhaps via the ACC.(Bissière et al., 2008; Jeon et al., 2010) Indeed, evidence for independent circuits specific to social or direct fear have already been discovered within the hypothalamus.(Silva et al., 2013) More generally, the absence of any effect of either acute or chronic oxytocin on fear conditioning requires us to re-evaluate the role that oxytocin plays in fear and anxiety.(Neumann & Slattery, 2016) The results of a number of other studies have suggested that oxytocin is anxiolytic, and therefore conforms to the hypothesis that oxytocin primarily modulates core emotional systems of the brain.(Ring et al., 2006; Waldherr & Neumann, 2007; Yoshida et al., 2009) In contrast, we showed that oxytocin or an oxytocin receptor antagonist enhance or block socially transmitted fear, respectively. A plausible resolution to these apparently paradoxical effects is that the reductions in fear and anxiety previously seen in rodent studies were secondary to the prosocial effects of oxytocin. In both humans and rodents, fear and anxiety can be buffered by the presence of another non-fearful individual.(Egliston & Rapee, 2007; Y. Guzmán, Tronson, & Guedea, 2009) Thus, the presence of a home-cage conspecific at the time that oxytocin is administered could have the incidental effect of ameliorating subsequent acquisition of fear. When this factor is controlled for, as in this study and others that are attuned to the effects of social cues(Choe et al., 2015), modulatory effects of oxytocin on fear acquisition are not seen.

Our findings extend those from the human literature highlighting the role of OXT in emotional recognition and empathy-like behavior. Using this mouse model of emotional "state-matching", we point to neural sequelae of sub-chronic administration and possible early-life contributions to such behavior. Our study is also the first to use chemogenetic techniques for evoking OXT release, showing significant effects on socially transmitted fear in unfamiliar male mice. As a whole, such findings contribute to our understanding of the evolutionarily conserved oxytocin system and provide a model system through which the role of oxytocin in normal and pathological components of empathy can be studied.

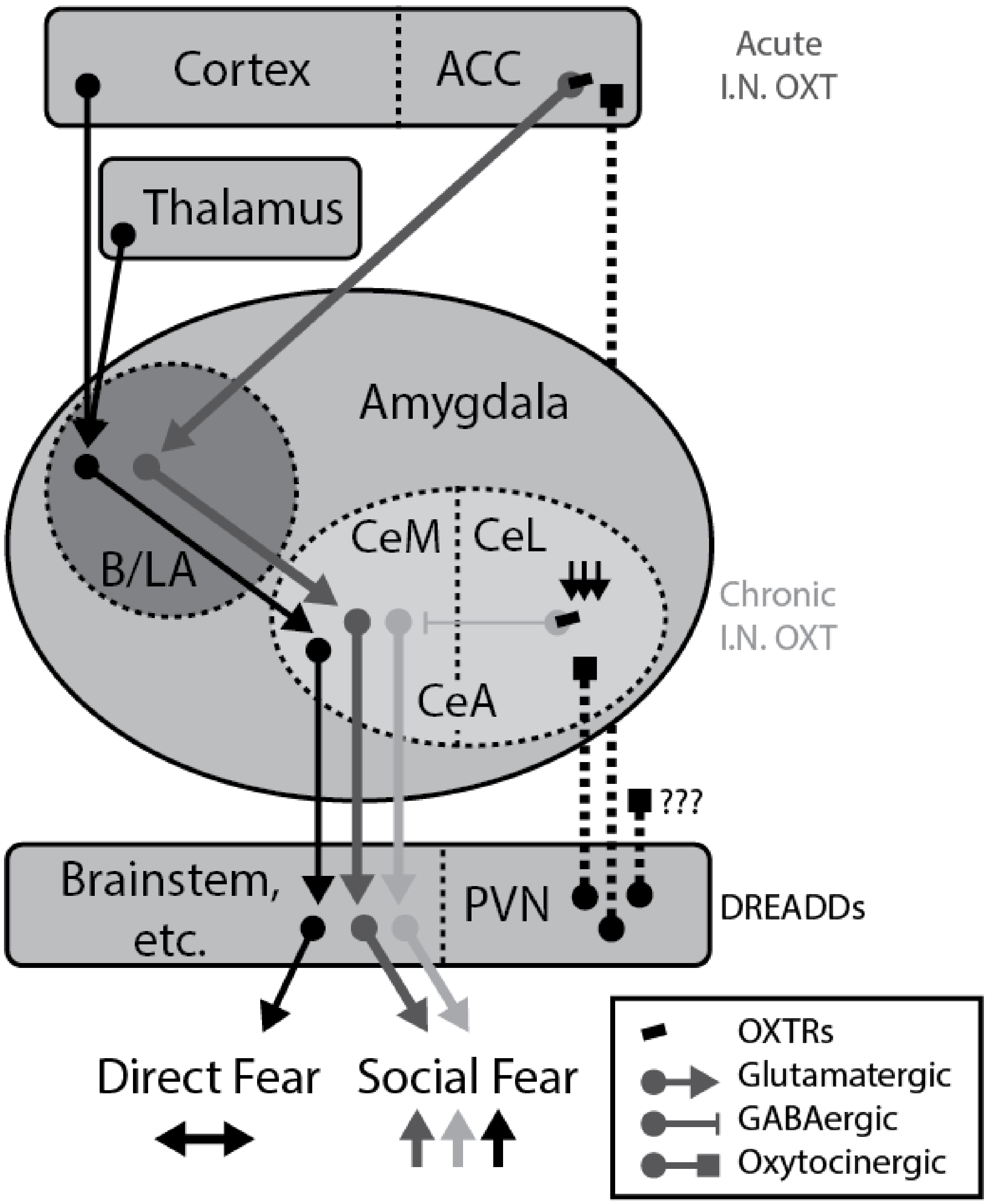

Figure 6. Circuitry model for the oxytocin effect on empathic behavior. *Normal fear circuitry (**black**) involves top-down excitatory (glutamatergic, **arrows**) projections from cortex and thalamus converging on the basolateral/lateral (B/LA) nuclei of the amygdala that then activate neurons of the central nucleus (CeA). These neurons activate various efferents of the brainstem, midbrain, and hypothalamus that produce fear behaviors. Acute intranasal oxytocin (**dark gray**) binds preferentially to oxytocin receptors (OXTRs) within the anterior cingulate cortex (ACC) that enhances social fear via a parallel thalamic-amygdalar-brainstem pathway. Chronic*

*intranasal oxytocin (**light gray**) causes downregulation of OXTRs of the central lateral nucleus (CeL), which depotentiates inhibitory projections to the central medial nucleus (CeM), and enhances social fear. These effects are consistent with those in the literature.*(Burkett et al., 2016; Huber et al., 2005; Viviani et al., 2011) *Chemogenetic activation of oxytocinergic neurons (**black, dashed**) of the paraventricular nucleus (PVN) releases oxytocin that putatively binds to receptors within the ACC, amygdala, or other central or peripheral targets to increase social fear. None of these manipulations of oxytocin signaling alter directly-acquired fear, which is proposed to be mediated by an independent, yet parallel, circuit.*

Chapter 4: The Chd5 epigenetic mouse model of ASDs

Introduction

The complex genetic basis of ASDs has been hypothesized to result from dysregulation of epigenetic mechanisms(Zhubi, Cook, Guidotti, & Grayson, 2014) that preferentially impact neurodevelopmental processes.(Mohan & Chaillet, 2012; Ronan, Wu, & Crabtree, 2013) To examine this etiological hypothesis, we focused on chromodomain helicase DNA-binding 5 (Chd5), a neural-specific chromatin remodeler. Chd5 associates with a nucleosome remodeling deacetylase (NuRD)-like complex to bind with histone lysine residues and thereby modulate gene accessibility and transcription.(Eberl et al., 2013) Expression of Chd5 is upregulated within the brain during early postnatal development in mice(Vestin & Mills, 2013) where it mediates neurogenesis(Egan et al., 2013) and influences expression of numerous neurodevelopmental genes.(Egan et al., 2013; Potts et al., 2011) Variants of the CHD5 gene have been identified in large genome-wide association studies of ASDs(Bucan et al., 2009), including the most recent and largest whole-exome sequencing study to date.(De Rubeis et al., 2014) These findings indicate a strong contribution of the Chd5 chromatin remodeler to neurodevelopmental processes and a putative role in the pathogenesis of ASDs.

To examine the role of the Chd5 chromatin remodeler in ASDs, we deleted the second exon of the Chd5 gene conditionally within neural tissue of mice. In these genetically modified mice, characterization of the cortex transcriptomic profile highlighted functional gene networks involved with cell signaling, neurodevelopment, and ASDs. To ascertain the effects of Chd5 on cortical neuron morphology, we then imaged pyramidal neurons dissociated from Chd5$^{-/-}$ cortical tissue at post-natal day (PND) 0-1. Pyramidal neurons at PND 12 from Chd5$^{-/-}$ mice exhibited reduced dendritic complexity at sites proximal to the soma, thereby indicating that Chd5 deletion altered the normal development of neuronal morphology.

Materials and Methods

Animals & Genotyping

Chd5$^{-/-}$ mice were generated using conditional (Chd5$^{fl/+}$) embryonic stem cells (C57BL/6J) obtained from the EUCOMM consortium. Mice were genotyped using the following Chd5 primers: Floxed allele (Forward (P1): TCTACAGAGCAAGTTCCAGGAC; Reverse (P2): GACCCCAAGCTGAGGAAAACC), deleted allele (Forward (P1): TCTACAGAGCAAGTTCCAGGAC; Reverse (P3): CCGTGTTGGTTTCTCTGACTTTC). Tissue was also genotyped for the nestin-Cre transgene using the following primers: Forward: GCGGTCTGGCAGTAAAAACTATC; Reverse: GTGAAACAGCATTGCTGTCACTT. TomR-GFP transgenic mice were obtained from Jackson Laboratories (#007676) and genotyped using the following primers: Forward: CTCTGCTGCCTCCTGGCTTCT; Wild-type Reverse: CGAGGCGGATCACAAGCAATA; Mutant Reverse: TCAATGGGCGGGGGTCGTT.

All mice were bred and maintained according to US National Institutes of Health guidelines for animal care and use and Institutional Animal Care and Use Committee of the University of Minnesota – Twin Cities. Mice were provided food and water *ad libitum* except during experimental testing, and housing lights were maintained on a 12:12hr light/dark cycle. All experimental mice were group-housed males.

Western Blot

Whole-brain tissue was collected rapidly, transferred to cold PBS, and homogenized in cold lysis buffer. Homogenate was exposed to three freeze-thaw cycles, spun at 10,000 RPM at 4C, and supernatant was extracted. Total protein was quantified using standard spectrophotometer methods. Forty micrograms of protein was loaded into a gel then transferred and incubated with Chd5 anti-rabbit primary antibody (1:1000), followed by Chd5 rabbit-anti-mouse secondary (1:500). Control antibodies included Gapdh primary (1:20,000) and secondary (1:500). Bands were visualized and quantified using the ImageQuant system (GE Healthcare Life Sciences).

RNA Sequencing

Total RNA was extracted from frontal cortex brain tissue of naïve, 8-weeks old male mice matched with same-sex littermates (3 wild-type; 3 Chd5$^{-/-}$). After collection, tissue was rapidly immersed in RNAlater (Qiagen) and total RNA was isolated using the RNeasy Mini Kit (Qiagen, 74104). RNA integrity numbers were calculated using a bioanalyzer to be >7 for all samples. Extracted mRNA was purified with poly-T oligo-attached magnetic beads, heat-fragmented, and both strands were synthesized and purified. The 3' ends were then polyadenylated and RNA-Seq libraries were prepared from samples using the Illumina TruSeq protocol, then barcoded and sequenced using a 50bp paired-end run on the Illumina HiSeq 2500. Roughly 20 million paired-end reads were generated for each run. Raw RNA sequencing data was uploaded to the Minnesota Supercomputer Institute Galaxy server. Quality control of raw data was completed using FastQC and all samples had base sequence quality values (Phred) >30 with no overrepresented sequences. FastQ files were then aligned to the mm10_canonical mouse genome and mapped using Tophat(Trapnell, Pachter, & Salzberg, 2009). A list of assembled transcripts for each replicate was then analyzed using Cufflinks(Trapnell et al., 2012) to estimate abundances (represented by fragments per kilo bases per million mapped reads (FPKM)) and tested for differential expression. Transcriptional differences between wild-type and Chd5$^{-/-}$ replicates were then analyzed in cummeRbund. The lists of significantly differentially regulated transcripts ($p < 0.05$, false-discovery rate (FDR) < 5%) were exported and analyzed using the Ingenuity Pathway Analysis software.

Quantitative RT-PCR

Total RNA was obtained from frontal cortex brain tissue of naïve, 8 weeks of age mice matched with same-sex littermates using the same protocol as for RNA sequencing. Isolated RNA was processed for DNAase digestion and reverse-transcribed using the GoScript reverse transcriptase (Promega) according to the manufacture’s manuals. The cDNA was diluted 1:7 before preparing the qRT-PCR reaction, which was then run using the GoTaq qPCR master mix (Promega). All PCR primers were designed at qPrimerDepot (http://mouseprimerdepot.nci.nih.gov/) and purchased through the

University of Minnesota's Genomics Center. Mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA was used as the endogenous control. For each sample, a duplex PCR reaction was set-up containing a target gene primer set and a GAPDH primer set. We conducted 3 replicate PCR reactions for each sample. The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60+/-2°C for 1 min on the CFX96 Real-Time System (BioRad, Inc.). The relative levels of gene expression were determined according to the standard curve methods described in the BioRad company manual. The expression value of the target gene in each well was first normalized by the expression value of the GAPDH gene in the same well. The median of 3 repeated reactions was used to represent the relative quantity of the target gene.

Neuronal Culturing

Primary neuronal cell cultures from Chd5$^{-/-}$ and wild-type control littermates were prepared as described previously.(Beaudoin et al., 2012) Frontal cortex tissue was dissected from post-natal day (PND) 0-1 pups, dissociated, sparsely transfected with a GFP-encoded construct, and plated on poly-L-lysine-coated glass coverslips (~100,000 cells/35mm plate) using plating media (MEM Eagle's (Invitrogen, #14175095), FBS (Invitrogen, #16140071), 20% glucose, sodium pyruvate (Invitrogen, #11360070), glutamine (Invitrogen, #25030081), penicillin/streptomycin (Invitrogen, #15140122)). Two hours after plating, media was replaced with maintenance media (Neurobasal medium (Invitrogen, #21103049), B-27 (Invitrogen, #17504044), 200mM glutamine

(Invitrogen, #25030081), penicillin/streptomycin (Invitrogen, #15140122)), and half the media was subsequently replenished twice per week.

Neuronal Morphology

At 12 days *in vitro* (DIV), primary neuronal cultures were fixed with 4% paraformaldehyde/PHEM/0.12M sucrose, then blocked with 3% RIA grade BSA in 1x PBS for 1 hour at room temperature. Cells were then permeabilized with 0.2% Triton-X in PBS at room temperature, and blocked again with 3% RIA grade BSA in PBS. Cells were stained with a primary anti-MAP2 antibody (1:1000; Abcam, #ab11268) in 1% RIA grade BSA/PBS overnight at 4°C, followed by secondary (1:100; Abcam) and 1x DAPI counterstain for thirty minutes at room temperature. Finally, cells were mounted with DABCO mountant, sealed with nail polish, and stored at 4°C. Fluorescent imaging of cells was performed using an Ursula microscope by a 20X objective and Micro-Manager microscopy software. Images of 15 randomly-selected pyramidal neurons were acquired per culture. Cells were analyzed for dendritic arborization using the Image J Sholl Analysis plug-in(Ferreira et al., 2014) with a 10um radius interval.

Results

Generation and validation of the Chd5$^{-/-}$ mouse model

To examine the role of Chd5 in neurodevelopment, epigenetic dysregulation, and ASD-like endophenotypes, we generated mice harboring a conditional deletion of exon two of the Chd5 gene (**Fig 7a**). Chd5 gene-targeted mice were firstly crossed with FlpE transgenic mice to remove the targeting/selection construct. Conditional ($Chd5^{fl/+}$) mice were then crossed with nestin-Cre transgenic mice, allowing for restricted Cre-recombinase activity and Chd5 allele deletion within neural tissue. This strategy was pursued in order to preserve Chd5 expression in the testes (a significant peripheral source of Chd5), and thereby avoiding dysregulated spermatogenesis and infertility(Li et al., 2014). A subset of $Chd5^{fl/+}$ mice was also crossed with double fluorescent tomR-GFP transgenic reporter mice(Muzumdar, Tasic, Miyamichi, Li, & Luo, 2007), whereby Cre recombination produced GFP expression exclusive to neural tissue (**Fig 7b**). $Chd5^{-/-}$ mice were identified via PCR of genomic tail-DNA (**Fig 7c**), and validated via PCR of genomic brain-DNA (**Fig 7d**) and brain fluorescence labeling (**Fig 7e**). $Chd5^{-/-}$ mice show a nearly complete loss of the Chd5 protein in whole brain extracts, whereas $Chd5^{+/-}$ mice show haplosufficiency and were therefore excluded from analyses (**Fig 7f**). $Chd5^{-/-}$ mice were embryonically viable and exhibited no gross physical abnormalities compared to littermate controls. This was expected in light of the dramatic increase in Chd5 protein expression during late adolescent development in the mouse.(Vestin & Mills, 2013)

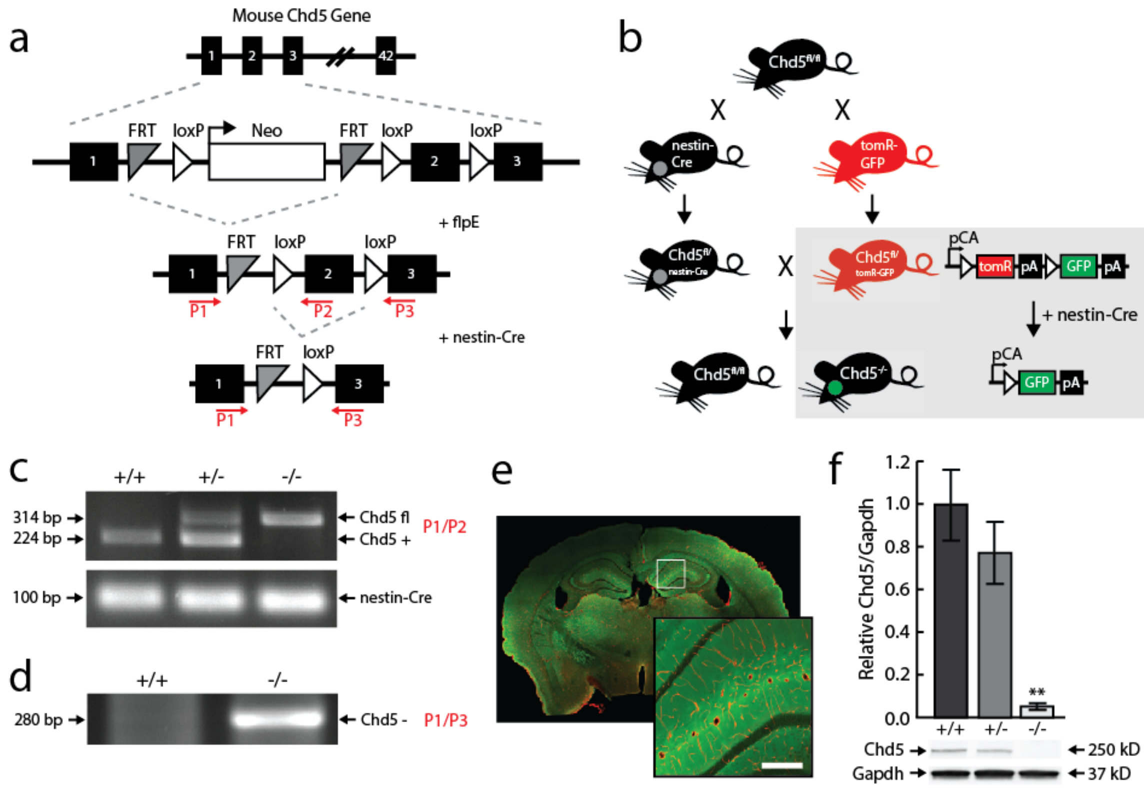

Figure 7. Generation and validation of the Chd5$^{-/-}$ mouse model of ASDs. *(**a**) Gene targeting strategy for generating the conditional Chd5$^{fl/+}$ allele in embryonic stem cells and null Chd5$^{-/-}$ allele in mice. See Methods for primer (P1-3) information. (**b**) Breeding strategy for generating brain-specific Chd5$^{-/-}$ mice with GFP reporter expression (**box**, schematic of tomR-GFP reporter gene expression). (**c**) PCR genotyping of genomic DNA from mouse tail biopsy for (**top**) Chd5 targeted allele and (**bottom**) nestin-Cre transgene allele. (**d**) PCR genotyping of genomic DNA from mouse whole-brain for Chd5 deletion. (**e**) Representative adult Chd5$^{-/-}$ coronal brain slice expressing TomR (**red**) in non-neural tissue and GFP (**green**) in neural tissue following Cre recombination. Inset represents magnified region. Scale bar, 100um. (**f**) Western blot analysis of whole brain extracts indicating the near absence of protein in Chd5$^{-/-}$ mice (two-tailed student's t test, $t(11) = 31.85$, $p = 0.0002$), but not Chd5$^{+/-}$ mice (two-tailed student's t test, $t(11) = 1.05$, $p = 0.33$), compared to Chd5$^{+/+}$ mice (**inset**, Chd5 protein bands over Gapdh controls). Error bars represent s.e.m. ** $p < 0.01$.*

Differential gene expression in the cortex of Chd5$^{-/-}$ mice

Chromatin remodeling exerts widespread effects on gene expression. To examine the transcriptional consequences of deletion of the Chd5 chromatin remodeler, we completed RNA sequencing of frontal cortex tissue collected from experiment-naïve mice. All significant differences in expression levels (FDR < 5%, $p < 0.05$) were examined between genotype groups. A highly restricted subset of all (23,284) transcripts showed significantly reduced (67) or elevated (77) expression in Chd5$^{-/-}$ mouse cortical tissue (**Fig 8a; Table 2,3**). The reduced transcript subset was enriched for functions related to cellular growth and proliferation ($p = 2.58 \times 10^{-10}$), cell death and survival ($p = 2.77 \times 10^{-8}$), cellular movement ($p = 7.33 \times 10^{-7}$), and gene expression ($p = 1.03 \times 10^{-6}$) (**Fig 8b; Table 2**); whereas the elevated transcript subset was enriched for molecular/cellular functions related to cell-to-cell signaling ($p = 1.64 \times 10^{-11}$), molecular transport ($p = 1.64 \times 10^{-11}$), small molecule biochemistry ($p = 1.64 \times 10^{-11}$), and vitamin/mineral metabolism ($p = 5.0 \times 10^{-9}$) (**Fig 8b; Table 3**).

In order to confirm differential expression of transcripts identified by RNA sequencing, we conducted qRT-PCR analyses in a select set of transcripts common to several molecular/cellular functions implicated in ASD pathophysiology (**Fig 8c**). RNA-sequencing and qRT-PCR values showed a statistically significant correlation, thereby validating gene expression differences in Chd5$^{-/-}$ mouse cortex (**Fig 8d**). These results confirm that deletion of the Chd5 chromatin remodeler induces targeted effects on cell signaling, development, and regulation of gene expression mechanisms.

As a putative mouse model of ASDs, we also compared the gene transcripts differentially expressed in the $Chd5^{-/-}$ mouse cortex with a set of candidate genes identified in humans with ASDs. Using the AutismKB nonsyndromic candidate gene list (2,136), we discovered 8 genes common to our list of 67 downregulated transcripts (**Table 4**), and 15 genes common to our list of 77 upregulated transcripts (**Table 5**). The overlapping list of upregulated gene transcripts was enriched significantly above chance (*Fisher's exact test; odds ratio = 2.8;* $p = 8.4 \times 10^{-4}$). This evidence suggests that the Chd5 chromatin remodeler may epigenetically influence candidate genes that putatively give rise to ASDs in humans.

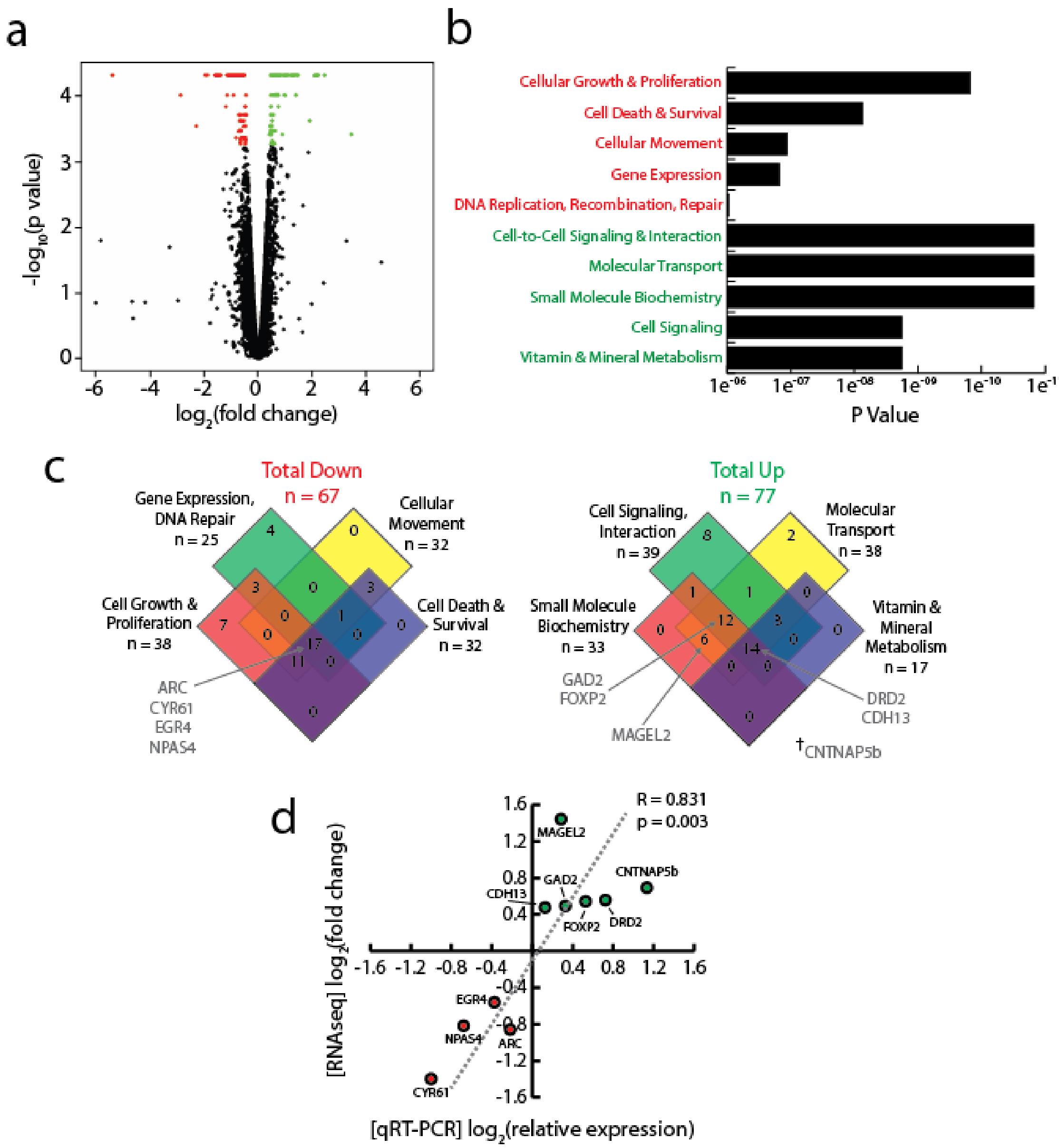

Figure 8. Differential gene expression in the cortex of Chd5$^{-/-}$ mice. *(**a**) Volcano plot illustration of significantly downregulated (**red**) and upregulated (**green**) gene transcripts in Chd5$^{-/-}$ cortical tissue (FDR < 5%). Gene transcripts with no-fold change were excluded. (**b**) The five most significant gene ontology (GO) terms for downregulated and upregulated gene transcripts from Ingenuity Pathway Analysis (IPA) (see Tables 2 & 3). (**c**) Venn diagram depictions of the most significantly (**left**) downregulated and (**right**) upregulated molecular/cellular functions. GO terms corresponding to each of the genes selected for qRT-PCR confirmation are indicated by arrows. † CNTNAP5b does not correspond to any of the highlighted GO terms. (**d**) qRT-PCR confirmation of RNAseq expression in a select set of transcripts (see **c**). ARC, activity-regulated cytoskeleton-associated protein; CNTNAP5b, contactin-associated protein-like 5b; CYR61, cysteine rich heparin-binding protein 61; DRD2, dopamine receptor 2; EGR4, early growth response 4; FOXP2, forkhead box protein P2; GAD2, glutamate decarboxylase 2; MAGEL2, MAGE-like 2; NPAS4, neuronal PAS domain protein 4; STAC2, SH3 and cysteine rich domain*

Dendritic alterations in cortical pyramidal neurons of Chd5$^{-/-}$ mice

Post-mortem studies of ASDs have identified cortical features consistent with aberrant neurodevelopment.(de la Torre-Ubieta et al., 2016) Chromatin remodeling mediates a panoply of neurodevelopmental processes(Ronan et al., 2013), and Chd5 in particular has been associated with early-life cortical development and neurogenesis.(Egan et al., 2013; Vestin & Mills, 2013) Another set of neuron-specific chromatin remodeling proteins – nBAF53a/b – have been previously shown to regulate dendritic outgrowth and complexity.(J. I. Wu et al., 2007) Suggesting a similar role in the regulation of neuronal morphology, our RNA sequencing findings from cortical tissue of Chd5$^{-/-}$ mice implicated dysregulation of transcripts associated with cellular growth and proliferation, and cell-to-cell signaling. To examine this feature of neurodevelopment, we dissociated and cultured cortical neurons from PND0-1 mice, immunofluorescently labeled dendritic processes with MAP2(Goedert, Crowther, & Garner, 1991), and quantified dendritic morphology of pyramidal neurons at 12 days *in vitro* (DIV) via Sholl analysis (**Fig 9a**). In Chd5$^{-/-}$ pyramidal neurons, we discovered an altered dendritic morphology profile that involved less intersections at sites proximal to the soma (**Fig 9b,c**). This reduced complexity of arborizations did not appear to influence the peak distance of dendrites from the soma (i.e., dendritic outgrowth) (**Fig 9d**).

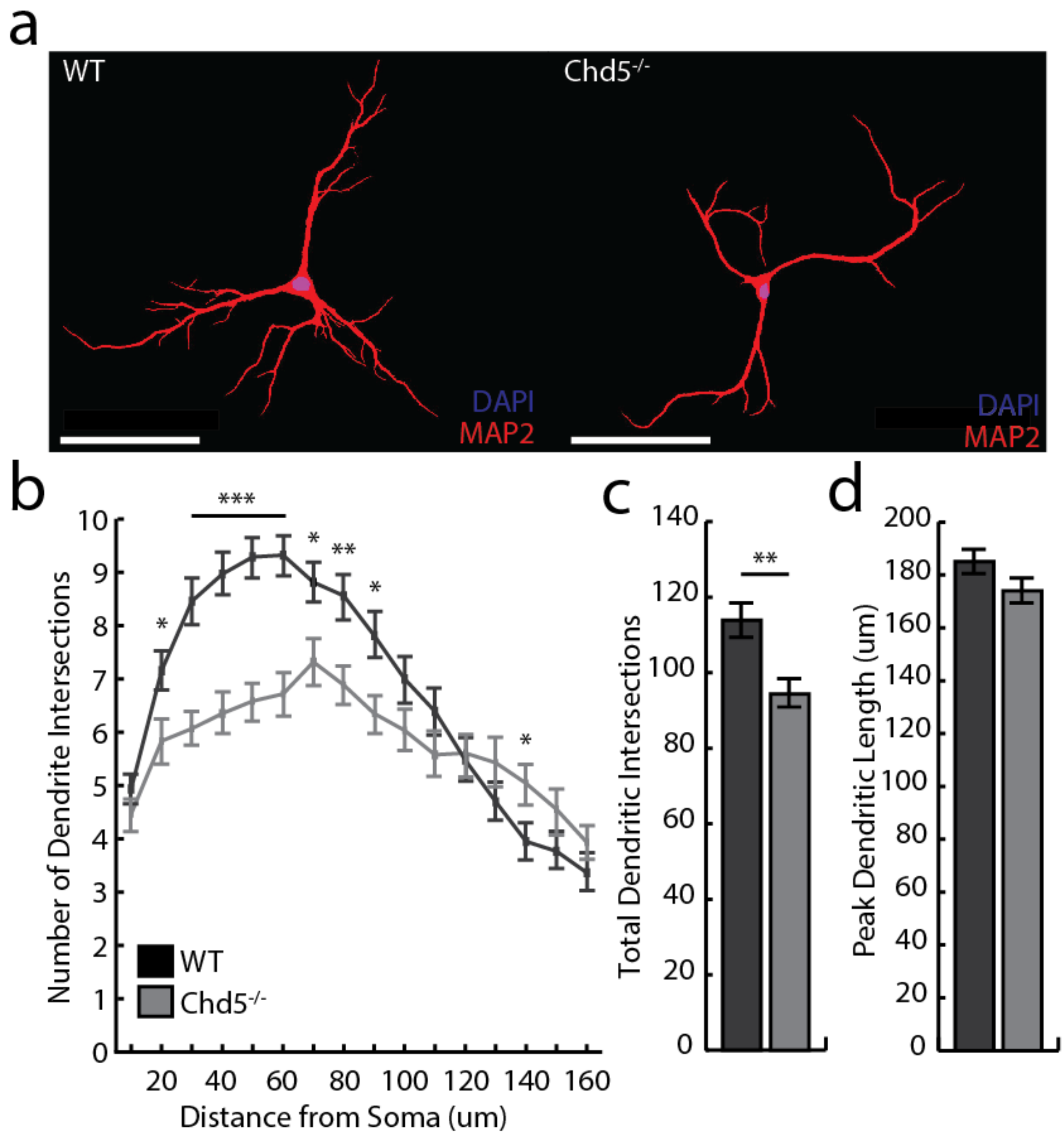

Figure 9. Dendritic alterations in cortical pyramidal neurons of $Chd5^{-/-}$ mice. *(**a**) Representative pyramidal neurons from (**left**) wild-type and (**right**) $Chd5^{-/-}$ mouse cortex immunofluorescently stained with MAP2 for dendritic processes (**red**) and DAPI for nuclei (**blue**) (scale bar, 100um). (**b**) Compared to wild-type (black, n = 4 mice, 60 neurons), pyramidal neurons from $Chd5^{-/-}$ cortex (gray, n = 4 mice, 60 neurons) showed significantly fewer dendritic intersections at sites more proximal to the soma. (**c**) The total number of dendritic intersections in these neurons were significantly less in $Chd5^{-/-}$ cortex compared to wild-type (two-tailed student's t test: $t(119)$: 3.14, $p = 0.002$). (**d**) $Chd5^{-/-}$ pyramidal neurons did not show differences in peak dendritic distance compared to wild-type (two-tailed student's t test: $t(119)$: 1.85, $p = 0.066$). Error bars represent s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

Discussion

We successfully engineered neural-specific deletion of the Chd5 chromatin remodeler in mice. Gene targeting and excision were confirmed via PCR, and protein depletion via western blot. A double-fluorescence transgenic cross further confirmed neuron-specific Cre-recombinase activity, and therefore restricted deletion of Chd5 in neuronal tissue.

Variations in frontal cortex gene expression, as measured by RNA sequencing and confirmed through qRT-PCR, indicated changes in gene expression linked to transcription, cell development, and signaling mechanisms. These canonical pathways are common to ASD etiology, and may therefore provide clues to the underlying pathophysiology of Chd5$^{-/-}$ mice.(De Rubeis et al., 2014) A strikingly high proportion of the genes differentially expressed by Chd5 deletion (23 of 144) are those implicated specifically in ASDs (e.g., Magel2, Cdh13) and/or socio-emotional functions (e.g., Foxp2, Drd2, Gad2). One of these, Magel2, is a prominent risk gene in Prader-Willi Syndrome (PWS), an ASD-associated condition caused by duplication of the 15q11 genomic region.(Schaaf, Gonzalez-Garay, & Xia, 2013) Mice harboring the homologous copy number variation display reduced novelty-based exploration and feeding, features akin to the neophobic behaviors observed in the Chd5 mouse model (see Chapter 5; Fig 10).(Tamada et al., 2010) Dysregulation of Magel2 by Chd5 deletion represents an example by which an epigenetic mechanism could impact expression of a highly penetrant risk gene.

Another particularly intriguing gene transcript downregulated following Chd5 deletion was Npas4. This transcription factor plays a critical role in activity-dependent inhibitory synapse formation, and thereby excitatory-inhibitory balance in the brain(Spiegel et al., 2014). Mice lacking the Npas4 transcription factor show behavioral characteristics indicative of psychiatric diseases, including reduced social novelty preference and cognitive functions, features reminiscent of the behavioral impairments of the Chd5$^{-/-}$ mice.(Coutellier, Beraki, Ardestani, Saw, & Shamloo, 2012) Interestingly, Npas4 mutations have not been directly implicated in ASDs via human GWAS (see (De Rubeis et al., 2014)). The current findings suggest an epigenetic pathway whereby this gene may be involved in the etiology of ASDs.

We also identified upregulation of dopamine receptor-encoding transcripts (Drd1a and Drd2) in Chd5$^{-/-}$ cortical tissue. Dopamine signaling, including specific polymorphisms in the Drd2 gene(Noble & Ozkaragoz, 1998), has been linked to novelty seeking behavior.(Suhara, Yasuno, Sudo, & Yamamoto, 2001) Therefore, the observed reduction in novelty preferences in Chd5$^{-/-}$ mice (see Chapter 5; Fig 10) could be attributed to the dysregulation of these transcripts. Lastly, we discovered upregulation of Foxp2, a transcription factor integral to speech/language development.(Lai, Fisher, & Hurst, 2001) Mice lacking the Foxp2 gene exhibit abnormal pup separation-induced USVs.(Shu et al., 2005) Similar to these findings, we detected changes in the number and type of USVs elicited by Chd5$^{-/-}$ pups (see Chapter 5; Fig 11). In sum, the known functions of a sizable proportion of the genes whose activity is affected by deletion of

Chd5 relate to preference for sameness and social cognition, and thus align with the behavioral impairments noted in Chd5$^{-/-}$ mouse (see Chapter 5).

Differential expression of transcripts related to cellular signaling, development, and disease could feasibly produce alterations in neuronal growth and structure. In agreement with this premise, we discovered variations in pyramidal neuron morphology within the cortex of Chd5$^{-/-}$ mice. Specifically, neurons cultured from frontal cortex displayed less branching at proximal dendrites that was normalized at more distal sites. One of the first documented neuropathological markers of ASDs(Raymond, Bauman, & Kemper, 1995), a reduction in dendritic complexity has since been recapitulated in mouse models following deletion of ASD risk genes, including MeCP2(Kishi & Macklis, 2010), SHANK3(Durand et al., 2012), and various neurexins/neuroligins.(S. X. Chen, Tari, She, & Haas, 2010) This cellular endophenotype of ASDs and various mouse models could indicate a common pathophysiological pathway underlying the disease. Further, such variations in cell structure likely alter functional signaling within the cortex and produce deleterious behavioral sequelae.

Chapter 5: Behavioral endophenotypes of Chd5$^{-/-}$ mice

Introduction

Having generated mice lacking Chd5 within neural tissue and subsequently examined transcriptional and neuron-level structural variations within the cortex, we next sought to characterize behavioral features of Chd5$^{-/-}$ mice. We firstly chose to adopt a classic phenotyping approach to identify features mirroring symptoms of ASDs. To characterize stereotypic/repetitive behavior or insistence on sameness, we performed open field and novel object recognition (NOR) tasks to measure anxiety-like and neophobia-like behaviors, respectively. To further characterize neophobia-like behavior and control for olfactory impairments that could confound assessment of social behaviors, we also completed an olfactory habituation-dishabituation task. Finally, to characterize socio-communicative abnormalities in Chd5$^{-/-}$ mice, we utilized a well-established three-chamber social approach assay in adult mice and isolation-induced ultrasonic vocalization (USV) measurements in early post-natal pups.

Expanding upon these classic phenotyping assays, we then used our socially transmitted fear and social conditioned place aversion (sCPA) paradigms to measure empathic behavior in Chd5$^{-/-}$ mice. Impairments in empathic behavior are established endophenotypes in ASDs(S Baron-Cohen et al., 1985; Lombardo, Barnes, Wheelwright, & Baron-Cohen, 2007; Sucksmith et al., 2013), but have yet to be identified in a rodent model of psychiatric disease. As endophenotypic features of ASDs, we hypothesized that Chd5$^{-/-}$ mice would exhibit impairments in these indices of empathy.

As predicted, $Chd5^{-/-}$ mice exhibited neophobic behaviors that spanned various domains (environmental, object-based, sensory, and social), and abnormal socio-communicative abilities as both pups and adults. More intriguing yet, we observed impairments in social fear behaviors of $Chd5^{-/-}$ mice, namely reduced freezing and escape, in response to the distress of a familiar conspecific. Levels of social fear were comparable those from unfamiliar wild-type mice, and were unable to be rescued with oxytocin treatment. Importantly, several other behavioral measures in mice were unaffected by deletion of Chd5, namely: motor activity (via startle reactivity, open field), sensorimotor gating (via prepulse inhibition), associative learning (via Pavlovian fear), and anxiety (via elevated plus-maze). Thus, these findings strongly link behavioral endophenotypes of ASDs to the $Chd5^{-/-}$ mouse model.

Materials and Methods

Open Field and Novel Object Recognition

The open field and novel object recognition tasks were conducted as previously described.(Bevins & Besheer, 2006) Six- to eight-week old male mice were handled for three consecutive days (5mins/day). On the first day of testing, individual mice were introduced to the open field arena (50x50x40cms) for 20mins. Distance traveled, velocity, and center time (defined as the center 50% of the arena area) were calculated using the TopScan software system (Clever Systems; Reston, VA). On the following day of testing, individual mice were placed into same arena with two identical (familiar) objects (solid-colored geometric shapes; e.g., red cubes) and allowed free exploration for

10mins. After one hour, mice were re-introduced to the same testing arena with one item replaced with a different (novel) object (e.g., blue pyramid) and allowed free exploration for 5mins. The time spent investigating each item (defined as when the mouse's head was orientated <1cm to the object) was scored manually by a trained, genotype-naive experimenter using Button Box 5.0 (Behavioral Research Solutions, LLC). A preference score was calculated as: [(novel time) / (novel time + familiar time)].

Olfactory Habituation-Dishabituation

The olfactory habituation-dishabituation test was conducted as described previously(Yang & Crawley, 2009). Male mice were presented with a control (water) odor followed by three distinct odors (vanilla, female bedding, male bedding) of three successive trials each. Each odor was presented using a cotton-tipped applicator suspended from the ceiling of a standard home cage. Animal odors were obtained by rubbing the applicator in soiled bedding from animals unfamiliar to the test mice. Each trial entailed a two-minute presentation of the applicator, followed by a one-minute inter-trial interval. Investigation of the applicator was scored by a genotype-naïve experimenter during testing using Button Box 5.0 (Behavioral Research Solutions, LLC). Time spent climbing on the applicator was excluded from final scoring. Habituation was defined as the reduction of investigation time over trials with the same odor; dishabitubation was defined as the increase in investigation when a novel odor was presented.

Pup Separation-Induced Ultrasonic Vocalizations

The mouse pup separation-induced ultrasonic vocalizations (USVs) task was conducted as previously described(Hofer, Shair, & Brunelli, 2001). Prior to testing, the litter was separated from the dam for 15mins in order to obtain steady-state conditions. During this time, a heating pad was used to maintain pups' basal temperature. Then, individual mice were removed from the litter and placed in a sound-attenuated chamber outfitted with a high-frequency microphone and data acquisition hardware (UltraSoundGate 416H, Avisoft Bioacoustics, Inc.). USVs were recorded for 5mins (Recorder USGH; Avisoft Bioacoustics, Inc.). Each mouse pup was tested on postnatal days (PNDs) 6, 8, 10, and 12. Recording chambers were cleaned with 70% ethanol between pups. For analysis, recordings were processed using a custom-built R script. In brief, spectrograms were generated from raw WAV files and band pass filtered (15-110kHz). A time-varying parameter, spectral purity, was computed as the fraction of total power within a single frequency bin, and filtered over 8ms. Identification of a vocalization bout was based on three parameters: minimal length (>5ms), spectral purity (>0.15), and holding time (<10ms), defined as the threshold time in which separate bouts were merged. Classification of each USV syllable was characterized by frequency and time parameters, and was defined as follows: NS, no frequency modulation, short duration (<25msec); NL, no frequency modulation, long duration (≥25msec); JU, frequency jump upwards (>7kHz); MJ, multiple frequency jumps.

Three-Chamber Social Approach

The three-chamber approach task was conducted as described previously(Crawley, 2004). Eight- to ten-week old male mice were habituated to handling for three consecutive days (5mins/day). On the subsequent day, mice underwent three 10min testing phases within an arena (20x25x45cms) partitioned into three chambers containing fresh corncob bedding and inverted wire mesh cups in opposite chambers. In the first (habituation) phase, individual test mice were placed into the arena and allowed to explore freely. In the second (social approach) phase, an age- and sex-matched conspecific (unfamiliar mouse) was enclosed under a cup within one chamber. In the third (social novelty) phase, another age- and sex-matched conspecific (novel mouse) was added to an identical cup within the opposite chamber. The test mouse was restricted to the center chamber between phases. For each phase, the time spent within each chamber for each test mouse was scored by a genotype-naïve experimenter using Button Box 5.0 (Behavioral Research Solutions, LLC). For the second (approach) and third (novelty) phases, the time the test mouse spent investigating each cup was also scored. Investigation was defined as when the mouse's head was orientated approximately <1cm to the cup. A ratio of time spent in either chamber or investigating either cup was calculated.

Social Fear Behaviors

See Chapter 2.

Results

Neophobic behavior of Chd5$^{-/-}$ mice

Individuals with ASDs frequently present with stereotyped motoric behavior, insistence for sameness, and comorbid anxiety(Association, 2013; Gillott, Furniss, & Walter, 2001). Therefore, we first sought to distinguish Chd5$^{-/-}$ from wild-type control mice in standard behavioral assays for locomotor activity and anxiety-like behavior. Chd5$^{-/-}$ mice were phenotypically normal in locomotor tests, including open field velocity (**Fig 10a, left**) and distance (**Fig 10a, middle**), and showed normal activity levels prior to startle reactivity testing ($t(37) = 1.15, p = 0.25$). Chd5$^{-/-}$ mice also showed typical levels of prepulse inhibition, a measure of sensorimotor gating ($F_{1,4} = 2.37, p = 0.13$). However, compared to wild-type littermates, Chd5$^{-/-}$ mice spent less time within the center area of an open arena (**Fig 10a, right**). This potential indicator of anxiety-like behavior was not replicated in measures of the elevated plus-maze (*open:closed arms time:* $t(25) = 0.19, p = 0.85$) or startle reactivity (*open arm entries:* $t(25) = 0.36, p = 0.72$). Since mice were not habituated to the open field arena prior to testing, the reduced center-time of Chd5$^{-/-}$ mice may instead represent a neophobic behavioral response to the testing environment. To further examine neophobic behavior in Chd5$^{-/-}$ mice, we tested the mice in a novel object recognition task(Bevins & Besheer, 2006; Lyon, Saksida, & Bussey, 2012). Both Chd5$^{-/-}$ and wild-type mice demonstrated equivalent time investigating two, identical objects (**Fig 10b, left**). When the same object was presented together with a novel object one hour later, wild-type mice displayed a significant species-typical preference for the novel object (**Fig 10b, right**). In contrast, Chd5$^{-/-}$ mice exhibited a preference for the familiar object that was significantly different from both wild-type mice and chance investigation. To further investigate this neophobia-like behavior, we conducted an olfactory habituation-dishabituation task, in which a series of unique social and non-social odors were presented to individual mice. $Chd5^{-/-}$ mice did not show gross deficits in recognition of olfactory cues, as indicated by investigatory habituation to repeatedly presented odors or dishabituation to disparate odors (**Fig 10c**). However, $Chd5^{-/-}$ mice exhibited less overall investigation of olfactory stimuli, evidence further supporting the neophobia-like behavior of these mice. Together with the findings from the open field and NOR, these data suggest that $Chd5^{-/-}$ mice exhibit a proclivity for familiarity that spans environmental contexts, and object-based and sensory stimuli.

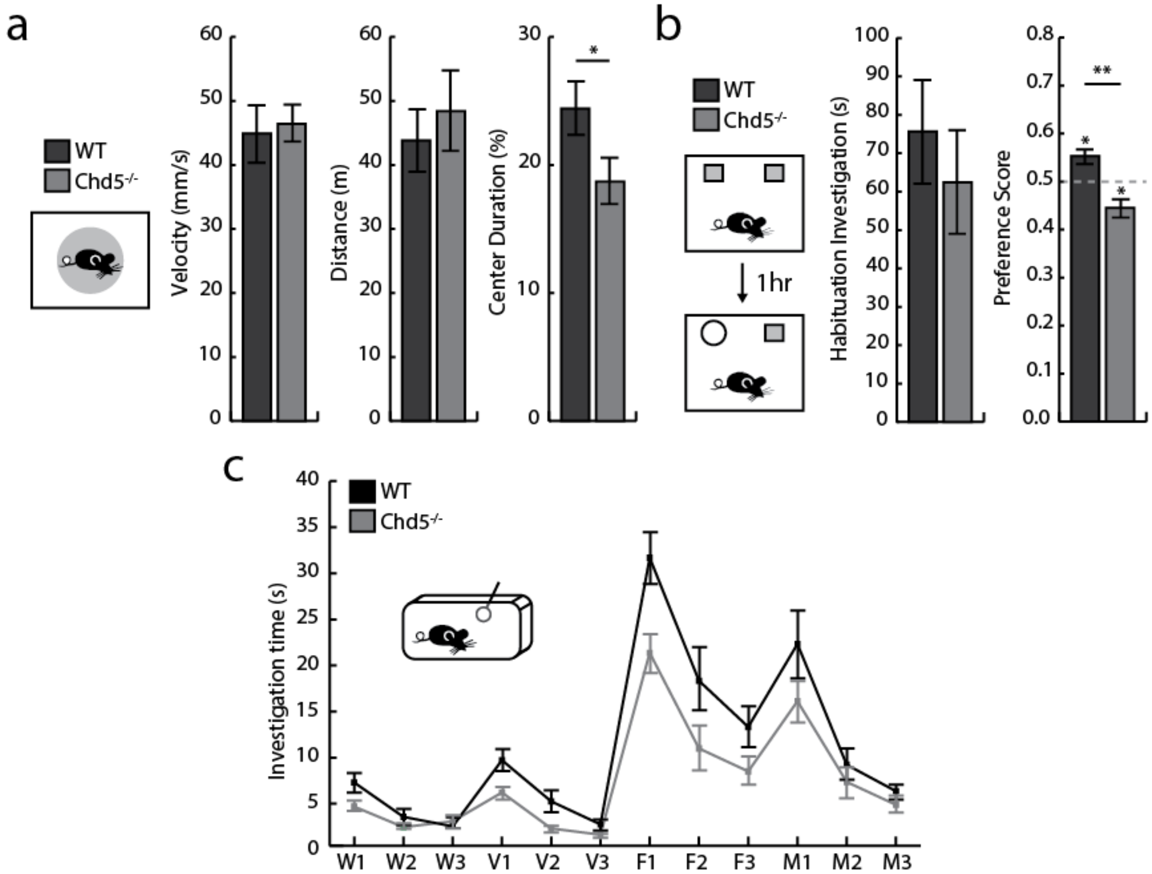

Figure 10. Neophobic behavior of Chd5$^{-/-}$ mice. *(**a**) (**right**) Male Chd5$^{-/-}$ mice (gray, n = 15) spent significantly less percentage time in the center of an open field compared to wild-type mice (black, n = 15) (two-tailed student's t test, t(29) = 2.10, p = 0.046) without differences in (**left**) velocity (student's t test, t(29) = 0.97, p = 0.34) or (**middle**) distance traveled (student's t test, t(29) = 0.69, p = 0.49). (**b**) Male Chd5$^{-/-}$ mice (gray, n = 15) exhibited (**right**) a preference for a familiar object in the novel object recognition task, whereas wild-type mice (black, n = 15) showed a preference for the novel object (two-tailed student's t test, t(29) = 4.45, p = 0.0001) compared with a chance preference score (Dunnett's post-hoc, p = 0.015). There were no differences between the groups (**left**) in total investigation time during training (two-tailed student's t test, t(29) = 0.69, p = 0.49). (**c**) Male Chd5$^{-/-}$ mice (gray, n = 16) exhibited normal habituation across trials (ANOVA (Chd5$^{-/-}$ only): effect of trial $F_{2,48}$ = 32.34, p < 0.0001) and dishabituation to various odors (ANOVA (Chd5$^{-/-}$ only): effect of odor $F_{3,48}$ = 40.28, p < 0.0001), yet less overall investigation compared to wild-type mice (black, n = 19; ANOVA: effect of genotype, $F_{8,419}$ = 15.48, p < 0.0001; genotype x odor interaction $F_{8,419}$ = 2.47, p < 0.043. Error bars represent s.e.m. * p < 0.05; ** p < 0.01; *** p < 0.001. W, water; V, vanilla; F, female; M, male.*

Abnormal socio-communicative behavior of Chd5$^{-/-}$ mice

One of the most profound impairments in ASDs relates to socio-communicative interaction(Association, 2013). We examined this behavioral domain in Chd5$^{-/-}$ mice using standard tasks, particularly pup separation-induced ultrasonic vocalizations (USVs) and adult three-chamber social approach(Silverman, Yang, Lord, & Crawley, 2011). In the pup separation-induced USVs task, male Chd5$^{-/-}$ pups produced significantly fewer USVs across early postnatal days (PNDs) when compared to littermate wild-type mice (**Fig 11a**). This effect was not exhibited in female mice (*repeated measure ANOVA effect of genotype, $F_{1,51} = 2.68$, $p = 0.11$).* Mice exhibit a complex repertoire of USVs(Grimsley, Monaghan, & Wenstrup, 2011); therefore, we further analyzed the types of USV syllables by time/frequency characteristics. Compared to wild-types, male Chd5$^{-/-}$ mice elicited a significantly smaller proportion of complex, frequency-modulated USVs (**Fig 11b**). While our understanding of the communicative purposes of pup USVs is rudimentary, these data suggest abnormal communicative functioning specific to male Chd5$^{-/-}$ mice. In the adult three-chamber social approach task, male wild-type and Chd5$^{-/-}$ mice displayed equivalent exploration time within adjoining chambers during a habituation phase (**Fig 11c**). When next exposed to an enclosed, unfamiliar conspecific mouse in one chamber, Chd5$^{-/-}$ mice exhibited no preference for the conspecific-containing chamber and spent significantly less time than wild-type mice directly investigating the mouse (**Fig 11d**). Following addition of a second, novel conspecific in the opposite chamber, Chd5$^{-/-}$ mice exhibited no preference for the novel conspecific-containing chamber and significantly less time than wild-type mice directly investigating the enclosed novel conspecific (**Fig 11e**). This lack of preference for social novelty coheres with the neophobia exhibited in the open arena, novel object recognition, and olfactory habituation-dishabituation tasks (see Fig 10).

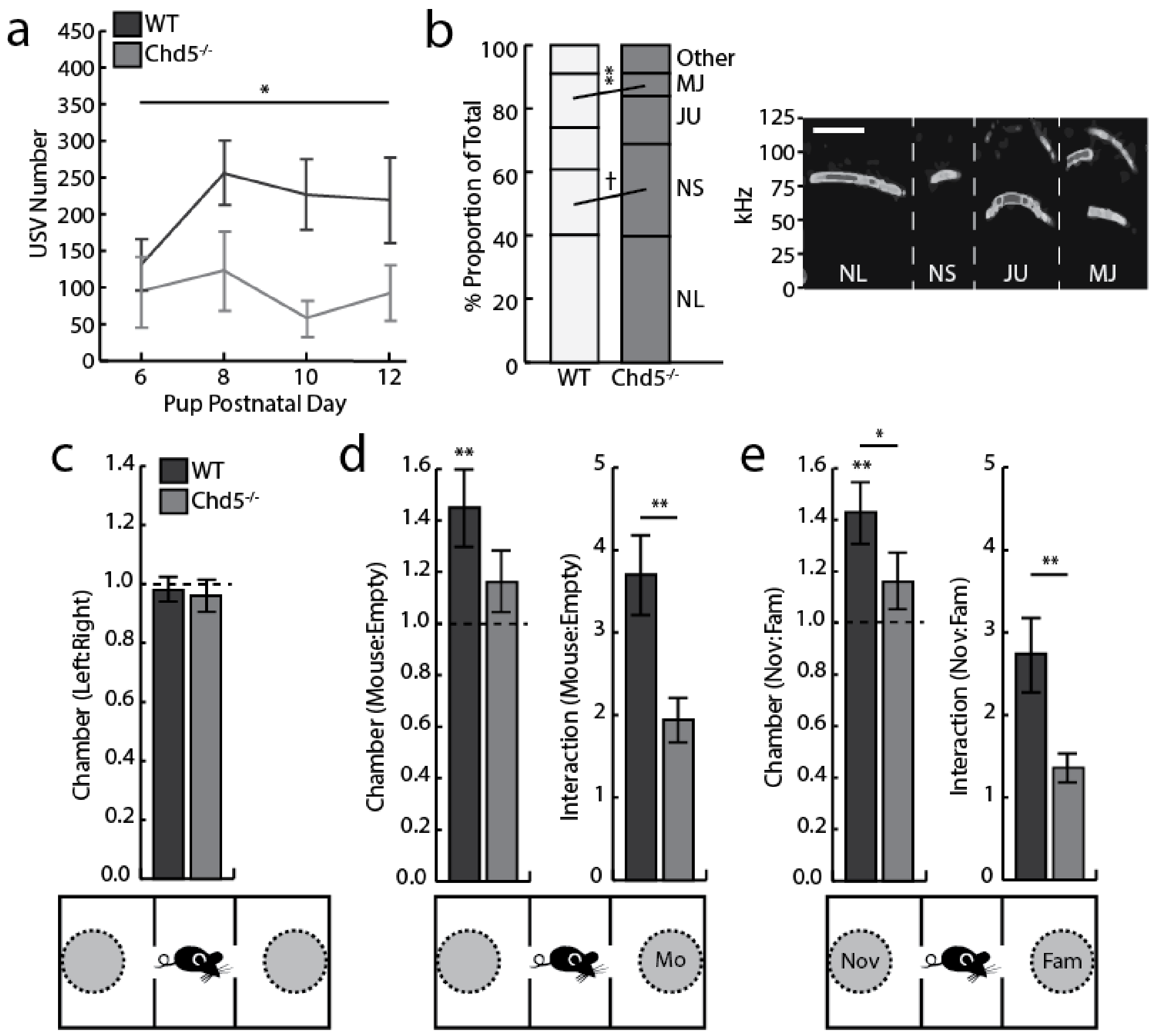

Figure 11. Socio-communicative abnormalities of Chd5$^{-/-}$ mice. *(**a**) Chd5$^{-/-}$ pups (n = 7) produce fewer separation-induced USVs across early postnatal life (PNDs 6-12) compared to wild-type pups (n = 14) (repeated measure ANOVA effect of genotype, $F_{1,86}$ = 5.77, p = 0.02). (**b**) (**left**) On PND6, Chd5$^{-/-}$ pups elicited a significantly smaller proportion of complex (MJ) USVs compared to wild-type pups (two-tailed student's t test, t(20) = 2.67, p = 0.01). (**right**) Spectrogram examples of USV types (scale bar, 25msec). (**c**) In the three-chamber social approach task, no difference was noted in ratio of time spent between adjoining chambers for adult Chd5$^{-/-}$ (n = 20) (Dunnett's post-hoc, p = 0.70) or wild-type mice (n = 20) (Dunnett's post-hoc, p = 0.94) during an initial habituation phase (two-tailed student's t test, t(39) = 0.35, p = 0.73). (**d**) (**left**) When*

*presented with an unfamiliar, enclosed conspecific, wild-type mice exhibited a significantly elevated ratio time in the conspecific-containing chamber (two-tailed student's t test, $t(39) = 3.04$, Dunnett's post-hoc, $p = 0.01$), whereas Chd5$^{-/-}$ mice showed no preference (two-tailed student's t test, $t(39) = 1.37$, Dunnett's post-hoc, $p = 0.47$). (**right**) Wild-type mice displayed a significantly higher ratio of time engaged in direct investigation of the conspecific-containing cage compared to Chd5$^{-/-}$ mice (two-tailed student's t test, $t(39) = 3.1$, $p = 0.002$); (**e**) (**left**) Chd5$^{-/-}$ mice exhibited approach to a novel conspecific that was impaired compared to wild-type mice (one-tailed student's t test, $t(39) = 1.97$, $p = 0.03$) and no difference from chance (Dunnett's post-hoc, $p = 0.38$). (**right**) Wild-type mice displayed a significantly higher ratio time in direct investigation of the novel conspecific-containing cage compared to Chd5$^{-/-}$ mice (two-tailed student's t test, $t(39) = 2.72$, $p = 0.01$). NS, no frequency modulation, short duration (<25msec); NL, no frequency modulation, long duration (≥25msec); JU, frequency jump upwards (>7kHz); MJ, multiple frequency jumps. Error bars represent s.e.m. * $p < 0.05$; ** $p < 0.01$; † $p = 0.06$.*

Impaired socially transmitted fear behaviors in Chd5$^{-/-}$ mice

Impairments in social cognition, including facial processing, emotional recognition and empathy, are prominent endophenotypes of ASDs (Adolphs, Spezio, Parlier, & Piven, 2008; Lombardo et al., 2007; Sucksmith et al., 2013). We investigated empathic behaviors of Chd5$^{-/-}$ mice using the socially transmitted fear paradigm (**Fig 12a**; see Chapter 2, Fig 2), in which foot shock applied to a demonstrator mouse elicits freezing in an observer mouse. Although basal activity levels were similar to familiar/wild-type mice, familiar/Chd5$^{-/-}$ observer mice exhibited significantly less freezing during conditioning of demonstrator mice (**Fig 12b**). Importantly, this difference was not attributable to differences in demonstrator vocalizations ($F_{2,47} = 0.680$, $p = 0.512$) or freezing behavior on control conditioning days (*noDem:* $F_{2,45} = 1.85$, $p = 0.17$; *noShock:* $F_{2,45} = 0.14$, $p = 0.87$), nor to the capacity to acquire ($t(31) = 1.05$, $p = 0.30$) or express ($t(45) = 0.002$, $p = 0.96$) Pavlovian fear conditioning behavior. This effect was also sex-specific. Female observers did not exhibit differences in freezing by genotype (*familiar/wild-type: 4.97+/- 0.52; familiar/Chd5$^{-/-}$: 4.58 +/- 0.57;* $t(21) = 0.49$, $p = 0.63$). Average locomotor activity measurements obtained immediately after demonstrator foot shock (measurement (M)1) indicated similar orientation responses for both genotypes and unfamiliar observer male mice (**Fig 12c**). However, only familiar/wild-type mice exhibited sustained freezing behavior throughout each trial (i.e., M2-12). This finding implies that sensory processing of cues emanating from demonstrator mice was intact in all three groups. In contrast to familiar/wild-type mice (see Fig 2f), familiar/Chd5$^{-/-}$ observer mice also did not exhibit graded increases in locomotor activity when examined across three conditioning days (**Fig 12d**), implying impairments in escape-specific social fear behavior.

Having previously shown that oxytocin enhances empathic behavior in unfamiliar male mice (see Chapter 3), we lastly investigated whether intranasal oxytocin could rescue the impaired empathic behavior in Chd5$^{-/-}$ mice. However, following both acute and chronic intranasal oxytocin administration in Chd5$^{-/-}$ observers, we failed to detect enhancements in freezing (**Fig 12e).**

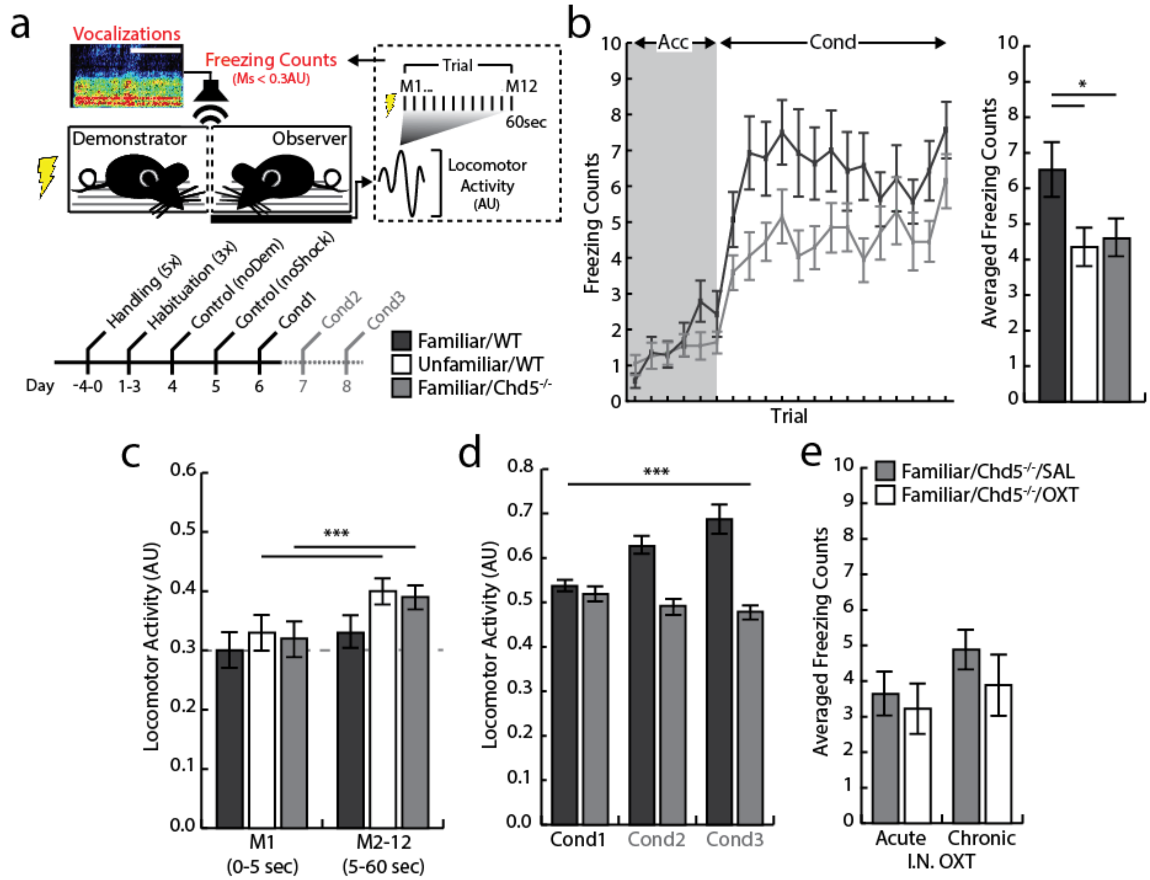

Figure 12. Socially transmitted fear impairments of Chd5$^{-/-}$ mice. (a) *Schematic of the socially transmitted fear paradigm (AU, arbitrary unit; M, measurement), and (**bottom**) schedule (Acc, acclimation; Cond, demonstrator conditioning; WT, wild-type). (**b**) (**left**) Familiar/wild-type observer mice (black, n = 14) exhibited significantly greater freezing behavior during demonstrator conditioning compared to familiar/Chd5$^{-/-}$ (gray, n = 20) and unfamiliar/wild-type (white, n = 14) observers as assessed by (**right**) averaged freezing counts during conditioning trials (T7-20) (unfamiliar/wild-type: two-tailed student's t test, t(27) = 2.30, p = 0.03; familiar/Chd5$^{-/-}$: two-tailed student's t test, t(33) = 2.05, p = 0.05). Gray box indicates acclimation period prior to demonstrator conditioning. (**c**) Observer mice activity measurements (M) recorded either immediately (M1; 0-5s) or subsequently (M2-12; 5-60s) after demonstrator conditioning indicated equivalent orientation-specific immobility behavior (M1), but sustained freezing (M2-12) only in familiar/wild-type mice (unfamiliar/wild-type (M1 vs M2-12): paired t test, t(13) = 4.57, p < 0.001; familiar/Chd5$^{-/-}$: paired t test, t(19) = 5.60, p < 0.001; familiar/wild-type (M1 vs M2-12): paired t test, t(13) = 2.00, p = 0.067); (**d**) Averaged locomotor activity of familiar/Chd5$^{-/-}$ observer mice during acclimation on subsequent conditioning days was not significantly different (ANOVA: $F_{2,56}$ = 1.41, p = 0.253), but was significantly different when compared to familiar/wild-type observer mice (repeated measure ANOVA: $F_{5,95}$ = 15.48, p < 0.0001; effect of genotype $F_{1,95}$ = 0.354, p = 0.553; effect of day $F_{2,95}$ = 3.56, p = 0.032; genotype x day interaction $F_{5,95}$ = 11.09, p < 0.0001). Familiar/wild-type data from Fig 2. (**e**) Familiar/Chd5$^{-/-}$ observer mice administered acute (white, n = 6; two-tailed student's t test, t(11) = 0.44, p = 0.67) or chronic (white, n = 6; two-tailed student's t test, t(11) = 0.95, p = 0.37)*

*intranasal oxytocin exhibit similar levels of freezing compared to saline-treated familiar/Chd5$^{-/-}$ controls (gray, n = 6/6, respectively). Error bars represent s.e.m. * p < 0.05; ** p < 0.01; *** p < 0.001*

Familiar/Chd5$^{-/-}$ mice failed to show increases in activity across repeated sessions of demonstrator conditioning. Therefore, we next examined escape behavior using the sCPA paradigm (**Fig 13a**; see Chapter 2, Fig 3). Although familiar/Chd5$^{-/-}$ mice showed significant escape-like behavior during acclimation on the second conditioning day, this behavior was not maintained during conditioning compared to non-conditioned controls (**Fig 13b**). Familiar/wild-type mice exhibited more typical escape behavior during demonstrator conditioning, and unfamiliar/wild-type mice did not show escape during either acclimation or conditioning. Side preferences were equivalent for all groups at pre-test, yet only familiar/wild-type mice exhibited a significant preference for the non-demonstrator side at the post-test (**Fig 13c**). Lastly, a normalized score of escape behavior, sCPA, indicated that only familiar/wild-type mice exhibited a significant conditioned avoidance of the demonstrator-containing side (**Fig 13d**).

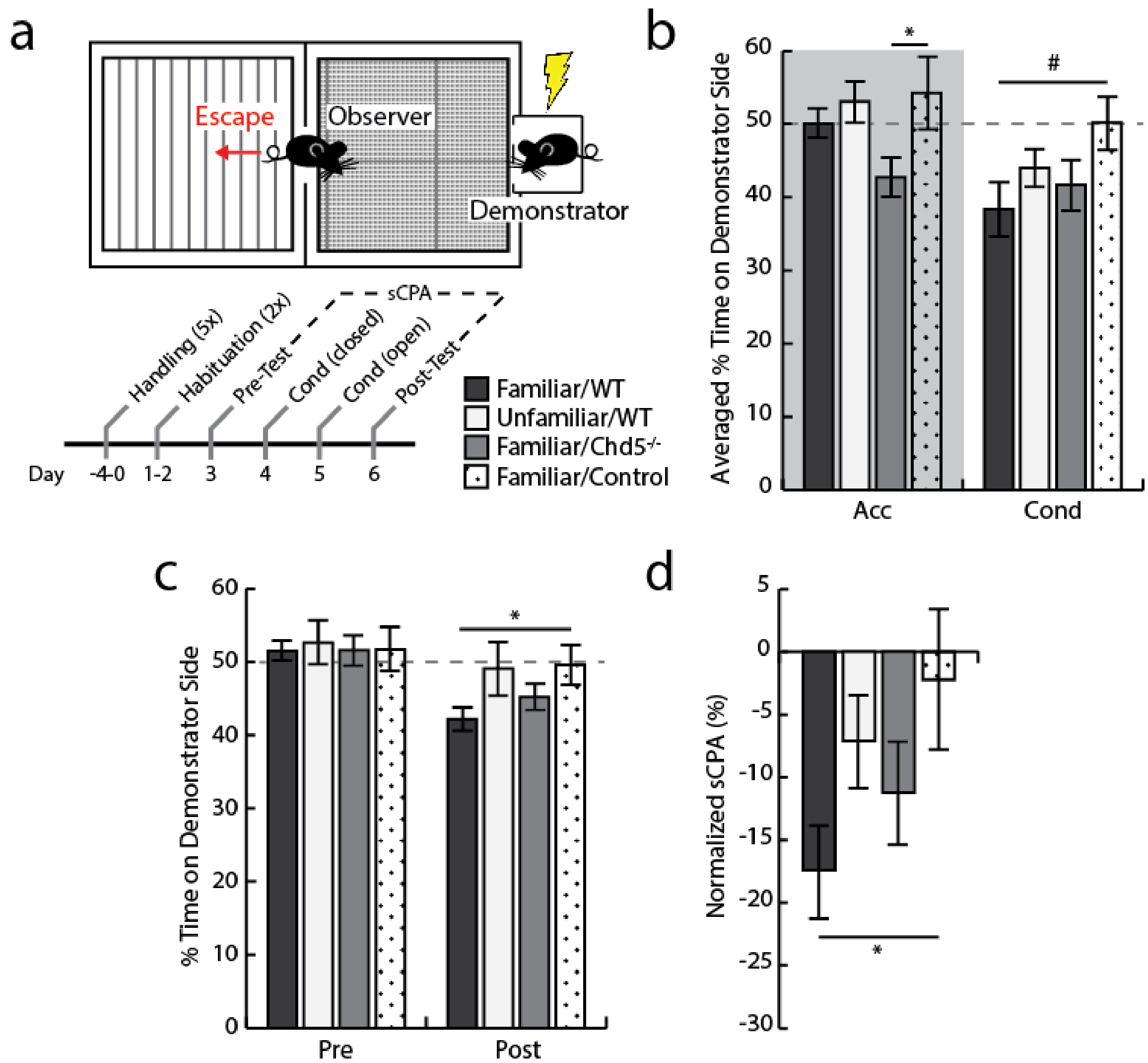

Figure 13. Social conditioned place aversion impairments of Chd5$^{-/-}$ mice. *(**a**) (**top**) Schematic of the sCPA paradigm, and (**bottom**) schedule (Acc, acclimation; Cond, demonstrator conditioning; WT, wild-type; Control, non-conditioned familiar wild-type). (**b**) Compared to non-conditioned controls (control, dots, n = 10), familiar/Chd5$^{-/-}$ mice (gray, n = 13) show initial escape behavior during acclimation (two-tailed student's t test: t(22) = 2.03, Dunnett's post-hoc, p = 0.035) that is not maintained through conditioning (two-tailed student's t test: t(22) = 1.69, Dunnett's post-hoc, p = 0.210); whereas familiar/wild-type (black, n = 13) show near-significant escape behavior during conditioning (two-tailed student's t test: t(22) = 2.26, Dunnett's post-hoc, p = 0.055) (**c**) Compared to non-conditioned controls, familiar/wild-type mice exhibited escape behavior at post-test (two-tailed student's t test: t(22) = 2.32, Dunnett's post-hoc, p = 0.093), whereas familiar/Chd5$^{-/-}$ mice (two-tailed student's t test: t(22) = 1.33, Dunnett's post-hoc, p = 0.439) did not. (**d**) Familiar/wild-type observer mice exhibited a significant difference in normalized sCPA scores compared to non-conditioned controls (two-tailed student's t test: t(22) = 2.26, Dunnett's post-hoc, p = 0.043), whereas familiar/Chd5$^{-/-}$ observer mice did not (two-tailed student's t test: t(22) = 1.30, Dunnett's post-hoc, p = 0.317). Error bars represent s.e.m. * p < 0.05; # p = 0.055.*

Discussion

Dysregulated epigenetic mechanisms have emerged as putative factors underlying psychiatric diseases.(Tsankova et al., 2007) Chromatin remodeling is critical for regulating gene expression necessary for neurodevelopment, and mutations in chromatin remodeling genes have been linked to various psychiatric diseases, including ASDs. The CHD genes encode a family of chromatin remodelers, several of which have been strongly implicated in sporadic cases of ASDs.(De Rubeis et al., 2014) Our study is the first to characterize transcriptional variations, neuron-level morphological alterations, and behavioral abnormalities resulting from deletion of the neuron-specific Chd5 chromatin remodeler in mice. RNA sequencing of frontal cortex tissue identified dysregulation of genes involved with gene expression and cellular development and signaling, as well as specific genes directly implicated in ASDs. Consistent with these findings, cultured $Chd5^{-/-}$ pyramidal neurons showed reduced dendritic arborizations, and the $Chd5^{-/-}$ mouse displayed behaviors characteristic of ASD endophenotypes, including abnormal socio-communicative functioning and reduced preference for novelty. This mouse model also failed to show species-typical responses to the distress of another mouse (i.e., freezing and escape), mirroring deficits in empathy that are highly characteristic of ASDs.(Lombardo et al., 2007) Given the degree of functional specificity of many of the genes affected, the observed transcriptional alterations provide possible mechanisms to explain the morphological alterations of cortical neurons and specific behavioral sequelae observed by deletion of Chd5. Characterizing the pathways between these transcriptional changes, neurodevelopment, and the aberrant behaviors identified in this study promises to offer novel targets for understanding the etiology of ASDs.

Our data highlight a consistent preference of these mice for familiarity over novelty. $Chd5^{-/-}$ mice exhibit neophobia to environmental contexts (open field), objects (novel object recognition), social stimuli (three chamber approach), and sensory stimuli (olfactory habituation-dishabituation) stimuli. Collectively, this reduction in novelty-seeking behavior parallels the restricted/repetitive symptomology characteristic of ASDs, including limited interests, inflexibility, and insistence on sameness. Indeed, several reports have highlighted abnormal behavioral, physiological, and electrophysiological responses to novel stimuli in ASD individuals(Gomot, Belmonte, Bullmore, Bernard, & Baron-Cohen, 2008; Sokhadze et al., 2009). The fact that reduced responses to novelty, and even a preference for sameness, was seen across multiple classes of stimuli strongly indicates a broad deficit in this endophenotype in the $Chd5^{-/-}$ mouse.

Empathy, a relatively high-level social cognition process in humans and core endophenotype of ASDs, has not typically been indexed in mice with mutations of genes implicated in these disorders. Our approach was to apply validated paradigms (see Chapter 1; Figs 2 & 3) that lend quantitative measures of social fear, a phenomenon only recently identified in rats(Kim et al., 2010) and mice(Q. Chen et al., 2009; Jeon et al., 2010). We found that whereas male $Chd5^{-/-}$ mice failed to respond to the distress of a familiar conspecific, this impairment was not evident in female $Chd5^{-/-}$ mice. This finding recapitulates the sex disparity in overall prevalence of ASDs, and suggests a sexually dichotomous mechanism underlying this endophenotypic deficit in $Chd5^{-/-}$ mice. Using the socially transmitted fear paradigm, we have also discriminated between immediate orientation responses (e.g., to demonstrator vocalizations) and sustained freezing behavior. That is, while familiar/wild-type and familiar/Chd5$^{-/-}$ observer mice exhibit similar levels of immobility immediately upon application of shock to the demonstrator, only familiar/wild-type observer mice exhibit sustained freezing throughout conditioning trials. Furthermore, deficits of Chd5$^{-/-}$ mice in escape behavior using the sCPA paradigm indicate that this mouse is not impaired in a single form of empathic behavior but rather in expressing the appropriate repertoire of emotional responses to the emotional cues of others.

Aiming to restore normal levels of socially transmitted fear in the Chd5$^{-/-}$ mice, we examined the effects of intranasal administration of oxytocin. This approach is an emerging treatment option for individuals with ASDs, and has already demonstrated promising clinical improvements.(Andari et al., 2010; Auyeung et al., 2015; Guastella et al., 2010; Hollander et al., 2007; Preti et al., 2014) While our group (Chapter 3; Fig 4b) and others(K L Bales et al., 2014; Freeman et al., 2016; Neumann et al., 2013) have validated the entry of intranasal oxytocin into the brain and corresponding improvements in empathic behavior of prototypical mice, comparable oxytocin regimens (i.e., acute and chronic) were unable to rescue deficits in Chd5$^{-/-}$ mice. This null effect may not be altogether surprising. ASDs involve complex and heterogeneous etiologies, all of which may not be ameliorated by a single therapeutic approach. In particular, deletion of Chd5 influences developmental pathways, which may be difficult to mitigate in adulthood. Moreover, our RNA sequencing experiments did not identify pathways implicating oxytocin in the Chd5$^{-/-}$ mouse. Based on our previous data (Chapter 3), an alternative explanation for this failed treatment may be that oxytocin does not bolster socially transmitted fear between familiar mice; rather remediating effects are specific to unfamiliar conspecifics. Collectively, these findings contribute to the notion that social cognition entails an evolutionarily conserved set of processes impaired within psychiatric disease.(J. Panksepp & Panksepp, 2013) Future experiments using the Chd5$^{-/-}$ mouse model will aim to elucidate the specific neurobiological correlates of oxytocin-independent impairments in empathy.

Chapter 6: Discussion & Conclusion

This dissertation research examined social cognition in both normal emotional states and using a mouse model of disease. The primary form of social cognition investigated was empathy, the capacity to infer the emotional state of another. Empathy was operationalized as emotional "state-matching" and measured as the social transmission of fear between two conspecifics. In Chapter 2, empathic behavior was shown to be modulated by sex, degree of social familiarity, and pre-weaning litter size of the observer mouse, and vocal communication elicited by the demonstrator conspecific. We further demonstrated a transition from freezing to escape behavior that was specific to familiar observer mice. Following validation of this behavioral approach and various factors contributing to its expression, Chapter 3 then detailed experiments demonstrating that the evolutionarily conserved neuropeptide, oxytocin, is both necessary and sufficient for normal empathic behavior. To investigate empathy within a disease state, Chapter 4 validated the generation of a mouse model of ASDs by genetically deleting the Chd5 chromatin remodeler, an epigenetic source of neurodevelopmental processes. Transcriptomic analyses in $Chd5^{-/-}$ cortical tissue demonstrated altered expression of neurodevelopmental and disease-relevant genes, and transcriptional effects were subsequently reflected in altered dendritic morphology of pyramidal cells of the cortex. Chapter 5 characterized several hallmark behavioral features of ASDs, including reduced novelty preferences among several domains and abnormal socio-communicative functioning. Lastly, impairments in social fear behaviors, namely freezing and escape, of

$Chd5^{-/-}$ mice were detailed. These findings of impaired empathic behavior in a mouse model of psychiatric disease are a first of their kind, and provide a platform for investigating the neural substrates of social cognition endophenotypes in mouse models of psychiatric disease.

While our investigation into the neural substrates of normal empathic behavior were restricted to oxytocin, several other neural systems also contribute to this socio-emotional facility. Ample evidence exists for a modulatory role of stress on empathy, particularly as operationalized in social fear paradigms. One study demonstrated that blocking glucocorticoid synthesis or receptor binding in unfamiliar humans and mice enhanced emotional "contagion".(Martin et al., 2015) A more recent study (Burkett et al., 2016), reported enhanced corticosterone levels in prairie voles observing a distressed conspecific. In alignment with the hypothesis concerning oxytocin's direct engagement of core emotional processes (e.g., fear and stress), other studies have shown that sub-chronic administration of oxytocin in rats was sufficient to decrease glucocorticoid receptor mRNA in the hippocampus.(Petersson & Uvnäs-Moberg, 2003) These findings suggest an interaction between the oxytocin and glucocorticoid systems and therefore a connection between empathy and stress systems.

Our findings regarding oxytocin leave open the possible additional involvement of vasopressin, a structurally and evolutionarily-similar neuropeptide to that of oxytocin.(Stoop, 2012) Vasopressin mediates a range of socio-emotional processes,(Meyer-Lindenberg et al., 2011) and dysregulation represents a potential source of ASD pathophysiology and related endophenotypes.(Francis 2016) Both oxytocin and vasopressin are neuropeptides with considerable sex differences, and it is conceivable that vasopressin exerts opposing effects on empathic behavior in male mice.(Viviani & Stoop, 2008) Sex differences in empathic behavior are particularly noteworthy, and further investigation is required. Our preliminary data (not shown) implicates the estrous cycle as a source of the heightened socially transmitted fear observed in females. Interestingly, the estrous cycle, including a key sex hormone, estradiol, modulates the oxytocin system.(De Kloet, Voorhuis, & Elands, 1985) During the peak of estrus, oxytocin mRNA levels within the hypothalamus are significantly elevated, and levels of the neuropeptide are reduced.(Van Tol, Bolwerk, Liu, & Burbach, 1988)

Our experiments using DREADDs to chemogenetically activate oxytocinergic neurons of the hypothalamus hint at the neural circuitry underlying empathy. Consistent with the literature(Burkett et al., 2016), our preliminary data suggests that oxytocin signaling within the ACC partially mediates socially transmitted fear. A more refined picture of the underlying circuitry requires additional investigation and neuromodulatory strategies. Brain region-specific infusion of DREADD ligands could be pursued to activate or inhibit other oxytocinergic projections from the hypothalamus. Based on our model (**Fig 6**), these regions would include the CeL amygdala, or – perhaps – the lateral septum, which has been implicated in social recognition and fear behaviors.(Y. F. Guzmán et al., 2013; Lukas, Toth, Veenema, & Neumann, 2013; Zoicas et al., 2014) Optogenetics may also be leveraged to elucidate this circuitry. Prolonged activation of oxytocinergic neurons (via DREADDs) may be more neurophysiologically appropriate, yet optogenetics have been previously used to evoke oxytocin release(Knobloch et al.,

2012) and several bi-stable step opsins have been developed to chronically modulate neuronal activity.(Yizhar, 2012; Yizhar et al., 2011) At putative downstream target of oxytocin release, the recently developed technique of fiber photometry could be applied to study the temporal dynamics of oxytocin signaling during empathic behaviors.(Gunaydin et al., 2014)

We have developed the sCPA paradigm as a tool for studying empathic behaviors in mice. This paradigm measures a second, ethologically-relevant form of fear: escape. The escape response corroborates our conclusion that observer mice express fear, rather than a form of motor mimicry in response to demonstrator freezing. Furthermore, these diametric motoric responses of demonstrator and observer mice relate to the "self-other distinction" feature of empathy. That is, observer mice recognize distress of a conspecific, yet engage a disparate motoric response to avoid their own exposure to distress. To the best of our knowledge, this finding is some of the first evidence that mice exhibit this feature of empathy. A similar paradigm – social conditioned place preference – has been used to study social reward,(J B Panksepp & Lahvis, 2007) and one study has identified a key modulatory role of oxytocin in this behavior.(Dölen, Darvishzadeh, Huang, & Malenka, 2013) Our paradigm involves the converse – a form of vicarious/social punishment – and could be used to further our understanding of social learning under this motivational valence. More fundamentally, sCPA could be used to studying empathic learning processes that are governed by higher-order brain circuitry and processes.(J. Panksepp, 2011b) We propose that the observed switch from freezing to escape could therefore reflect these respective reflexive and learned components of empathy nested within the sub-cortical and cortical structures of the mammalian brain.

Our study is the first to investigate empathy using social fear paradigms in a mouse model of psychiatric disease. Impairments in empathy are common endophenotypes across psychiatric disease, including ASDs, schizophrenia, and antisocial personality disorder,(S Baron-Cohen et al., 1985; Decety et al., 2013; Derntl et al., 2009) and therefore represent a vital investigatory measure in other rodent models of disease. Importantly, our preliminary data (not shown) in the serine racemase mouse model of schizophrenia demonstrate an intact repertoire of empathic behaviors. Therefore, impairments in this endophenotypic measure are likely disease- or model-specific, rather than a result of gross deficits in sensory or emotional processing. Our approach departs from classic, symptom-based characterizations and moves towards a more endophenotypic approach. Thus, these behavioral tools allow for a characterization that is closer to the genetic source of psychiatric disease.

There remain several open avenues of investigation with the Chd5$^{-/-}$ mouse model of ASDs. Future transcriptional analyses could be refined by the use of chromatin immunoprecipitation to identify Chd5-bound genomic sequences. Moreover, although Chd5 is expressed solely in neurons, cell sorting prior to sequencing could provide more detailed understanding of Chd5 epigenetic regulation across cell types and within specific brain regions. While our morphological analyses revealed dendritic abnormalities in cortical Chd5$^{-/-}$ pyramidal neurons, future experiments may also look at spine formation/pruning *ex vivo.* Variations in dendritic spines represent a long-standing neural hallmark of ASDs, and similar evidence in mice lacking Chd5 could further our understanding of epigenetic regulation of neural plasticity. Another neural hallmark of ASDs, excitatory-inhibitory imbalance, could be investigated in the Chd5 model using electrophysiological techniques.(de la Torre-Ubieta et al., 2016) For instance, *in vivo* field recordings during social interactions or transmitted fear could reveal neural correlates underlying these impairments in Chd5$^{-/-}$ mice. Lastly, at the macroscopic level, magnetic resonance imaging techniques could be applied to understanding structural or functional variations within the Chd5$^{-/-}$ mouse brain.

Given the centrality of human interpersonal relations to well-being – the rewarding nature of social interaction and the impairments evident in psychiatric disease – characterizing the neural bases of social cognition – particularly empathy – represents an important step for understanding the development and manifestation of both adaptive and maladaptive mental states. "Fear contagion" in humans correlates positively with several other measures of empathy, including those with no obvious murine homologs.(Corden, Critchley, Skuse, & Dolan, 2006) In this study we have demonstrated the importance of the brain oxytocin system in the social transmission of fear among mice. Further, we have fully characterized endophenotypic features of a novel mouse model of ASDs, including impaired empathic behavior. The effects of deletion in mice of the neuron-specific Chd5 chromatin remodeler suggest that development of empathy is under epigenetic control. Thus, socially transmitted fear would appear to be well suited as a behavior for investigating genetic, epigenetic, developmental, and neural substrates of empathy in mouse models. With this knowledge, the stage is set for dissecting the circuitry and neurophysiology of empathy in both typical and pathological states. This avenue of research will lead to improved understanding of empathy systems of the brain, and promises to advance treatments for the prevention and amelioration of cases of psychiatric and developmental diseases, such as ASDs, in which reduced empathy processes are prominent endophenotypic features.

Table 1. Litter sizes for experimental groups.

	Fam/Male	***Unfam/Male***	***Fam/Female***	***Unfam/Female***	***Unfam/Acute OXT***	***Unfam/Acute SAL***	***Unfam/Chronic OXT***	***Unfam/Chronic SAL***	***Unfam/CNO***	***Unfam/SAL***	***Fam/SAL***	***Fam/5mg/kg OXTA***	***Fam/10mg/kg OXTA***
Average	**8.00**	***8.15***	***8.30***	***7.60***	*	*	***7.63***	***5.66***	***7.80***	***7.70***	***7.50***	***7.66***	***7.90***
S.E.	*0.73*	*0.37*	*0.33*	*0.58*	*	*	*0.43*	*0.92*	*0.53*	*0.58*	*0.43*	*0.16*	*0.58*
Sig?	*n.s.*		*n.s.*		*n.s.*		*n.s.*		*n.s.*		*n.s.*		

** Data not available.*

Table 2. Pathway analysis for downregulated transcripts in Chd5$^{-/-}$ mice

	P value	***IPA Score***
Associated Network Function		
Neurological Disease, Cancer, Gastrointestinal Disease		***73***
Neurological Disease, Gene Expression, Nervous System Development and Function		***26***
Cell-To-Cell Signaling and Interaction, Behavior, Metabolic Disease		***18***
Embryonic Development, Organismal Development, Tissue Development		***16***
Connective Tissue Disorders, Developmental Disorder, Hereditary Disorder		***2***
Molecular and Cellular Functions		
Cellular Growth and Proliferation	2.58E-10 – 9.20E-03	***38***
Cell Death and Survival	2.77E-08 – 9.31E-03	***32***
Cellular Movement	7.33E-07 – 8.59E-03	***24***
Gene Expression	1.03E-06 – 8.30E-03	***25***
DNA Replication, Recombination, and Repair	9.26E-06 – 9.26E-06	***9***
Physiological System Development and Function		
Organismal Survival	2.15E-09 – 2.15E-09	***30***

Connective Tissue Development and Function	2.62E-09 – 9.03E-03	***25***
Tissue Morphology	2.62E-09 – 9.31E-03	***30***
Organ Development	1.05E-05 – 9.31E-03	***10***
Skeletal and Muscular System Development and Function	1.05E-05 – 6.21E-03	***15***

Table 3. Pathway analysis for upregulated transcripts in Chd5$^{-/-}$ mice

	P value	***IPA Score***
Associated Network Function		
Gene Expression, Embryonic Development, Organismal Development		***32***
Endocrine System Development and Function, Molecular Transport, Small Molecule Biochemistry		***31***
Behavior, Cell Signaling, Molecular Transport		***26***
Cell Death and Survival, Cellular Assembly and Organization, Cellular Function and Maintenance		***25***
Psychological Disorders, Nutritional Disease, Connective Tissue Disorders		***23***
Molecular and Cellular Functions		
Cell-To-Cell Signaling and Interaction	1.64E-11 – 3.63E-03	***38***
Molecular Transport	1.64E-11 – 3.63E-03	***38***
Small Molecule Biochemistry	1.64E-11 – 3.63E-03	***33***
Cell Signaling	5.00E-09 – 2.29E-03	***21***
Vitamin and Mineral Metabolism	5.00E-09 – 9.46E-04	***17***
Physiological System Development and Function		
Behavior	3.96E-16 – 3.63E-03	***31***
Digestive System Development and Function	3.96E-16 – 1.92E-03	***19***
Nervous System Development and Function	2.09E-09 – 3.63E-03	***34***
Organ Morphology	7.48E-07 – 3.63E-03	***18***

Organismal Development	7.48E-07 – 3.63E-03	***24***

Table 4. Downregulated transcript overlap with AutismKB gene database

ID	**Name**	**Function**
ADRA1B	adrenoceptor alpha 1B	GPCR, activates mitogenic responses and regulates cell growth and proliferation
ARC	activity-regulated cytoskeleton-associated protein	synaptic plasticity
CLDN5	claudin 5	membrane protein in tight junction
CYR61	cysteine-rich angiogenic inducer 61	growth factor, promotes adhesion of endothelial cells; interacts with proteoglycan and extracellular matrix
JUNB	jun B proto-oncogene	DNA-binding activity
MGP	matrix GLa protein	inhibitor of bone formation
ROBO3	roundabout, axon guidance receptor homolog 3	controls neurite outgrowth, growth cone guidance
SCN1B	sodium channel, voltage-gated, type 1, beta	Mediates action potentials

Table 5. Upregulated transcript overlap with AutismKB gene database

ID	**Name**	**Function**
ADORA2A	adenosine receptor subtype A2	Adenosine receptor
AHI1	abelson helper integration site 1	required for cerebellar and cortical development
AQP4	aquaporin 4	intrinsic membrane protein forming water channel found predominantly in the brain
CACNA2D2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	associated with early infantile epileptic encephalopathy
CSMD3	CUB and sushi multiple domains 3	???
DACH1	dachshund family transcription factor	chromatin-associated protein; binds with other transcription factors; cell fate determination during development
DRD2	dopamine receptor D2	dopamine receptor subtype
FOXP2	forkhead box P2	transcription factor
GAD2	glutamate decarboxylase 2	catalyzes GABA
GLRA2	glycine receptor alpha 2	glycine receptor subtype
GPRASP2	g protein-coupled receptor	interacts with several GPCRs,

	associated sorting protein 2	including M1 muscarinic acetylcholine receptor.
GPRIN3	GPRIN family member 3	???
HTR2C	serotonin receptor 2C GPCR	Serotonin metabotropic receptor
NTS	neurotensin	precursor to two neuropeptides, neuromedin N and neurotensin; neurotransmitter
TMEM130	transmembrane protein 130	???

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