NEURODEVELOPMENTAL OUTCOMES IN THE FRAGILE X MOUSE

NEURODEVELOPMENTAL OUTCOMES IN THE FRAGILE X MOUSE

BY

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Lay Abstract

Autism spectrum disorder (ASD) is a diagnosis based on observed behaviours: impaired communication and repetitive actions. However, there are genetic and other behavioural differences in ASD patients that are not shared among the group. It is important to tease apart this group since current treatments for ASD do not target the biological problems or the core impairments. This thesis focuses on Fragile X Syndrome, the leading genetic condition that results in ASD in order to understand the biological basis of ASD. Using a mouse model, compared to healthy mice, these studies report changes in behaviours, in the size of different brain regions, and in molecules involved in connecting brain cells during development. These findings shed light on the molecular story underlying ASD. By understanding the nature of influences on the developing brain, the type and timing of interventions can be designed to keep the brain on a healthy trajectory.

Abstract

Fragile X Syndrome (FXS) is a neurodevelopmental disorder and the most common heritable single gene cause of Autism Spectrum Disorder (ASD). The Fragile X (*FMR1-KO*) mouse model has been used to understand the pathophysiology of the disease. However, the majority of studies have been done in adult mice and early life outcomes have yet to be explored. Therefore, in order to contribute to the knowledge of the neurodevelopmental processes associated with brain disorders, *this thesis examines postnatal outcomes in the Fragile X Mouse Model: early life behaviours, the developmental trajectory of a set of ASD risk genes, and neuroanatomical phenotype.* The first study examined ultrasonic vocalizations in pups and showed a transient increase in calls in *FMR1-KO* mice. To understand the relationship between early life behaviours, the second study examined outcomes in the pre-pubertal period in these mice when challenged with lipopolysaccharide and maternal separation. The results showed genotype and treatment interactions affecting sexually dimorphic behavioural outcomes and developmental milestones. In the third study, possible underpinnings of behavioural differences were explored by examining mRNA expression of the neuroligins and neurexins. In *FMR1-KO* mice, changes were transient and sex-specific, suggesting these as molecular effectors in the disease. Lastly, using structural brain imaging, the fourth study examined regional volume differences that may be related to behavioural differences. Differences in regions affected in FXS patients were observed and genetic background was shown to affect the neuroanatomical phenotype. Overall, this thesis demonstrates that the FXS model recapitulates some outcomes in other ASD mouse models and shows that this single gene has multiple interactions with sex, strain, and postnatal challenge which manifests at specific ages at molecular, brain structure and behavioural levels. This work contributes to the efforts elucidating the neurobiology of ASD and reverse translation approaches to identify therapeutic targets for neurodevelopment disorders.

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5-HT	5-hydroxytryptamine
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
ASD	autism spectrum disorder
ATP	adenosine triphosphate
B6	C56Bl/6
BDNF	brain derived neurotrophic factor
CA1	*Cornu Ammonis* 1
CA3	*Cornu Ammonis* 3
CNS	central nervous system
CNTNAP	contactin-like associated protein
CNV	copy number variation
CON	control
CPM	counts per minute
DISC	disrupted in schizophrenia complex
DG	dentate gyrus
DNA	deoxyribonucleic acid
DPM	disintergrations per minute

DSM	diagnostic and statistical manual
DTI	diffusion tensor imaging
E	embryonic day
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalography
EPM	elevated plus maze
EPSC	excitatory postsynaptic current
ERP	evoked response potential
FMR1	Fragile X mental retardation 1
FMR1-KO	Fragile X mental retardation 1 gene knock out
fMRI	functional magnetic resonance imaging
FMRP	Fragile X mental retardation protein
FXS	Fragile X Syndrome
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GSK	glycogen synthase kinase
HIP	hippocampus
HPA	hypothalamic pituitary adrenal
HPLC	high performance liquid chromatography
i.p.	intraperoneal

ICI	intercall interval
ICR	Institute for Cancer Research
IPSC	inhibitory postsynaptic current
ITG	integrin
KCC2	potassium-chloride symporter member 5
KO	knock out
LPS	lipopolysacchride
LRRTM	leucine-rich repeat transmembrane protein
LTD	long term depression
LTP	long term potential
MAGE	melanoma antigen gene
MALTT	multiple autistic-like trait transgenic
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MW	molecular weight
MYO	myogenin
NGF	nerve growth factor
NIH	National Institutes of Health
NKCC	sodium-potassium-chloride co-tranporter
NLGN	neuroligin

NLGN1	neuroligin-1
NLGN2	neuroligin-2
NLGN3	neuroligin-3
NLGN4	neuroligin-4
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NOS	nitrous oxide synthase
NRXN	neurexin
NRXN1	neurexin-1
NRXN2	neurexin-2
NRXN3	neurexin-3
NSERC	Natural Sciences and Engineering Research Council
OBI	Ontario Brain Institute
OF	open field
P	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDD	pervasive developmental disorder
PDZ	Post synaptic density protein 95- Drosophila disc large tumor suppressor 1-Zonula occludens 1 protein

PFA	paraformaldehyde
POND	Province of Ontario Neurodevelopmental Disorders
PSD	postsynaptic density
PTEN	phosphatase and tensin homolog
RELN	reelin
RNA	ribonucleic acid
S1	primary somatosensory cortex
SAL	saline
SEM	standard error of the mean
SERT	serotonin transporter
SHANK	SH3 and multiple ankyrin repeat domains
SNP	single nucleotide polymorphism
SNV	single nucleotide variation
SRY	sex-determining region on Y chromosome
SSRI	selective serotonin reuptake inhibitor
TEA	triethanolamine
TNF	tumor necrosis factor
mTOR	mammalian target of rapamycin
TSC	tuberous sclerosis complex
USV	ultrasonic vocalization

UTP	uridine-5'-*triphosphate*
VPA	valproic acid
YAC	yeast artificial chromosome

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Chapter 1: Introduction

Fragile X Syndrome

Fragile X Syndrome (FXS) is a neurodevelopmental disorder. It was first described in 1943 as Martin-Bell Syndrome (Martin and Bell, 1943) and is currently thought to be the most common heritable form of intellectual disability (Lubs et al., 2012). It is also the most common single gene cause of autism spectrum disorder (ASD) (Chonchaiya et al., 2009, Hagerman et al., 2014). In 1969, a "fragile" site at the end of the X chromosome was described which lead to its present alluding designation as Fragile X Syndrome (Lubs, 1969, reviewed in Krueger and Bear, 2011). In 1991, the fragile site was mapped to the Fragile X Mental Retardation 1 (*FMR1*) gene (Verkerk et al., 1991, Yu et al., 1991). The disease is caused by 200 or more CGG trinucleotide repeats in the FMR1 gene, compared to less than 50 repeats in the general population (Fu et al., 1991), which silences its transcription through epigenetic mechanisms (Fu et al., 1991, Oostra and Verkerk, 1992, Sutcliffe et al., 1992, Verheij et al., 1993). The prevalence of *FMR1* full mutation is approximately 1 in 7000 males and 1 in 11,000 females (Hunter et al., 2014). The physical hallmarks of the disease include macroorchidism, large ears, an elongated face and a prominent jaw (Hagerman, 2002). The behavioural phenotype of patients with FXS includes intellectual disability, anxiety, social deficits, hyperarousal, abnormalities in communication, abnormal sensory reactivity, gaze aversion, inattention, impulsivity, aggression, hyperactivity, and self-injurious behaviour (Cohen et al., 1991, Hagerman, 2002, Kaufmann et al., 2004, Budimirovic et al., 2006, Bailey et al., 2008, Hessl et al., 2008).

Below, I review the current literature on neuroanatomical and synaptic changes in FXS, followed by the behavioural comorbidities with ASD which point to underlying shared neurobiology of FXS and ASD at the synapse. The current literature on the role of neuroligins and neurexins in synaptic development, which are a family of synaptic adhesion molecules recently identified as susceptibility genes for ASD, is reviewed as a possible mechanism for the shared

neurobiology of FXS and ASD. Following this, a current understanding of the etiology of FXS will be presented through reviewing studies of the FXS mouse model and its phenotype at the synaptic, behavioural, and neuroanatomical levels.

The Neuroanatomical Phenotype of Fragile X Syndrome

Imaging studies report volume changes in various brain regions in FXS patients (Barnea-Goraly et al., 2003, Haas et al., 2009), consistently showing enlargement of the caudate nuclei (Reiss et al., 1995, Eliez et al., 2001, Hoeft et al., 2008, Bray et al., 2011, Hallahan et al., 2011) and the ventricles (Reiss et al., 1991, Reiss et al., 1995, Eliez et al., 2001, Lee et al., 2007) as well as reductions in volume of the cerebellar vermis (Reiss et al., 1991, Kaufmann et al., 2003, Lee et al., 2007, Gothelf et al., 2008). The functions of these areas have been well-established and can be linked to the behavioural phenotype of patients with FXS. For instance, the frontostriatal circuitry – which includes the caudate – is a network involved in the behaviour phenotype of the disorder, namely, integration of information that elicits or inhibits a response (Bonelli and Cummings, 2006). Lesions of the dorsolateral or ventrolateral areas of the prefrontal cortex, or the efferent projections in the respective anterodorsal and ventrolateral areas of the caudate nucleus, result in deficits in delayed-response tasks (Divac et al., 1967) and changes in response inhibition (Eagle et al., 2008, Fox et al., 2010). Both of these behavioural signs have been reported in patients (Hoeft et al., 2007). In addition, decreased volume in the left frontal lobe, another region in this circuit, has also been reported (Hallahan et al., 2011). The cerebellar vermis is involved in the coordination of emotional responses into autonomic and motor behaviour (Baldacara et al., 2008, Sacchetti et al., 2009). Heightened autonomic responses to emotional stimuli have been reported in children with FXS (Hessl et al., 2004, Baranek et al., 2008, Roberts et al., 2009b, Roberts et al., 2012, Cohen et al., 2013). Furthermore, volume differences in the insula, an area also involved in emotional response regulation and awareness (Jones et al., 2010) (Craig, 2009, Gu et al., 2013), have been reported in patients as well (Cohen et al., 2011).

Direct associations of brain volume and behavioural phenotype further strengthen the utility of imaging as a biomarker for a certain behavioral phenotype. For example, changes in superior and medial prefrontal gyri volumes correlate with cognitive outcomes in spatial relations and verbal fluency scores in adolescences with FXS (Bray et al., 2011). Increases in caudate volume in patients are associated with repetitive behaviours (Wolff et al., 2013) as well as cognitive deficits (Peng et al., 2014). Furthermore, whole brain expression analysis of FMRP in monkeys has shown that regions such as the striatum, cerebellum and temporal lobes highly express FMRP (Zangenehpour et al., 2009). These are also structures that are involved in the behavioural and cognitive impairments seen in the disorder. Overall, these studies show regional brain changes are related to functional impairments within the clinical setting in FXS patients.

Neuroanatomical volume changes in FXS children vary by regional trajectories across development. For example, a study examining boys with FXS from 1-4 years old reported smaller orbitofrontal and medial prefrontal cortices, superior temporal gyri, parietal lobes and insular cortices while having enlarged caudate nuclei, fusiform gyri, hypothalami, thalami and dorsal cingular regions compared to controls at the 1 year time point (Hoeft et al., 2008, Hoeft et al., 2011). However, some of the regions that were different at 1 year were not different at 3 years. Only the increases in the caudate and fusiform gyri and decreases in the cerebellar vermis were consistent at 1 and 3 years relative to controls (Hoeft et al., 2010). Those differences, based on another study, continue on to adolescence (Bray et al., 2011). In contrast, enlargements of the orbital gyri, basal forebrain, and thalamus were only seen at 3 years but not the initial 1 year time point (Hoeft et al., 2010). These regional specific trajectories may be based on the rate of maturation of the area, which in turn is affected locally by the lack of expression of FMRP and its downstream effects on synapse structure and function.

The Synaptic Phenotype of Fragile X Syndrome

Synaptic changes in the shape and number of dendritic spines are present in FXS patients. Dendritic spines provide a specialized postsynaptic compartment for glutamatergic

synaptic transmission (Nimchinsky et al., 2002, Sala and Segal, 2014). The shape and size of dendritic spines vary from small heads with long necks, to large heads with short necks. Studies examining spine morphology in post mortem brain tissues from FXS patients across the lifespan showed normal neuronal number and size but an increase in long, thin spines in multiple cortical areas (Rudelli et al., 1984, Wisniewski et al., 1990, Hinton et al., 1991, Irwin et al., 2000). Spines morphology is dynamic, ranging from thin and long spines that lack synaptic inputs and postsynaptic densities (Ziv and Smith, 1996, Jontes and Smith, 2000) to spines with large heads and short necks that have functional synaptic inputs and postsynaptic densities (Bourne and Harris, 2007). In addition, some studies have reported an overall higher density of spines in FXS patients in addition to the morphological changes (Irwin et al., 2000, Irwin et al., 2001), perhaps due to a lack of synaptic pruning or a downstream effect of excitatory-inhibitory balance and unstable synapses (Portera-Cailliau, 2012). Overall, this evidence suggests that synaptic alterations are present in FXS brains, and several investigators suggest that synaptic dysfunction is shared by many other neurodevelopmental disorders, including ASD (Qiu et al., 2012, Spooren et al., 2012, Carter and Scherer, 2013, Banerjee et al., 2014, Pinto et al., 2014).

The Autistic Phenotype of Fragile X Syndrome

ASD is a complex neurodevelopmental disorder with variable clinical presentation (Anagnostou et al., 2014), including functional impairment in social communication, language and sensory motor outcomes. Diagnostic criteria from the DSM 5.0 define autism spectrum disorder (formerly, autism and Asperger's) with two core symptom dimensions: (1) impairments in social communication and (2) repetitive behaviours (American Psychiatric Association, 2013). This phenomenologically defined behavioural disorder is heterogeneous in presentation (Lord et al., 2000, Shevell et al., 2001, Amaral et al., 2008, Mandell, 2011) and in etiology (Gibson et al., 2008, Gutierrez et al., 2009, Etherton et al., 2011), likely a result of multigenic interactions and environmental contributions (Freitag, 2007, Abrahams and Geschwind, 2008, Hallmayer et al., 2011). Both ASD and FXS share deficits in social interactions and communication (Kaufmann et

al., 2004, Belmonte and Bourgeron, 2006). 25% of males and 6% of females with FXS meet clinical criteria for ASD (Hatton et al., 2006) while up to 90% of FXS patients display autistic symptoms. 1–2% of people diagnosed with ASD have FXS (Bailey et al., 2008), making FXS the leading heritable single gene cause of ASD. This association, to a large extent, is due to impairments in the quality of social interaction (Kaufmann et al., 2004, Hernandez et al., 2009, Roberts et al., 2009a). Many of these autistic behaviours remain stable in FXS patients (Sabaratnam et al., 2003). *Therefore, understanding the neurobiology of Fragile X Syndrome is an avenue to understanding the processes gone awry in ASD in particular and neurodevelopmental disorders in general.*

Key Points:

- ✓ Regional brain volume changes are related to functional impairments within the clinical setting in FXS patients.
- ✓ Neuroanatomical volume changes in FXS children vary by regional trajectories across development.
- ✓ Differences in the morphology and density of dendritic spines are observed in FXS patients.
- ✓ Individuals with ASD and FXS have impairments in social interactions; comorbidity of these conditions suggest that better understanding FXS will also lend greater understanding of ASD

Synaptic Genes in Neurodevelopmental Disorders

The biological basis of traits such as the shared behavioral symptoms mentioned above can be demonstrated by examining the data showing that genetics and psychiatric disorders are related (Insel and Lehner, 2007). In particular, the heritability of ASD is high, with a 60-80% concordance rate in monozygotic twins and less than 10% in dizygotic twins (Folstein and Rutter, 1977b, a, Steffenburg et al., 1989, Bailey et al., 1995, Nordenbaek et al., 2014). This genetic component has been investigated through linkage studies and has demonstrated that ASD involves multiple genetic loci of small effect size (Abrahams and Geschwind, 2008, Anney et al., 2010, Klei et al., 2012). Nevertheless, the linkage studies generally have not been replicable, since the common variant effect size is small and thus obtaining a sufficient sample size is the

primary barrier to date (Murdoch and State, 2013). Studies examining both copy number variations and single nucleotide polymorphisms have found rare variants of larger effect size (Pinto et al., 2010, Sanders et al., 2011, Devlin and Scherer, 2012, Pinto et al., 2014), both transmitted and *de novo* (Levy et al., 2011, Sanders et al., 2011, Iossifov et al., 2012). These genes have pointed to a common theme in neurodevelopmental disorders: disruptions in connectivity and circuit wiring (Hammock and Levitt, 2006, Garber, 2007, Cook and Scherer, 2008, Rapin and Tuchman, 2008, Tommerdahl et al., 2008, Zoghbi and Bear, 2012), or a "neuropsychiatric diseasome" (term from Woodbury, 2012, personal communication) with mutations in the same genes involved in several different disorders. Researchers have discovered a network of genes involved in the formation and function of synapses that are mutated, deleted or duplicated in ASD (Moessner et al., 2007, Marshall et al., 2008, Sudhof, 2008, Betancur et al., 2009, Bourgeron, 2009, Glessner et al., 2009, Pinto et al., 2010, Gilman et al., 2011, Levy et al., 2011, Sanders et al., 2011, O'Roak et al., 2012). These include synaptic cell adhesion molecules that provide scaffolding between the pre- and post-synaptic terminals, suggesting that ASD may be a syndrome of aberrant connectivity (Geschwind and Levitt, 2007). Each of these mutations may be a different entry point into this common problem that manifests ultimately in the two core domains of autistic behaviours. To elucidate the cellular gap between the clinical genetic studies and presenting symptoms of individuals, one must look at the molecular and cellular underpinnings of the disorder. In this thesis, FXS is proposed to be a model to explore gene interaction with genetic hits implicated in ASD. Since the majority of genes point to synaptic dysfunction as a core pathway and synaptic changes are reported in Fragile X patients (discussed above), one may hypothesize that there may be a coalescing of molecular biology between the two disorders.

Neuroligins and Neurexins

The neuroligin (NLGN) and neurexin (NRXN) family were among the earliest rare penetrant genes to be linked with autism in family studies. *De novo* chromosomal deletions in

NLGN3 (Jamain et al., 2003) and heritable base pair deletions of NLGN4 (Laumonnier et al., 2004, Yan et al., 2005) were first reported in patients with ASD a decade ago. More recent studies have identified de novo CNVs or SNVs in the NLGN1 (Glessner et al., 2009, Pinto et al., 2010), NLGN3 (Pinto et al., 2010, Yu et al., 2011), NLGN4X (Pinto et al., 2010), NRXN1 (Feng et al., 2006, Kim et al., 2008, Yan et al., 2008, Sanders et al., 2011, Iossifov et al., 2012, Bena et al., 2013), NRXN2 (Gauthier et al., 2011), and NRXN3 (Vaags et al., 2012) that are associated with ASD. These genes are involved in structural and functional integrity of mature synapses (Scheiffele, 2003, Craig and Kang, 2007). Furthermore, recently, NLGN3 and NRXN1, among other ASD candidate genes, have been shown to bind to FMRP (Darnell et al., 2011, Iossifov et al., 2012). Therefore, it is possible that the expression of specific neuroligins and neurexins are altered in FXS.

Neuroligin-1 is predominantly expressed at glutamatergic synapses (Song et al., 1999, Chubykin et al., 2007). The early expression of N-methyl-D-aspartate (NMDA) receptors is mediated through co-transport with NLGN1 to nascent synapses, followed by more extensive recruitment of those receptors through its interaction with PSD95 (Barrow et al., 2009) while overexpression of NLGN1 increases NMDA/AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) ratio (Chubykin et al., 2007). This allows for NMDA-dependent stabilization of the synapses to occur (Chen et al., 2010) and modulates synapse size (Ko et al., 2009), partly through movement of AMPA receptors and PSD95 between synapses (Mondin et al., 2011). Conversely, the expression of NLGN1 is decreased by knockdown of PSD95 (Levinson et al., 2010) or inducing changes in plasticity (Schapitz et al., 2010). Overall, these data suggest that NLGN1 may regulate the localization and stabilization of NMDA receptors.

Neuroligin-2 is expressed at gamma-aminobutyric acid (GABA)-ergic synapses (Graf et al., 2004, Chubykin et al., 2007) with a collybistin and gephyrin postsynaptic protein (Poulopoulos et al., 2009, Pettem et al., 2013). NLGN2 localizes to specific types of GABAergic synapses, as NLGN2 knockout mice have decreased transmission from fast spiking interneurons, but not

somatostatin positive ones (Gibson et al., 2009). Neuroligin-2 knockout mice have decreased GABA receptor clusters, decreased gamma burst (indicative of GABAergic network inhibition) and increased excitation in the dentate gyrus during paired pulse stimulation (Jedlicka et al., 2011). Overexpression of NLGN2 in mice has been reported to show altered synaptic transmission and an autistic-like behavioural phenotype including repetitive behaviour, anxiety-like behaviour, and deficits in social behaviour (Hines et al., 2008). Neuroligin-2 expression leads to KCC2 expression (Sun et al., 2013), which is involved in the switch from excitatory GABAergic function to adult inhibitory function early in development (Cancedda et al., 2007). Since inhibitory networks are important for development (Marin, 2012) and network synchrony is perturbed in models of both ASD (Gutierrez et al., 2009, Testa-Silva et al., 2012) and FXS (Paluszkiewicz et al., 2011, Goncalves et al., 2013), one may conceive that neuroligin-2 levels may be altered at synapses in these disorders.

Neuroligin-3 is present at both glutamatergic and GABAergic synapses and form heterodimers with NLGN1 or NLGN2 at those synapses respectively (Budreck and Scheiffele, 2007). Introduction of the human ASD-related NLGN3-R451C gene into mice resulted in a 90% decrease in neuroligin-3 protein that was associated with impaired social behaviour and an increase in basal inhibitory synapse transmission (Tabuchi et al., 2007). However, another study reported no differences in social, cognitive and spatial abilities with the same mutation; only a male-specific decrease in in ultrasonic vocalizations (Chadman et al., 2008). Another mutation, R471C, resulted in a decreased number of inhibitory synapses *in vitro* whereas NLGN3 overexpression in the same system resulted in increased spontaneous burst activity compared to WT (Gutierrez et al., 2009). When another human mutation, NLGN3-R704C was inserted into the cytoplasmic sequence of all NLGNs, there were no changes in synapse formation, but a specific decrease in AMPAR transmission (Etherton et al., 2011). However, knockout of NLGN3 gene in mice show reduced ultrasonic vocalizations and a lack of social novelty preference with no differences in learning and memory (Radyushkin et al., 2009). Recent work suggests that

synaptic context is important to understand the function of these molecules and may explain some of the discrepancies in the findings. For instance, parvalbumin-positive and cholecystokinin-positive interneurons in the hippocampus function differently in respects to NLGN3-R451C knock-in where synapses of the former on pyramidal cells have reduced release probability, but the later have decreased release probability (Foldy et al., 2013). In addition, knockout of NLGN3 at parvalbumin-positive neurons have no effect whereas knockout in the cholecystokinin-positive neurons have a similar effect as the NLGN3-R451C knock-in. These differences and variations may reflect the multiple etiologies underlying a single disease risk gene, since specific mutations may alter different processes (Comoletti et al., 2004, Yamakawa et al., 2007). Thus, one would expect that the expression levels of these genes are tightly regulated in spatial and temporal patterns.

The NRXNs are the presynaptic binding partners of the NLGNs and are composed of three genes, each with α and β transcripts, making up six isoforms and thousands of splice variants (Ullrich et al., 1995, Missler and Sudhof, 1998, Craig and Kang, 2007, Tanaka et al., 2011). α-NRXNs have much longer extracellular domains than the β- NRXNs, but share the same C-terminus (Missler and Sudhof, 1998). *In vitro* transfection of NLGN1 increases frequency of excitatory post-synaptic currents (EPSCs) whereas NLGN2 increases frequency of both EPSCs and inhibitory post-synaptic currents (IPSCs) (Futai et al., 2013). Again, the role of synaptic context was significant. These differences were due to the differential binding of NRXN1β in pyramidal neurons to NLGN1 and NRXN1α in interneurons binding NLGN2 (Futai et al., 2013). The mRNA of all six isoforms are expressed in both glutamatergic and GABAergic neurons (Ullrich et al., 1995), suggesting that influence on postsynaptic differentiation may not be as simple as a correspondence of a single isoform to a single synapse type. For example, in the cerebellum, neurexin expression is specific to parallel fibres but not climbing fibre inputs (Pregno et al., 2013). Furthermore, there is specificity in NRXN action; NRXN1β and NRXN2β binding to

Cbln1 mediates the size of the active zone in the inhibitory parallel fibre-Purkinje cell synapse (Mishina et al., 2012).

In vitro studies show that α-neurexin expression results in the assembly of GABAergic postsynaptic proteins (Chih et al., 2005, Nam and Chen, 2005, Kang et al., 2008). Triple and double knockouts of α-NRXNs die within one day and one week respectively, due to lack of synapse formation in key respiratory centres in the brain (Missler et al., 2003). α-NRXNs have a role in recruiting calcium channels for release (Missler et al., 2003) and, not surprisingly, NRXN1α knockout mice have decreased neurotransmitter release due to impairments in calcium signalling (Zhang et al., 2005, Etherton et al., 2009). It is known that specific variants in α-NRXNs regulate its ability to bind the NLGNs (Tanaka et al., 2011).

The developmental expression of NLGNs and NRXNs in the rat shows that levels increase during times of functional synapse maturation. Neuroligin-1 is present in low amounts in rat embryonic brains, increases at birth and peaks at P5-8 until levels again increase in adulthood (Song et al., 1999). Neuroligin-2 is present at E16, peaks at P15-P21 and expression is maintained in adulthood (Varoqueaux et al., 2004). Neuroligin-3 protein expression is present *in utero*, increases and peaks at P14, and is maintained in adulthood (Budreck and Scheiffele, 2007). Neurexin proteins in the cerebellum co-localize with GABA at higher levels at P5-P21 than at P30 (Pregno et al., 2013), and are associated with NLGN2. Overall, the NLGN and NRXN family are regulated in a spatial and temporal fashion that may contribute to synaptic maturation and function, as suggested by the increased risk for ASD in their absence. These genes may have a role in the molecular processes underlying the deficits in neurodevelopmental disorders. Specifically, from the clinical reports of shared traits in ASD and FXS, one can postulate that gene-gene interactions may exist between *FMR1* and synaptic genes. However, due to the transient nature of developmental processes and the invasive procedures needed to understand these interactions, one may utilize mouse models to examine underlying biology.

Key Points:

- ✓ A common theme in neurodevelopmental disorders is disruption in connectivity and circuit wiring, with mutations in the same genes involved in several different disorders.
- ✓ Researchers have discovered a network of genes involved in the formation and function of synapses are affected in autism and other neurodevelopmental disorders.
- ✓ The neuroligins and neurexins family are involved in structural and functional integrity of mature synapses, and their loss is a risk for autism. There is also evidence that the expression of specific neuroligins and neurexins are altered in FXS.

Mouse Models of Neurodevelopmental Disorders

In behavioural neuroscience, animal models can help map out the biological processes that correspond to the symptomology of a psychiatric disorder (Szechtman and Eilam, 2005). Because detailed and descriptive results can come from invasive procedures and manipulations, experimental demonstration of biological causation can be investigated (Teitelbaum and Pellis, 1992). Historically, the validity of animal models has included the following criteria: 1) construct validity - matching biological dysfunction of human disorder 2) face validity - resemblance of human phenotype 3) predictive validity – similar response to treatments as in human (McKinney and Bunney, 1969).

Although mouse models of neurodevelopmental disorders have been developed, due to difficulties in the diagnostic criteria at the clinical level, researchers must choose strategies to figure out what specifically to model. Additionally, since disorders such as ASD have multiple etiologies and phenotypic diversity, it is likely that there is not a single animal model that will be able to capture all the levels of the disease state (Flint and Shifman, 2008, Moy and Nadler, 2008).

Genetic Models

Currently, preclinical animal models of neurodevelopmental disorders are mostly based on genetic manipulations and validated by behavioural phenotype (Moy et al., 2006). Studies use models that mimic known etiology of diseases, assuming that some of the underlying

pathophysiology is shared. Examples include the methyl CpG binding protein 2 (*MeCP2*) KO (Picker et al., 2006, Allan et al., 2008, Schaevitz et al., 2010) and *FMR1-KO* mouse (Bernardet and Crusio, 2006, McNaughton et al., 2008). Other genetic models of neurodevelopmental disorders include NLGN2 knockout (Hines et al., 2008, Blundell et al., 2009), phosphatase and tensin homolog (*PTEN*) KO (Kwon et al., 2006), SH3 and multiple ankyrin repeat domains 3 (SHANK3) mutation (Bangash et al., 2011), NLGN3 mutations (Radyushkin et al., 2009), NLGN4 mutations (Jamain et al., 2008) and engrailed-2 (*En-2*) KO (Cheh et al., 2006) are used to further understand the role of these genes. The genetic approach uses targeted mutations in mice to define mechanisms regulated by genes considered important for neurodevelopment.

Environmental Models

Early-life challenge models are also used to explore the mechanisms behind environmental exposures that are potential risk factors for neurodevelopmental disorders. In the 1960s, follow-up studies of a rubella epidemic in the US showed that maternal infection resulted in congenital malformation (Chess, 1970) and higher incidences of ASD (Chess, 1977). In addition, studies showed that exposure to valproic acid (VPA), an anti-epileptic medication, led to increased risk of congenital malformations, developmental delay, reduced cognitive function (Meador et al., 2003, Ornoy, 2009, Nadebaum et al., 2011, Shallcross et al., 2011) as well as a higher incidence of ASD or autistic behaviours in the offspring, particularly if exposed in the first trimester (Christianson et al., 1994, Williams and Hersh, 1997, Williams et al., 2001, Rasalam et al., 2005, Bromley et al., 2008). These two environmental insults led to the establishment of models investigating the role of prenatal exposures in neurodevelopment using polyinosinic:polycytidylic acid (poly (I:C)) (Patterson, 2002, Shi et al., 2003, Meyer et al., 2008, Li et al., 2009) and VPA respectively (Rodier et al., 1996, Narita et al., 2002, Schneider and Przewlocki, 2005, Wagner et al., 2006, Markram et al., 2008, Schneider et al., 2008, Kolozsi et al., 2009, Gandal et al., 2010, Roullet et al., 2010, Kataoka et al., 2013).

The activation of the maternal immune response through exposure to poly (I:C), a viral mimic, has also been used to investigate the role of maternal immune function in neurodevelopment. Studies examining poly (I:C) mice have found deficits in exploratory behavior, social behavior, and sensorimotor gating (Shi et al., 2003, Shi et al., 2005, Smith et al., 2007) as well as anatomical and molecular changes, cerebellar abnormalities, changes in dopaminergic circuitry (Shi et al., 2003), and increased expression of neurotrophic factors (Gilmore et al., 2005).

The *in utero* VPA exposure is a rodent model that has strong predictive validity for ASD, even as studies vary in regard to their timing of exposure and dose (see (Roullet et al., 2013) for review). After pregnant dams are exposed to VPA, there is a clear impact on the offspring including deficits in sensorimotor gating, decreased exploratory behaviour, increased anxiety-like behaviour and decreased social behaviour (Schneider and Przewlocki, 2005, Markram et al., 2008, Roullet et al., 2010, Kataoka et al., 2013). Furthermore, sex-related differences presented as greater impairments in males (Schneider et al., 2008). Of relevance, our lab has found that mRNA expression of NLGN3 is decreased in the somatosensory cortex and hippocampus of VPA-treated mice (Kolozsi et al., 2009). Interestingly, in a separate study, levels of NLGN3 protein were positively correlated with EEG gamma band oscillations in response to auditory stimulation (Gandal et al., 2010), suggesting the involvement of NLGN3 in the refinement of GABAergic networks after VPA exposure. Overall, mouse models that explore both genetic and environment risk factors of neurodevelopmental disorders enable the study of cellular processes and circuitry (Krueger et al., 2011) as well as alterations in behavioural outcomes (Silverman et al., 2010).

Key Points:

- ✓ Animal models can help map out the biological processes that correspond to the symptomology of a psychiatric disorder
- ✓ Since disorders such as autism have multiple etiologies and phenotypic diversity, there is thus far no single animal model that can capture all the levels of the disease state.
- ✓ A variety of models exist, such as models that mimic known etiology of the disease, harbor associated genetic mutations, utilize environmental exposures that are risk factor, and investigate in the role of maternal immune response on neurodevelopment.

Etiology and Pathophysiology of FXS

In order to understand the etiology and develop treatments for Fragile X Syndrome, the *FMR1-KO* mouse was developed by the Dutch-Belgium Fragile X Consortium in 1994 (The Dutch-Belgian Fragile X Consortium, 1994). In humans, the silencing of the *FMR1* gene, due to the trinucleotide expansion, results in the lack of expression of the protein product, Fragile X Mental Retardation Protein (FMRP), an RNA binding protein (Ashley et al., 1993). The mouse model differs from the human condition in that the *FMR1* gene is knocked out, however, the protein product, FMRP, is missing in both cases.

Through studies using the *FMR1-KO* mouse, we know that FMRP is expressed in dendritic spines (Weiler et al., 1997) and it is involved in various steps in the translation process of its mRNA targets by repressing and stalling ribosomes (Darnell et al., 2011, Maurin et al., 2014), inhibiting translation initiation (Napoli et al., 2008), and regulating the stability of other mRNAs (Zalfa et al., 2007, Zhang et al., 2007) necessary for experience-dependent plasticity. These include synaptic scaffolding proteins (Zalfa et al., 2007, Darnell et al., 2011), receptors (Darnell et al., 2011) and various voltage-gated channels (Brown et al., 2001, Darnell et al., 2001, Chen et al., 2003, Strumbos et al., 2010, Darnell et al., 2011, Gross et al., 2011, Lee et al., 2011, Brager and Johnston, 2014), all of which affect the developmental processes that govern the dynamics of the brain circuit. Functionally, a lack of FMRP leads to up-regulation of mGluR5 receptors and altered plasticity (Huber et al., 2002) through processes such as mGluR5-

dependent internalization of AMPAR (Nakamoto et al., 2007), reviewed in (Bear et al., 2004, Dolen and Bear, 2008). Indeed, crossing mGluR5-KO mouse with the *FMR1-KO* mouse rescued the impaired long-term depression, elevated the dendritic spine densities in the hippocampus, and attenuated seizures (Dolen et al., 2007). Overall, the loss of FMRP leads to dysregulation of processes critical for fidelity in information integration and transfer across the neural circuitry, leading to morphological and functional changes, which result in the defects in learning and behavioural impairments observed in the disorder.

The Developmental Synaptic Phenotype of the FMR1-KO Mouse

At a molecular level, synaptic level morphological changes have been reported over the course of postnatal development in the *FMR1-KO* mouse (Cruz-Martin et al., 2010, Meredith et al., 2012). In mice, invasive procedures such as Golgi-Cox staining and electrophysiology recording *ex vivo* have respectively shown morphological and functional changes occurring in various brain regions and in different types of neurons over different windows of time (Bureau et al., 2008, Gibson et al., 2008, Pan, 2010). For example, spine morphology changes are reported in the first two weeks of life in the cortex, which then normalize at P28 and reappear at P75 (Nimchinsky et al., 2001, Galvez and Greenough, 2005). Layer-specific changes in functional synapse maturation and structural spine morphology have been observed at different ages, for example, afferents from layer 4 pyramidal neurons to layers 2/3 are immature at P14, but not P21 (Bureau et al., 2008) while spines from layers 2/3 pyramidal have delayed maturation at P14 but not P7 or P21 (Cruz-Martin et al., 2010). Changes vary across brain region as well; in the hippocampus, compared to WT controls, spines are longer at P10, no different at P20, shorter at P30, and, again, no different at P40 (Grossman et al., 2006). Lastly, changes are input specific; for example, connections from layer IV pyramidals to fast-spiking interneurons are altered at P14 but not P28 (Gibson et al., 2008). Overall, changes in postsynaptic composition, plasticity and input strength are specific to age, input, region, and layer (Monyer et al., 1994, Pilpel et al., 2009, Adusei et al., 2010, Edbauer et al., 2010, Harlow et al., 2010, Meredith et al., 2011), perhaps a

sign of global imbalance of stable and dynamic connections (Portera-Cailliau, 2012). Interpretations of these studies are confounded by the fact that FMRP expression itself is regionally and developmentally regulated in both mice and humans (Hinds et al., 1993). In mice, peak FMRP expression is between P7 and 14 in the barrel cortex (Harlow et al., 2010), when synaptic connections are forming. In the hippocampus, expression peaks at P7 to P28 (Lu et al., 2004). Overall, these data suggest that the lack of FMRP disturbs the typically highly coordinated process for synapse maturation and stability. Considering the developmental profiles of genes that are involved in this process would be useful in understanding the effects of *FMR1* silencing during brain development.

The Behavioural Phenotype of the FMR1-KO Mouse

Behavioural outcomes in mouse models are useful to examine neurodevelopmental disorders (Bolivar et al., 2007, Moy et al., 2007, Moy and Nadler, 2008, Ryan et al., 2010)(reviewed in (Ricceri et al., 2007, Silverman et al., 2010). The behavioural manifestations of FXS found in humans are well-represented in the *FMR1-KO* mouse model (Bernardet and Crusio, 2006, Oddi et al., 2013). Studies to date have identified behavioural deficits in domains including sensorimotor (Chen and Toth, 2001, Nielsen et al., 2002, Pietropaolo et al., 2011), activity (Nielsen et al., 2002) (Peier et al., 2000, Ventura et al., 2004, Restivo et al., 2005), learning (Frankland et al., 2004), sociability (Spencer et al., 2005, Mineur et al., 2006, McNaughton et al., 2008, Spencer et al., 2008, Mines et al., 2010, Pietropaolo et al., 2011, Spencer et al., 2011, Gantois et al., 2013, Heitzer et al., 2013), and anxiety-like behaviour (Peier et al., 2000, Spencer et al., 2005, Heulens et al., 2012), however, inconsistences exist in the literature (Van Dam et al., 2000, Nielsen et al., 2002, Spencer et al., 2008, Moy et al., 2009, Qin et al., 2011, Spencer et al., 2011). This may be due in part to external experimental setup and procedures (Bailey et al., 2005); furthermore, many of these contradicting findings may be explained by differences in strain and age (Pietropaolo et al., 2011). Differences in behaviour in *FMR1-KO* mice due to background strain were first shown by Dobkin (2000) in learning in the Morris water maze (Dobkin et al.,

1999). Additionally, these results suggest that strain-gene interactions must be taken into consideration when examining the *FMR1–KO* behaviour phenotype.

Social behaviour testing is commonly used in mouse models of neurodevelopmental disorders since the quality of social interactions is a core domain in ASD and many associated neurodevelopmental disorders. The literature reports differences in social behaviour in *FMR1-KO* mice that are dependent on both age and strain. Prior to that discussion, a brief review of findings on traits that may affect the sociability phenotype, activity and anxiety-like behaviour, of *FMR1-KO* mice on pure C57Bl/6 and FVB background strains, will take place.

Activity in an open field has been used to assess overall locomotor activity in *FMR1-KO* mice on both strains and a primary tool for this trait. In adult *FMR1-KO* on a C57Bl/6 background, studies report an increase in overall activity (Peier et al., 2000, Mineur et al., 2002, Restivo et al., 2005, Spencer et al., 2005) compared to WT C57Bl/6 mice. One study reported no difference due to genotype (Nielsen et al., 2002), however, in this study, a battery of behavioural tests was done on consecutive days, which could influence the results. In adult *FMR1-KO* mice on a FVB background, Ventura (2004) has reported an increase in locomotor activity compared to WT mice (Ventura et al., 2004), while Qin (2005) reported an increase compared to WT mice that appeared after the first 15 minutes in a 30 minute test, likely due to a lack of habituation (Qin et al., 2005). Again, as with the C57Bl/6 mice, Nielsen (2002) reported no difference (Nielsen et al., 2002). Overall, considering similar methodologies, *FMR1-KO* mice on both FVB and C57Bl/6 strains have increased activity measures.

Anxiety-like behaviour is another trait that may affect social behaviour phenotype. Results suggest that both strain and age are factors that affect this trait. For example, on the elevated plus maze, *FMR1-KO* on a C57Bl/6 background were reported to be no different (Mineur et al., 2002, Nielsen et al., 2002, Moy et al., 2009) or have increased anxiety-like behaviour (Heulens et al., 2012) at 2-3 months but decreased at 3-4 months (Peier et al., 2000, Spencer et al., 2005) compared to WT C57Bl/6 mice using that test. In the light-dark box, the anxiety-like behaviour

increases with age in mice on a C57Bl/6 background. *FMR1-KO* C57Bl/6 mice have decreased anxiety-like behaviour at 2-3 months (Spencer et al., 2008) and increased anxiety-like behaviour at 3-4 months (Peier et al., 2000) compared to WT mice using that test. In the open field, the proportion of time spent in the periphery compared to the centre of the field is a measure of anxiety-like behaviour and studies show that *FMR1-KO* C57Bl/6 mice have either decreased time spent in that area (Peier et al., 2000, Spencer et al., 2005) or are not different (Mineur et al., 2002) compared to WT mice. On the FVB background strain, anxiety-like behaviour in *FMR1-KO* mice has been reported to be increased at 3 weeks old (Bilousova et al., 2009) and not different at older ages (Moy et al., 2009, Qin et al., 2011) compared to WT FVB mice on the EPM. This is similar to what is seen on the C57Bl/6 background at the older ages. However, on the elevated zero maze, Liu (2009) reports a decrease in anxiety-like behaviour in *FMR1-KO* adult mice (Liu and Smith, 2009). Overall, these studies demonstrate that anxiety-like behaviour is dependent on age and strain – on an FVB background, increased in early life but no different than WT mice later in life.

Social behaviour has been assessed in mouse models of ASD primarily using the 3-chambered apparatus developed by Crawley (Silverman et al., 2010). This test measures the amount of time spent in 3 compartments as an index of social approach or sociability. The side chambers contain a naïve “stranger” mouse underneath an inverted cup and an empty inverted cup. The middle chamber is empty and allows for access to both side chambers. Other social tests include free interaction with the naïve stranger mouse in an open field and time spend in proximity as well as scoring of specific behaviours, such as aggression, grooming, and sniffing.

On a C57Bl/6 background, results regarding social behaviour are varied. The majority of studies report no differences in sociability at 2-3 months (Mines et al., 2010, Pietropaolo et al., 2011, Spencer et al., 2011) and at 5-7 months (Heitzer et al., 2013), while one shows an increase at 3 months (Gantois et al., 2013). In social interaction, *FMR1-KO* mice on a C57Bl/6 background have increased interaction at both 2-3 months (Spencer et al., 2011) and at 3-4 months (Spencer

et al., 2005, Spencer et al., 2008) while another group report decreased interactions at 3 months (Mineur et al., 2006) as well as more aggressive behaviours (Pietropaolo et al., 2011) at that age compared to WT C57Bl/6 controls. On an FVB background, there is no difference in sociability in *FMR1-KO* mice compared to WT controls. Liu (2009) reported normal social preference for the chamber with the mouse over the empty chamber in *FMR1-KO*, but no genotype difference in time spent with the stranger mouse (Liu and Smith, 2009). However, they also observed decreased sniffing investigations of the mouse. Similarly, Pietropaolo (2011) reported no difference in sociability due to genotype. In social interaction, one study reports no difference in the quantity or type of interactions with a stranger, in this case, juvenile mouse, in *FMR1-KO* mice compared to WT FVB controls at 3 months of age (Pietropaolo et al., 2011). Overall, sociability is not different in *FMR1-KO* mice on either background, although in free social interaction and increases in interaction time have been reported in *FMR1-KO* mice, only when on a C57Bl/6 background.

These data demonstrate that strain and age considerations are crucial when evaluating the validity of a model. These discrepancies at the behavioural level suggest possible background genetic interactions with the *FMR1* gene that may vary by age. These factors ought not to be seen as hindrances to modelling the disorder, but to have utility by reflecting clinically relevant genome heterogeneity. Exploring the interactions and modifiers may be necessary to understand the variability in symptom presentation of FXS and other neurodevelopmental disorders.

Key Points:

- ✓ FMRP is expressed in dendritic spines, and has a critical effect on the developmental processes that govern the dynamics of the brain circuit. A lack of FMRP disturbs synapse maturation and stability with many downstream effects.
- ✓ There are discrepancies at the behavioural level of the *FMR1-KO* mouse, which suggest possible background genetic interactions with the FMR1 gene; these interactions may also vary by age.

Brain Imaging in Mice

Similar to behavioural phenotyping, preclinical magnetic resonance imaging (MRI) has been used to validate mouse models through detection of similar neuropathological features reported in human disorders (Meyding-Lamade et al., 1998, Kooy et al., 2001, Helpern et al., 2004, Francis et al., 2006). Furthermore, as a shift in our understanding of psychiatric disorders goes from a symptomology-based to a circuit-driven brain-based model, the translational value of neuroimaging increases. Beyond identifying similar neuroanatomical changes to the clinical pictures, preclinical imaging has been able to describe how each model affects specific regions of the brain (Cyr et al., 2005, Fatemi et al., 2008b, Chen et al., 2009, Mercer et al., 2009). Specifically, in neurodevelopmental disorders, neuroanatomical phenotyping has identified regional brain volume differences in models of prenatal alcohol exposure (O'Leary-Moore et al. 2010), ASD (Ellegood et al., 2010, Ellegood et al., 2011, Horev et al., 2011, Ellegood et al., 2012, Ellegood et al., 2013) and schizophrenia (Ellegood et al., 2014b). Mouse models of ASD have been assessed using high resolution MRI including the 15q11-13$^{patdp/+}$ (Nakatani et al., 2009), BTBR T+tf/J (McFarlane et al., 2008, Ellegood et al., 2013), contactin-associated protein-like 2 (CNTNAP2) (Penagarikano et al., 2011), NLGN3-R451C knock-in (Ellegood et al., 2011), integrinβ3 (ITGβ3) -/- (Ellegood et al., 2012), SHANK3-/+ and -/- (Peca et al., 2011), NRXN1α-/- (Etherton et al., 2009), En2-/- (Brielmaier et al., 2012), Balb/C (Kumar et al., 2012), neonatal hyperoxia (Ramani et al., 2013), maternal immune activation (Fatemi et al., 2008a), and MAGE-like 2 (magel2) -/- (Mercer et al., 2009). The brains of these models are heterogeneous, with few common differences between all models in direction or magnitude. The most commonly affected areas include the striatum, cerebellar cortex, hypothalamus and parieto-temporal lobe – similar to what has been reported in clinical studies (Stanfield et al., 2008, Kurth et al., 2011). Additionally, differences in white matter volumes include the corpus callosum, cerebral peduncle and internal capsule. In an attempt to cluster a subset of these ASD models based on commonalities similar to clustering behavioural phenotypes (Ey et al., 2011), Ellegood et al., (2014) showed 3 distinct groups that appeared with regions that covary – an inferior colliculus-cerebellar group, a cortical-thalamic-striatal group and a group of limbic areas involved in autonomic and social processing

(Ellegood et al., 2014a). Thus, through structural imaging, our understanding of the relationship between risk genes and brain phenotype has been enhanced. Therefore, preclinical MRI may guide the way to more anatomically relevant studies of risk genes or early-life exposures at other levels.

Cellular Correlates of Imaging

Changes in brain volume can reflect various cellular level changes (Zatorre et al., 2012, Mills and Tamnes, 2014) and MRI is not selective enough to reveal specific neurobiology (Kooy et al., 2001, Hoyer et al., 2014). Metrics for voxel-based morphology are affected by multiple tissue properties including cell size, number, and characteristics of the cell morphology. Furthermore, these changes could be specific to neurons, glial, myelin or vasculature. Neuronal changes that may contribute to changes in the grey matter are thought to be a result of neurogenesis (Kee et al., 2007) and apoptosis (Fossati et al., 2004); although recent evidence suggests that morphologic changes such as dendritic branching (Vincenzo De et al., 2006), pruning (Lendvai (Lendvai et al., 2000, Holtmaat et al., 2005), and axonal sprouting (Lerch et al., 2011) may play a larger role. The notion of neurogenesis and apoptosis in respectively maintaining and reducing brain volume came primarily from studies of depressive patients having smaller hippocampal volumes (Huang et al., 2013). It was thought that since neurotrophic factors were decreased in preclinical models of depression and patients with depression and that these were anti-apoptotic in nature, the decreased volume would be due to a loss of cells (Fossati et al., 2004). However, more recent studies show that these changes are likely morphological, rather than being caused by the number of neurons and this is supported by the role of stress on dendritic morphology and the possibility of recovery (Czeh and Lucassen, 2007). Although neurogenesis does occur in the adult hippocampus (Song et al., 2002), the relative number is small and would not be a significant contribution to the magnitude of the volume changes reported (Aimone et al., 2009). In contrast, changes in morphology have been reported directly through pairing imaging and histology (Lerch et al., 2011, Mills and Tamnes, 2014). For example, volume changes in the hippocampus has

been shown to be due to increased axonal sprouting (measured with GAP-43) rather than cell size or number after learning in adult mice (Lerch et al., 2011). Other possible cellular correlates of differences in regional grey matter volume include changes in glial cell size and number. Glial cells outnumber neurons and are dynamic in adults (Rakic, 2002), playing a role in learning and experience (Dong and Greenough, 2004). Thus, the role of astrocytes enabling and enhancing neuronal function suggests their own plasticity would impact volume changes. Studies have shown that astrocyte number is dynamic (Haber et al., 2006) and may serve as a proxy for brain activity (Theodosis et al., 2008, Koehler et al., 2009, Wang et al., 2009, Giaume et al., 2010). Changes in astrocytic volume in rats are transient compared to increases in neuronal synapse number after learning (Kleim et al., 2007). Lastly, angiogenesis in the grey matter may contribute to changes in volume as well (Pereira et al., 2007). In white matter, changes in volume could be attributed to changes in myelination (Pujol et al., 2004) as well as axonal fibre packing density, diameter and permeability (Concha et al., 2010, Paus, 2010). For example, a study demonstrated that increases in volume in the corpus callosum after enrichment in rodents was due to astrocytic processes and axonal branching, but not changes in myelin (Markham et al., 2009). Altogether, the cellular processes underlying the changes in brain volume seen in MRI must be elucidated through other tools.

Although MRI resolution does not offer explanatory power at the cellular level, the value of identifying regional changes cannot be discarded – rather, it may guide further understanding of those processes and how they may relate to behavioural and cognitive deficits. Neuroanatomical differences in mice have been shown to relate to behavioural abnormalities in various models (Nieman et al., 2006, Lerch et al., 2011, Capossela et al., 2012, Kumar et al., 2012, Ellegood et al., 2013, Ramani et al., 2013). For example, spatial maze training changes hippocampal volume while cued training results in growth in the striatum specifically (Lerch et al., 2011) and time spent in self grooming behaviour has been associated with the volume of the globus pallidus (Ellegood et al., 2013). Associations between brain volume and behaviour will

increase our understanding of the functional significance of various brain regions in mouse models.

The Neuroanatomical Phenotype of the FMR1-KO Mouse

Neuroanatomical phenotyping on the *FMR1-KO* mouse has been performed previously in two studies. *FMR1-KO* mice on an FVB/n.129 background at P70 showed no differences in brain volume compared to WT, although clinical differences were reported (Kooy et al., 1999), however the technology available at the time did not allow for a high-resolution examination of regional brain changes. More recently, *FMR1-KO* male mice on a C57Bl/6 background at P30 had decreased cerebellar nuclei volume (fastigial nucleus and nucleus interpositus) with no differences in other regions (Ellegood et al., 2010). Given that background genetic differences in mouse strains are a differentiating factor in brain volume changes (Chen et al., 2006, Nieman et al., 2006, Spring et al., 2007, Ellegood et al., 2013), there may be gene modifiers that influence neuroanatomical differences, similar to the differences in behavioural phenotype (discussed above). For example, the BTBR strain has volume differences in cerebral white matter, cerebellum, and brainstem, among other regions, compared to C57Bl/6 and FVB strains (Ellegood et al., 2013). Therefore, in this thesis, differences in the neuroanatomical phenotype of the *FMR1-KO* on an FVB strain will be compared to previous findings.

Key Points:

- ✓ Through structural imaging, our understanding of the relationship between risk genes and brain phenotype has been enhanced
- ✓ Although MRI cannot reveal specific neurobiology, associations between brain volume and behaviour will increase our understanding of the functional significance of various regions in mouse models.
- ✓ Neuroanatomical phenotyping on the *FMR1-KO* mouse has been performed previously in two studies. My thesis will explore differences in neuroanatomical phenotype in relation to behaviour, age, and strain and compare these results to previous findings in *FMR1-KO* mice.

Developmental Trajectories

Traits in Neurodevelopmental Disorders

Uncovering the trajectory of core traits across the lifespan will allow us to better understand processes in neurodevelopmental disorders (Richler et al., 2010, Fountain et al., 2012) as well as identify time windows during which treatments and therapies can be most effective (Dawson et al., 2012, Daniels et al., 2014). In ASD and other PDDs, both neuroanatomical and behavioural profiles have identified distinct trajectories in populations on the autism spectrum (Hodapp et al., 1991, Hernandez et al., 2009, Szatmari et al., 2009, Travers et al., 2012, Dennis and Thompson, 2013, Doyle-Thomas et al., 2013, Mak-Fan et al., 2013). Longitudinal studies of children with FXS have identified different developmental trajectories of behaviour and neuroanatomical changes. For example, both autistic traits (Hernandez et al., 2009) and deficits in sensory processing (Baranek et al., 2008) are detected early in life in individuals with FXS. As early as 12 months, infants with FXS have prolonged visual attention that correlates with the severity of autistic symptoms and deficits in fine motor tasks (Roberts et al., 2012), similar to the delays in neural response seen in infants with ASD (Webb et al., 2011). The lack of inhibition in eye movements in toddlers with FXS (Scerif et al., 2005) is consistent with the lack of inhibitory control later in life (Munir et al., 1999, Cornish et al., 2000). In addition, IQ and adaptive behaviour scores decrease from 4-16 years, with IQ declining more in males than females in FXS (Fisch et al., 2010). Altogether, these studies, in recognition of developmental trajectories, demonstrate that examining phenotype across the lifespan is important.

Early Life Outcomes in Mouse Models

Recent reviews have advocated the utility of high throughput phenotyping over the lifespan in psychiatric disorders (Gould and Gottesman, 2006, Kalueff et al., 2008). At the same time, there is a dearth of studies examining behavioural phenotypes in the early life period when neurodevelopmental disorders are known to first exhibit symptoms. The studies to date have shown changes in the early life period but with no overarching framework. For instance,

developmental milestones such as grasping reflex and eye opening were shown to be delayed in NLGN2-KO mice (Wohr et al., 2013), a genetic model of ASD. In contrast, in the BTBR mouse strain, proposed to be a model of ASD, an acceleration of achieving the righting reflex milestone is reported compared to C57Bl/6 strain (Scattoni et al., 2008). Thus, clarity is needed regarding the utility of these assays and their association with biological pathways as well as with other behavioural outcomes. Furthermore, the majority of early life behaviours in mice have compared and reported differences due to genetic background strains. For example, compared to C57Bl/6 mice, BTBR mice are hyperactive at 6 weeks but not at 4 and 8 weeks (Molenhuis et al., 2014). This is in contrast to 128Sv and AJ mice which showed overall low levels of activity and self-grooming throughout the lifespan. The C58/J strain have increased activity beginning at P6 but decreased male sociability as well as increased repetitive behaviours compared to C57Bl/6 mice at 3 weeks (Ryan et al., 2010). Some behavioural measures, such as ultrasonic vocalizations, are consistently different through the first two postnatal weeks between strains (Scattoni et al., 2011). Therefore, epistatic interactions between background strains are an important part of the early life phenotype as well. Overall, there is a lack of studies examining behaviours and developmental milestones in the early life period of mouse models of neurodevelopmental disorders.

Key Points:

- ✓ Uncovering the trajectory of core traits across the lifespan will allow us to understand processes in neurodevelopmental disorders better and identify windows during which treatments and therapies can be most effective
- ✓ There is a lack of studies examining behaviours and developmental milestones in the early life period.

Chapter 2: Objective, Hypothesis, and Specific Aims

The *objective* of this thesis is to further our understanding of the etiologies of neurodevelopmental disorders using molecular, behavioural and neuroanatomical techniques in the FXS mouse. This work examines the interaction between the FMR1 gene and factors such as genetic background, age, sex and environmental exposures through various assays during the early life period.

The *central hypothesis* is that the biology and phenotype of the FXS mouse model is shared across neurodevelopmental disorders. This hypothesis is addressed by the following four specific aims.

1) To examine changes in ultrasonic vocalizations in *FMR1-KO* mice in early life. The working hypothesis for this aim is that the absence of the FMR1 gene will result in an early life social communication deficit.

2) To examine behavioural outcomes in the first four weeks of life in *FMR1-KO* mice exposed to early-life stressors. The working hypothesis for this aim is that the absence of the FMR1 gene will result in changes in early life behavioural outcomes in a manner that is sex-specific and influenced by postnatal adverse experience.

3) To examine the gene expression of the neuroligins and neurexins in *FMR1-KO* mice. The working hypothesis for this aim is that the absence of the FMR1 gene will modify the expression of ASD susceptibility genes in the brain.

4) To examine brain volume differences in *FMR1-KO* male mice on a FVB background strain compared to WT FVB mice. The working hypothesis for this aim is that the absence of the FMR1 gene will result in regional differences in brain volume.

Chapter 3: Temporal and Spectral Differences in the Ultrasonic Vocalizations of Fragile X Knock Out Mice During Postnatal Development

Chapter Link

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There are very few behavioural assays in the early life period of a mouse related to ASDs. Collaboration between Dr. Jane A. Foster and Dr. Paul A. Faure had previously shown differences in USV calls in the *in utero* VPA and poly(I:C) challenge mouse models of ASD. Differences in the number of calls were detected at P10 but not at P7 and P14 in both models. In contrast, other studies (reviewed in this chapter) have shown that P7 was the age at which the numbers of calls peaked in other mouse models of ASD. Therefore, the following study was performed in this continual collaboration to see if USVs were affected in *FMR1-KO* mice and at which age calls would be influenced.

Temporal and Spectral Differences in the Ultrasonic Vocalizations of Fragile X Knock Out Mice During Postnatal Development

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Abstract

The *fmr1* knock out (KO) mouse has been a useful animal model to understand pathology and treatment of FXS, both anatomically and behaviourally. Ultrasonic vocalizations (USVs) are a behavioural tool to assess early life communication deficits in mice. Here, we report on the temporal and spectral features of USVs emitted after maternal separation in wild type (FVB/N) and *fmr1 KO* pups at postnatal days (P) P4, P7 and P10. The results show changes in the number and duration of calls in *fmr1 KO* pups and wild type pups were dependent on age and call type. *Fmr1 KO* pups showed an increased number of USVs at P7 but not at P4 or P10. This increase was specific to Frequency Jump calls. In addition, *fmr1* KO mice showed a developmental shift in the temporal distribution of calls, with P10 mice calling in distinct bout patterns. Overall, these findings provide evidence that changes in USV outcomes were specific to certain call types and ages in *fmr1 KO* mice. Because early postnatal life is a window during which multiple neural systems activate and become established, behavioural measures such as using USVs as a measure of communication, may be useful as a predictor of brain changes and later developmental behavioural changes. Work is needed to better understand the functional outcomes of altered development of USVs and how these changes contribute to later emergence of autistic-like behaviours in animal models of autism.

1. Introduction

Fragile X Syndrome (FXS) is a human neurodevelopmental disorder affecting approximately 1 in 7000 males and 1 in 11,000 females [1-4]. To date, this disorder is the most common single gene cause of autism [5, 6]. Between 40 and 60% of patients with FXS meet DSM-IV criteria for autism spectrum disorders (ASDs); 21% have autism, and up to 90% present with autistic symptoms [7-10]. The overlapping diagnoses lie in the shared behavioural traits of impairments in social communication and cognitive deficits. Specific initial symptoms of children with FXS are similar to autism and include poor eye contact, hand flapping, and social deficits [11-13]; shared symptoms that manifest late in life include shyness, social avoidance, anxiety, hyperactivity, inattention, impulsivity, tactile defensiveness, self-injurious behaviour, aggression, irritability, and inflexible decision making processes [8, 9, 14]. Fragile X mental retardation 1 knockout mice (*fmr1 KO)* are used to investigate mechanisms of disease associated with loss of the fragile X mental retardation protein (FMRP) [15-17]. Behavioural and phenotypic manifestations of FXS in humans are well represented [18-23]. *Fmr1* KO mice demonstrate transient synaptic changes during postnatal development [20, 24-26]. Functional and morphological changes occur in various brain regions, in different neurons and/or neural compartments over different time windows [26-31]. For example, in the cortex, there is increased plasticity of thalamic afferents to cortical layer IV pyramidal neurons at P7, the typical end of a critical period of plasticity at this synapse, but not at P14 and P21 [29]. The projections from layer IV to layer II/III, however, have increased plasticity at P14, but not P21 [27]. In addition, the lack of FMRP modulates inhibitory projections from layer IV specifically to fast-spiking interneurons at P14 but not P28 [28]. Transient changes in cortical spine morphology reflect this laminar nature as well. Spines in layer II/III are thinner and less mature only at P14 but not P7 or P21 [25, 26]. In layer V, spines are changed at P7 and P14 [30], normal at P28 [30, 31] and changed again at P75 [31]. Therefore, *fmr1 KO* mice are useful in understanding the effects of FMR1 gene silencing on development.

Behavioural outcomes in mouse models are useful to examine neurodevelopmental disorders ([32-35]; reviewed in [36, 37]). Ultrasonic vocalizations (USVs) are communication sounds emitted by mouse pups in the first two weeks of life in response to maternal separation [38-41]. Changes in USV production are a key communication-related health outcome measure in early life [42-44], and differences have been detected in multiple mouse models of neurodevelopmental disorders (see Table 1). Several studies have examined USVs across multiple time points during postnatal development [45-54] by comparing temporal characteristics such as call number and duration [55] and the pattern of call production. For example, compared to single calls, bouts of calling are more frequent within a 2 min test in the first postnatal week of development [56]. By the end of the second week, pups emit very few USVs. Strain differences in the number of calls emitted have also been reported [57]. The most common outcome measures include call number and call duration. Frequency (spectral) differences have also been reported [46-48, 51, 58-65] and in a small number of reports calls have been categorized based on their spectral pattern [51, 59, 64, 66, 67].

In this study, we recorded USVs emitted after maternal separation in wild type (WT-FVB/N) and *fmr1* KO pups at postnatal (P) days 4, 7 and 10 and conducted a detailed temporal and spectral analysis to characterize how USV production changes in *fmr1 KO* and WT mice during development.

2. Methods

2.1. Animals and Housing

Wild type FVB/N (WT) and FVB/N/*fmr1* (*fmr1 KO*) mice were housed and bred in McMaster University's Central Animal Facility. All procedures were in accordance with the guide to the care and use of experimental animals by the Canadian Council on Animal Care, and were approved by the Animal Research Ethics Board of McMaster University.

2.2. Experimental Setup and Recording Procedure

Sounds emitted by P4, P7, and P10 pups were recorded during 3 min of maternal separation between 0800 and 1130 h in a procedure room whose temperature (21°C) was similar to that of the mouse holding room. For each litter, the dam was removed from the home cage 10 min prior to the start of pup recordings. The home cage with the isolated litter rested on a heating pad set to 37°C throughout testing. Individual pups were placed into the center of a 34 x 29 x 15 cm polypropylene chamber whose walls were lined with 5 cm thick acoustic foam (Sonex COC-2, Acoustical Solutions, Inc.). A towel was placed on the bottom of the chamber to reduce the amplitude of scratching noises produced during pup movement. The towel was changed between recordings of different litters to prevent odor transfer between pups.

Pup USVs were recorded with a CM 16 condensor microphone connected to a UltraSoundGate 116 digitizer (Avisoft Bioacoustics, Berlin, Germany) and monitored with a laptop computer running Avisoft Recorder. The microphone was clamped to a retort stand and situated 17.5 cm above the center of the recording chamber.

2.3. Data Analysis

Recordings were analyzed with Avisoft Sound Analysis and Synthesis Laboratory Professional software (SASLab Pro v 5.1.20). For each comparison group, the calls from up to five pups per litter were analyzed. In the WT group, there were 15 pups from 3 litters at P4, 21 pups from 5 litters at P7, and 18 pups from 4 litters at P10. In the *fmr1 KO* group, there were 23 pups from 5 litters at P4, 17 pups from 5 litters at P7, and 8 pups from 2 litters at P10. Calls were identified and classified by an individual observer whose was blind to treatment group. The onset and offset of each call was labeled automatically, although manual labels were added when needed. Call duration was calculated as the difference between the onset and offset times, whereas the intercall interval (ICI) was calculated as the difference in time between the onset of consecutive calls.

In addition to the above temporal outcome measures, USVs were classified spectrally into six categories of subtypes first described by Branchi et al. [68] (see Table 2 for a comparison with other research groups). These USV subtypes were defined by variation in frequency or bandwidth, and by their frequency modulation patterns. The subtypes we recorded included: composite (C), quasi-constant (QC) frequency, frequency jump (FJ), frequency modulated (FM), frequency jump plus composite (FJ+C), and short (S) call (Fig. 1).

Bouts of calling were identified based on an age-dependent minimum value in the distribution of the natural log of the ICI [67]. This minimum ICI value was considered a threshold, with bouts of calling to the left of this threshold and single USV calls to the right.

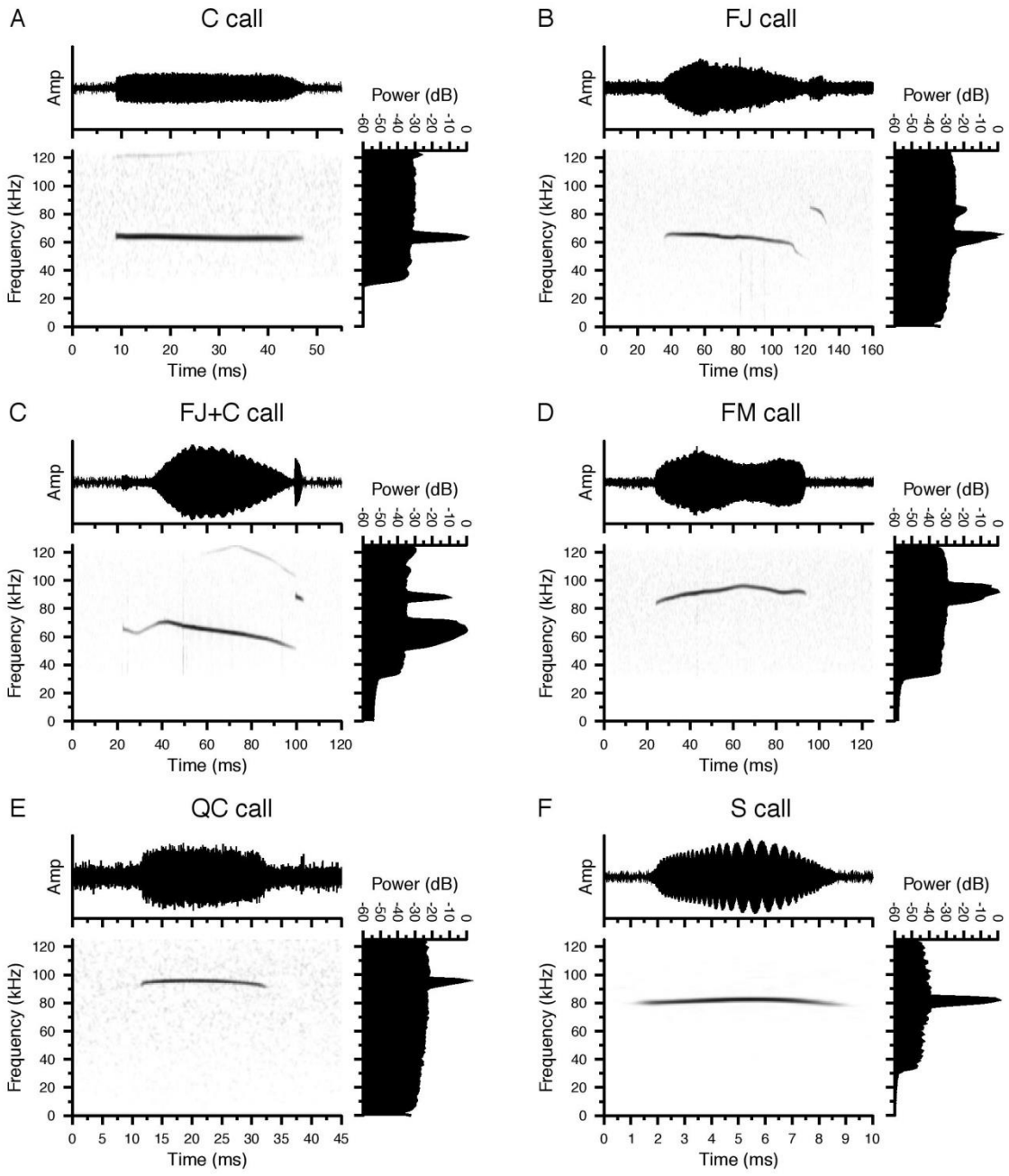

Figure 3-1. Categories of USV call types defined by the variation in frequency range, bandwidth, frequency modulation (FM) pattern and call duration. (A) Composite (C) – pure tone calls with overtones (harmonics) at integer multiples of the fundamental frequency; (B) frequency jump (FJ) – calls with an instantaneous discontinuity in frequency (i.e. a gap) that occurs within <0.1 ms; (C) frequency jump plus composite (FJ+C) – a combination of C and FJ calls with overlapping

frequencies and a frequency gap <0.1 ms, (D) frequency modulated (FM) – continuous calls with a change in spectral bandwidth >10 kHz; (E) quasi-constant (QC) frequency – continuous calls with a change in spectral bandwidth ≤10 kHz; (F) short (S) – punctuated vocalizations with a duration <10 ms.

2.4. Statistics

Data analyses were performed in GraphPad Prism (v5b, GraphPad Software, San Diego, CA). Differences between treatment groups were detected with a two-way analysis of variance (ANOVA) with genotype and age as main factors followed by Bonferroni's post-hoc tests. A p-value of <0.05 was considered significant. All data are expressed as the mean ± standard error of the mean (SEM).

3. Results

3.1. Total Call Number and Call Duration

We used a detailed temporal and spectral analysis to characterize the USVs emitted by WT and *fmr1 KO* mice at P4, P7 and P10. First, we first calculated the total number of calls, average call duration and average ICI per pup. There was a significant effect of age on the mean number of calls per pup ($F[2,98] = 4.48$, $p=0.014$), and post-hoc testing revealed an increase at P7 compared to P4 and P10 in *fmr1 KO* pups (Fig. 2A). While there was no main effect of genotype ($p>0.05$), there was a significant interaction between age and genotype on number of calls emitted ($F[2,98] = 3.26$, $p=0.04$), and post-hoc tests revealed that the number of calls emitted by *fmr1 KO* mice on P7 was higher than by WT mice (Fig. 2A). There was no effect of genotype on call duration ($p>0.05$); however, there was a significant effect of age ($F[2,94] = 16.6$, $p<0.0001$) and post-hoc analyses revealed an increase in call duration at P7 compared to P4 and P10 in both WT and *fmr1 KO* pups (Fig. 2B). There was no effect of genotype or age on ICI ($p>0.05$).

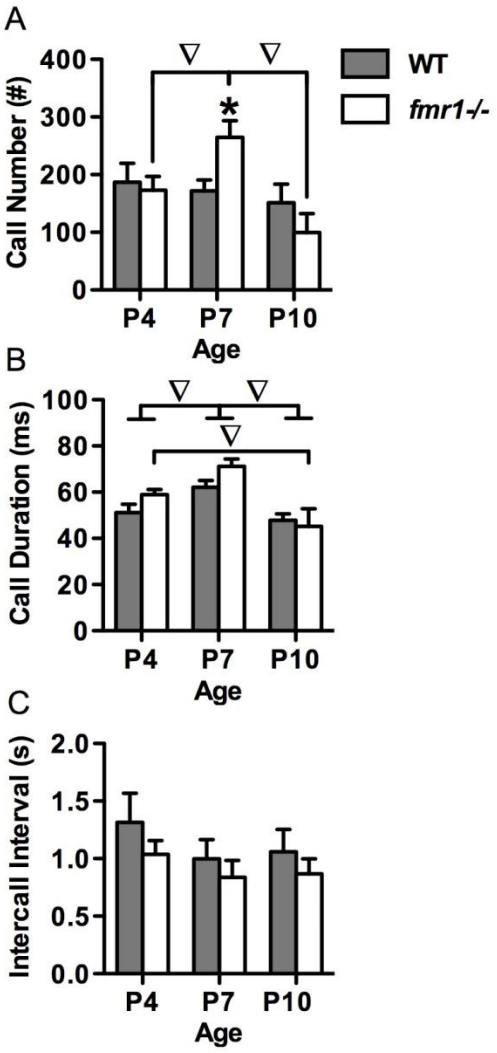

Figure 3-2. Temporal parameters of USV call production in WT and *fmr1 KO* pups. Shown are the mean ± SEM of the (A) number of calls, (B) call duration, and (C) intercall interval per group at P4, P7 and P10. * indicates genotype-related differences and ∇ indicates age-related differences between P7 and both P4 and P10; between P4 and P10; and between P7 and P10.

3.2. Temporal Characteristics of Calls

We plotted the number of calls detected in each 30 s time bin over the 3 min recording period to obtain a more detailed picture of the temporal spacing of USV emissions. Consistent with the increase in total call number in *fmr1 KO* at P7 (Fig. 2A), there was a significant effect of genotype on the distribution of calls at P7 ($F[1,210] = 14.42$, $p=0.0002$) (Fig. 3). Post-hoc tests revealed an increase in the number of calls by *fmr1 KO* mice at P7 was specific to the initial 30 s (Fig. 3B).

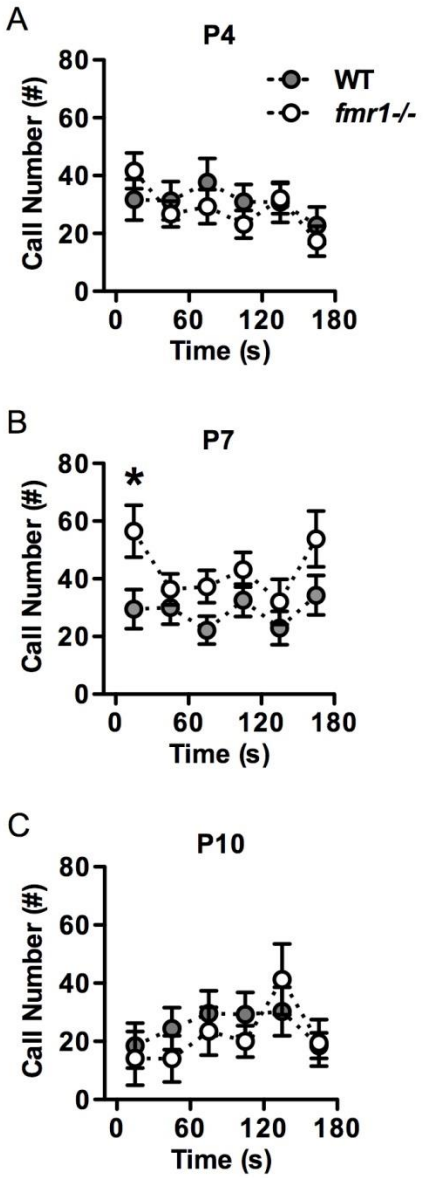

Figure 3-3. Detailed analysis on number of USVs emitted by WT and *fmr1 KO* pups at postnatal days (A) P4, (B) P7 and (C) P10. Each panel shows the mean ± SE number of calls per 30 s interval during 3 min recording. There was a significant effect (* $p<0.05$) of genotype on the number of USVs, with *fmr1 KO* mice emitting more calls than WT mice during the initial 30 s of maternal separation at P7 (B).

3.3. Spectral Characteristics of Calls

After examining temporal changes in USV emissions between WT and *fmr1 KO* mice, we looked at changes in the use of different call types. Calls were categorized based on the definitions set out in Table 2 and shown in Figure 1. First, we compared the number of calls per USV call type between WT and *fmr1 KO* mice (Fig. 4A-C), and found that there was an effect of genotype at P7 ($F[1,210] = 10.93$, $p=0.0011$). Post-hoc tests revealed that this difference at P7 was specific to an increase of the number of FJ calls emitted by *fmr1 KO* mice (Fig. 4B). When we examined the distribution of FJ calls in 30 s time bins (data not shown), we found there was an increase in FJ calls during the first 30 s bin but then a decrease in FJ calls in the 90-120 s bin in *fmr1 KO* mice (main effect of genotype: $F[1,160] = 6.572$, $p=0.0153$). This finding corroborates our previous result where we found an increase in the total number of USVs emitted during the first 30 s in *fmr1 KO* but not WT mice at P7 (see Fig. 3B).

We then examined the effect of genotype and age on the duration of each call type. There was a main effect of genotype on the average USV duration of specific call types at P4 ($F[1, 217] = 5.187$, $p=0.0237$; Fig. 4). Post hoc tests revealed that this effect was specific to a significant increase in the duration of FJ calls in the *fmr1 KO* group at P4 (Fig. 4D).

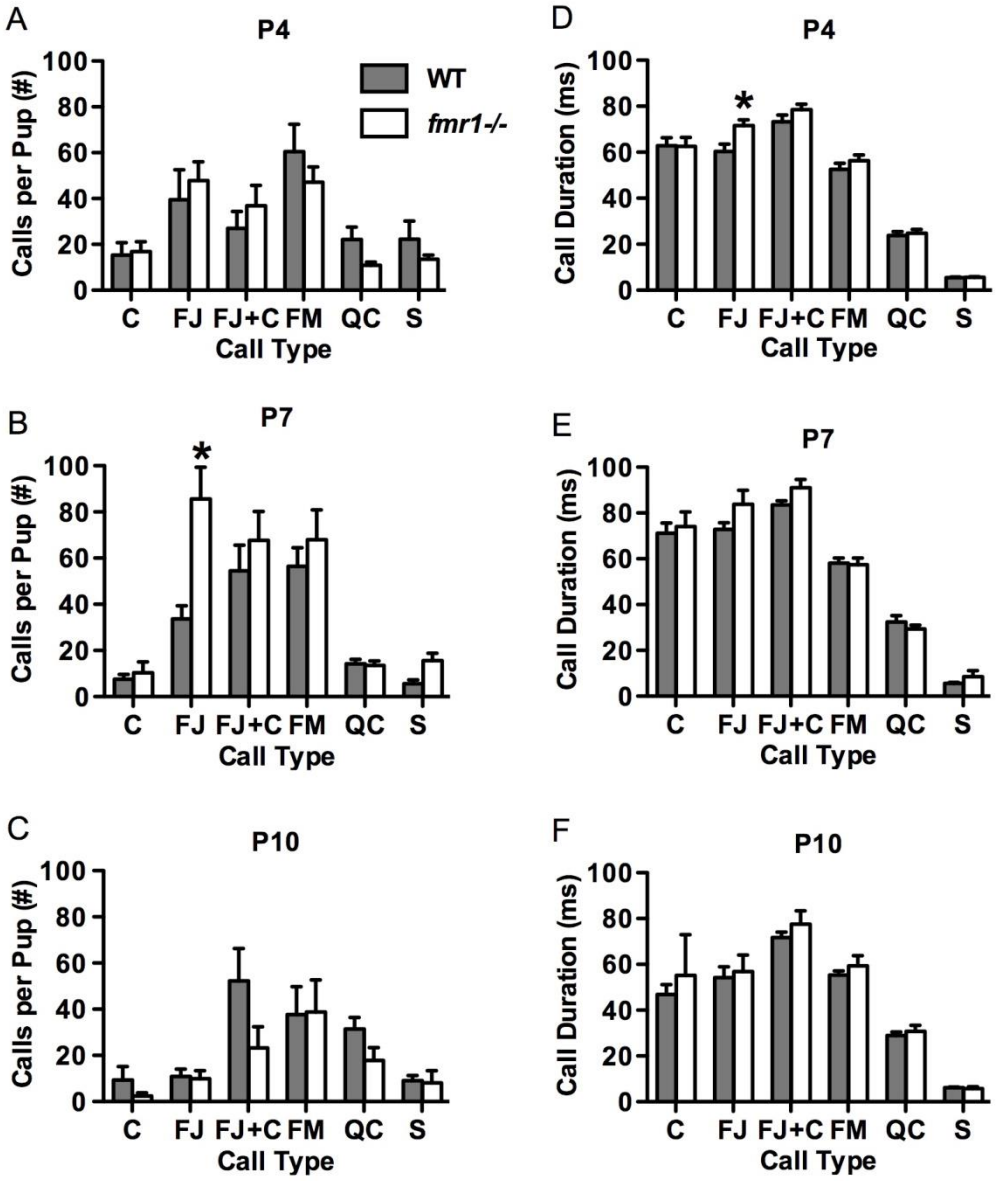

Figure 3-4. Distribution of call number and duration of USVs classified by call type. There was no effect of genotype on call number at P4 (A) or P10 (C), but there was a significant effect of genotype on the number of calls at P7 (B) that was specific to an increase in the number of FJ calls in *fmr1 KO* mice. The mean duration of FJ calls at P4 (D) also increased in *fmr1 KO* mice. No differences were detected at the other ages in call number (P4-A, P10-C) or duration (P4-E, P10-F).

3.3. Characteristics of Bout Calls

With regard to the temporal patterning of USV emissions, we noticed some pups emitted calls in bouts of quick succession with short ICIs, while others emitted calls that were more temporally spaced (i.e. single USVs) during recording and analysis. To test for temporal patterning differences in call emissions, we constructed a histogram and plotted the natural logarithm of ICI in our recordings. The distribution of ICIs was bimodal at P10, but not P4 and P7, and contained a local minimum that has been previously described [67] (Fig. 5A-C). This minimum was used as a threshold to separate calls with short ICIs that were emitted within a 'bout' *versus* 'single' call emissions with longer ICI values. No threshold values were identified in the P4 and P7 datasets; thus, bouts and single calls could not be systematically distinguished. In the P10 dataset, there were no effects of genotype on the number of bouts, bout duration, number of calls per bout, and bout rate at P10 (data not shown). We then examined the number of calls per bout and the number of single calls as a function of USV call type. There was no effect of genotype on the number of calls in a bout or the number of single calls of each call type. However, the proportion of bout calls and single calls as a function of call type were different in WT pups (interaction effect: $F[1,204] = 18.47$, $p = 0.0001$; Fig. 5D) and post-hoc tests revealed that the bouts had a higher proportion of FJ+C calls and a lower proportion of QC calls compared to single calls). These differences in the call type profile of bout calls and single calls were not present in the *fmr1 KO* pups (Fig. 5E).

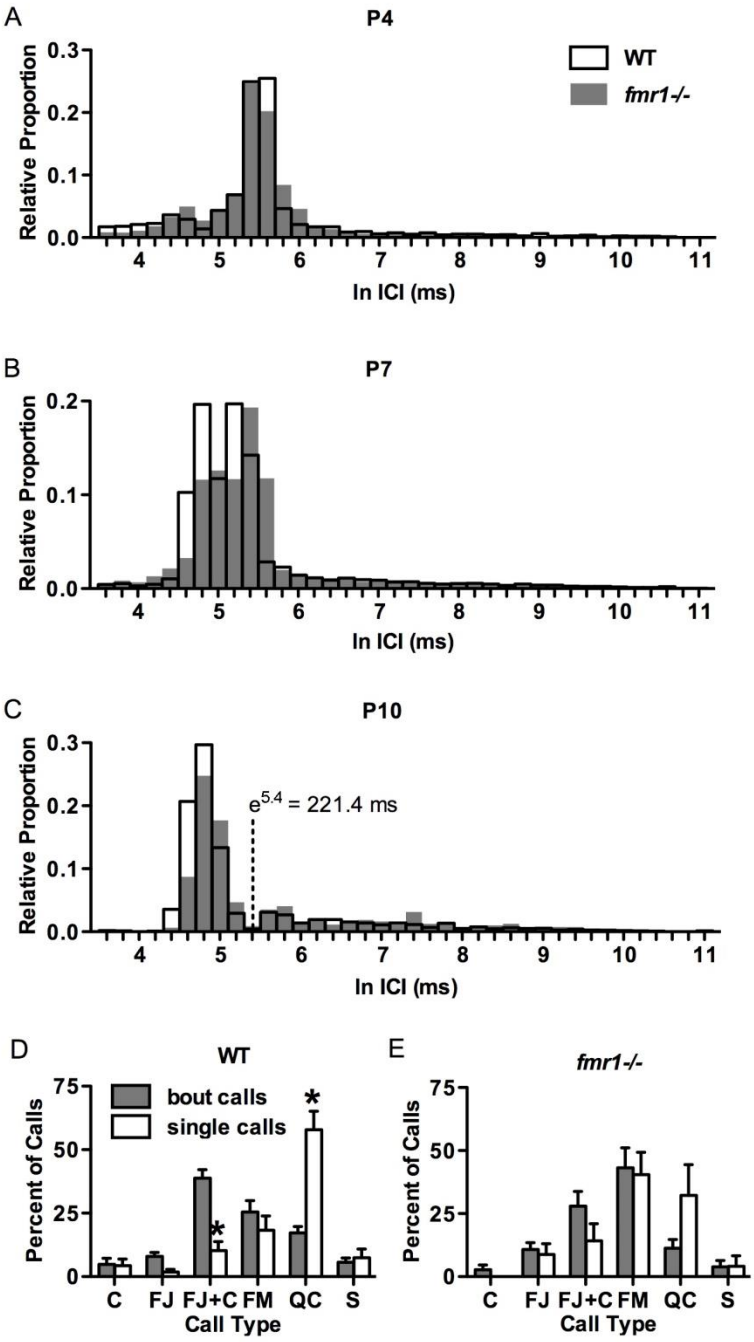

Figure 3-5. Temporal patterning of calling in WT and fmr1 KO pups. Panels A-C shows the natural logarithm of the ICI distribution at P4 (A), P7 (B) and P10 (C). An ICI threshold for distinguishing calling bouts *vs* single calls was identified at P10 (C) and is indicated by a dotted

vertical line. The threshold was similar in WT and *fmr1 KO* pups. There was no effect of genotype on the number of bouts, bout duration, number of calls per bout, and bout rate (data not shown). The profile of call types in bout calls and single calls were different in WT pups, where bouts had a higher proportion of FJ+C calls and a lower proportion of QC calls compared to single calls (D). These differences in the call type profile of bout calls and single calls were not present in the *fmr1 KO* pups (E).

4. Discussion

4.1 Summary of Results

In our study, *fmr1 KO* pups exhibited both temporal and spectral changes in USV call production compared to WT mice. We observed that *fmr1 KO* pups emitted more calls on P7, particularly FJ calls. In addition, *fmr1* KO mice showed a developmental shift in the temporal distribution of calls, with P10 mice calling in distinct bout patterns. This study provides a more nuanced understanding of USVs based on strain and background genetic differences as well as specific call types. We have shown for the first time, a developmental trajectory of the temporal characteristics of calls in which burst patterns appear and are a distinct population of calls.

4.2 Methodological Contrasts to a Recent Study

Our results are in contrast to a recent study by Roy et al. (2012) that showed no effect of the *fmr1 KO* genotype on call number or USV duration [59]. Differences between the two studies include the age and background strain of the pups tested; we recorded FVB/N strain pups at P4, P7, P10 whereas Roy et al. (2012) examined C67Bl/6 strain pups from P8. Our analysis found that the main difference in emitted USV types was an increase in the number of FJ calls, whereas Roy et al. (2012) reported a decrease in the relative proportion of downward FM calls but no differences in call number [59]. In addition to differences in defining call type categories, we used the number of calls in a fixed time window (3 min) rather than the relative proportion of call types. Finally, in our study we removed the dam and placed the litter on a heating pad for 10 minutes prior to recording whereas Roy et al. (2012) recorded USVs immediately following separation from the dam.

4.3 Effect of Genotype on Number of Calls at P7

In our study, *fmr1 KO* pups called more than WT pups at P7. This is in contrast to a majority of autism mouse model studies that have reported decreases in the number of USV emissions. For example, decrease in call number have been reported at P8 in knockout mice for:

the serotonin receptor-1A (*Htr1a*) [45], the mu-opioid receptor (*Oprm1*) [69], oxytocin (*Oxt*) [62] and the oxytocin receptor (*Oxtr*) [70], *Shank1* [65] and the neuroligin-3 mutant (*Nlgn3-R451C*) [71]. Some studies that have looked at multiple developmental ages have reported a decrease in the number of calls. For example, *Foxp2*-/- mice show reduced call numbers at both P6 and P10 [49], and *Oprm1*-/- mice have consistently fewer calls from P4 to P12 [69]. In contrast, the 15q12 deletion mouse model, which results in a knockout of *Ube3a* (ubiquitin protein ligase E3A), *Gabrb3* (GABA-A receptor beta 3) and *Atp10a* (ATPase-10A), all show reduced calling at P6 but increased USV emissions at P12 and no differences at P8 and P10 [54]. In our study, we observe a similar trajectory pattern between WT and *fmr1 KO* pups with an increase in the total number of calls at certain ages (P7) but not others (P4 and P10). A few studies have reported an increase in call number in autism mouse models. Pups with a paternal duplication of 15q11-13 emitted more calls on P7 and P14 [48]. The multiple autistic-like trait transgenic (*MALTT*) mouse showed increased USV production during its second week of postnatal life. Interestingly, this change in production was accompanied by a decrease in average call duration [50].

An increase in USV call number in early life could be indicative of increased stress reactivity or disrupted social communication. Increased USV call number in early life has been linked to increased anxiety-like behaviour as adults using the open field and elevated plus maze in rat [72]. In addition, administration of benzodiazepines (anxiolytics) in P7 mice resulted in decreased USV call production [73]. These data suggest that USVs could be a readout for early-life anxiety. However, there is a need for studies to identify the utility of USVs and specific calls types in their relationship to other social and stress-related phenotypes in mice. This would be particularly informative as FMR1 mice have decreased generalized anxiety-like behaviour in the open field and elevated zero maze [74, 75] but may have increased social anxiety in certain conditions [19, 74, 76-78].

Table 3-1. Differences in Ultrasonic Vocalizations in Genetic Mouse Models of ASDs

Model	Strain	USV Finding	Age	Call Types	Ref.
Htr1a-/-	C57BL6	reduced call number at P8; reduced call duration at P4; reduced call duration of +/- pups with maternal transmitted gene at P12	4, 8, 12	N	[45]
Asic3+/-	CD-1	no differences were detected	1-3	N	[95]
Avpr1b+/-, -/-	C57BL/6. 129/SvJ	no differences were detected upon separation; increased call number was not present upon potentiation (second separation) at P9	3, 6, 9, 12	N	[96]
Cadm1-/-	C57BL/6	decreased number of whistle/pure tone calls with a corresponding increase in click-type calls	8	N	[97]
Cacna1c-G406R	C57BL/6	shorter call duration at P6, 8, and 10; no differences in call number, peak frequency or peak amplitudes	2, 4, 6, 8, 10, 12	N	[47]
15q11-13 patDp/+	C57BL/6	patDp/+ pups emitted more calls on P7 and P14 and had calls over 70kHZ	5, 7, 14, 21	N	[48]
Fgf17-/-	C57BL/6	decreased calls in both +/- and -/-, -/- emitting the lowest number of calls	8	N	[89]
FMR1-KO	B6.129	reduced number of downward calls; frequency range was increased in all of the 4 types of frequency modulated calls; higher frequency of flat calls; no genotype effect on duration of calls for any call subtype	8	Y	[59]
FMR1-KO	FVB	decreased calling rate; no differences in duration, average frequency or range	3-4 months	N	[60]
Foxp2-/-	C57BL/6	reduced number of calls at P6 during the 6 min of recording; significantly reduced number of whistles and clicks at P10	6, 10	N	[49]
MALTT	FVB	increased call number in males on P8-11, 13; 4 day delay in males, 2 day delay in females;more calls with shorter duration	3-14	N	[50]
Mecp2-KO	mixed BALB/C.129. C57BL/6	MeCP2y/- exhibited an increase in call number at P5 and +/- (females) had increased call number at P7	3, 4, 5, 6, 7	N	[52]
Ext1C-/-	C57BL/6	reduced call number; call duration and peak amplitude	not stated	N	[79]
Nlgn2-/-	C57BL/6.129S6/SvEv Tac.129S2/SvPasCrlf	reduced call repetition rate; no genotype differences in latency to call, call duration or peak frequency	7	N	[61]
Nlgn3-/-	C57BL/6	decreased call number and latency to first call	14-17 weeks	N	[98]
Nlgn3-R451C	C57BL/6	decreased number of calls at P8	4, 6, 8, 11	N	[71]

Nlgn4-/-	C57BL/6	increased latency to call, reduced call number	3 months	N	[99]
Nlgn4-/-	C57BL/6	no differences were detected	2, 4, 6, 8, 10, 12	Y	[66]
Oprm1-/-	C57BL/6	fewer calls at P4, P8 and P12 after isolation; decreased number of calls in response to coldness, male's cues and other social stimuli at P8	4, 8, 12	N	[69]
Oxt-/-	C57BL/6	decreased call number and calls of higher frequencies	7-8	N	[62]
Oxtr-/-	C57BL/6	decreased call number with increased levels of locomotor activity during the test	7	N	[70]
CD38-/-	ICR	decreased call number; no change in frequency or duration	7	N	[63]
Relnrl	B6C3Fe	-/- emitted less calls than +/- or +/+	7	N	[100]
Shank1-/-	C57BL/6.129	reduced call number, total call time; a higher frequency of calls; no difference in the latency to first call, call duration or peak amplitude	8	N	[65]
BTBR T+tf/J	C57BL/6, FVB, 129	increased call number on P2, 4, 6, 8 than 3 other strains (B6, 129x1 and FVB); longer call duration within the first two weeks than B6 but shorter compared to FVB from P2-P8; lower frequency at P6 and P8; calls from BTBR and B6 pups were lower in amplitude than FVB and 129X1 on P2	2, 4, 6, 8, 12	Y	[51]
Tbx1+/-	C57BL/6	reduced number of complex, two-syllable, composite, frequency steps and flat calls; shorter duration in harmonics, two-syllable, composite and frequency steps calls	7-8	Y	[64]
Tsc2+/-	C57BL/6	Increased call number and duration; no differences in latency to call; increase proportion of multi-component calls	10	Y	[67]
15q12 deletion	C57BL/6	decreased call number on P6 and increased on P12	6, 8, 10, 12	N	[54]

Abbreviations: *Htr1a*, 5-hydroxytryptamine (serotonin) receptor 1A, G protein-coupled; *Asic3*, acid-sensing (proton-gated) ion channel 3; *Avpr1b*, arginine vasopressin receptor 1B; *Cadm1*, cell adhesion molecule 1; *Cacna1c*, calcium channel, voltage-dependent, L type, alpha 1C subunit; patDp, paternal duplication; *Fgf17*, fibroblast growth factor 17; *Fmr1*, fragile X mental retardation 1; *Foxp2*, forkhead box P2; MALTT, multiple autistic-like trait transgenic (formerly OVE876B); *Mecp2*,methyl CpG binding protein 2 (Rett syndrome); *Ext1*, exostosin 1; *Nlgn2*, neuroligin 2; *Nlgn3*, neuroligin 3; *Nlgn4*, neuroligin 4; Orpm, opioid receptor, mu 1; *Oxt*, oxytocin/neurophysin I prepropeptide; *Oxtr*, oxytocin receptor; *CD38*, CD38 molecule; Reln^{rl}, reelin; *Shank1*, SH3 and multiple ankyrin repeat domains 1; *Tbx1*, T-box 1; *Tsc2*, tuberous sclerosis 2.

4.4. No Effect of Genotype on the Duration of Calls

Our study did not observe an overall change in call duration in *fmr1* KO compared to WT mice—a finding that agrees with the majority of studies employing animal models of autism [61, 63, 65, 66]. A few mouse models exhibit decreases in USV duration—the *Cacna1c-G406R* [47] , MALTT [50], *Htr1a-/-* [45] and *Ext1C-/-* [79]—and in the latter two instances the reduction in call duration was accompanied by a reduction in total number of calls. In contrast, mice heterozygous for tuberous sclerosis-2 (*Tsc2*+/-) showed an increase in both call number and call duration [67].

4.5 Effect of Genotype Specifically on the Calls in the First 30 Seconds

The increase in call number observed in *fmr1 KO* mice at P7 was specific to the first 30 s of recording. It is well-established that USVs serve a communicative function [80] by eliciting maternal responses such as searching, nest building, licking, feeding and retrieval behaviours [39, 44, 81, 82], and the latency and effectiveness of such behaviours may influence the temporal distritbution of calling by the pups. For example, in contrast to our results, *Shank1-/-* mice show an increase in call number but only during the last 120 s of a 5 minute recording [65]. What is unclear to date is the relationship between pup calls and maternal responsiveness; therefore future studies should examine the maternal responsiveness to recorded calls from various genetic models to determine if differences in pup calling over time actually influence maternal behaviour.

4.6 Effect of Genotype on Call Types

We observe specific changes in the emission of certain USV types as a function of age and genotype. Overall, there was a shift in the profile of specific calls types, both in call number and duration. The increase in total number of calls in *fmr1 KO* mice at P7 was primarily due to an increase in FJ calls. FJ calls were also longer in duration in *fmr1 KO* compared to WT mice at P4. Other studies have classified calls with a larger number of call type categories than we employed, and Table 2 provides an overview of how these different classification systems compare [66-68,

83]. Reported changes in *BTBR T+tf/J* mice included an increased number of harmonic, two-syllable, C calls and a decreased number of FJ and upward FM calls [83]. In the *Tbx1*+/- strain, pups decreased the number of complex, two-syllable C calls, frequency steps, and the number of flat calls. Shorter duration harmonics on the two-syllable C calls with frequency steps were also observed [64]. These mice also exhibited decreased social interactions. At least one study reported an increased proportion of multi-component calls in *Tsc2*+/- pups [67]. When using call type categories that differentiate between pure tone and the broadband noisy components of USVs, there were no differences in the number of different call types in *Nlgn2*-/- mice [66].

Table 3-2. Comparison of Categories of Ultrasonic Vocalizations Call Types Used in Neonatal Mice Studies

<table>
<tr><th colspan="2">Current Study</th><th colspan="2">Scattoni et al., 2008 [83]</th><th colspan="2">Ey et al., 2012 [66]</th><th>Young et al., 2010 [67]</th></tr>
<tr><th>Call Type</th><th>Description</th><th>Call Type</th><th>Description</th><th>Call Type</th><th>Description</th><th>Call Type</th></tr>
<tr><td rowspan="2">Frequency Jump (FJ)</td><td rowspan="2">a gap in frequency with a time delay <0.1 ms</td><td>Two-syllable</td><td>a main call with a punctuated component towards the end</td><td>One Frequency Jump</td><td>two frequency components with a jump in frequency</td><td>Two-syllable</td></tr>
<tr><td>Frequency Step</td><td>instantaneous frequency changes appearing as a vertically discontinuous “step” with no interruption in time</td><td>Multiple Frequency Jumps</td><td>multiple frequency components with more than one jump in frequency</td><td>Frequency Step</td></tr>
<tr><td>Composite (C)</td><td>multiple frequencies at the same time</td><td>Composite</td><td>two harmonically independent components, emitted simultaneously</td><td rowspan="2">Complex</td><td rowspan="2">multiple frequency components</td><td>Composite</td></tr>
<tr><td rowspan="2">Frequency Jump and Composite (FJ+C)</td><td rowspan="2">a combination of overlapping frequencies and gaps in frequency</td><td rowspan="2">Harmonic</td><td rowspan="2">one main complex call surrounded by additional calls of different frequencies</td><td>Single Harmonic</td></tr>
<tr><td>Mixed</td><td>pure tone call with a noisy component</td><td rowspan="2">Harmonic Steps</td></tr>
<tr><td rowspan="3">Frequency Modulated (FM)</td><td rowspan="3">continuous with a bandwidth of >10 KHz</td><td>Upward</td><td>a continuous increase in pitch ≥12.5 kHz and >6.25 kHz difference from start to finish</td><td>Other</td><td>miscellaneous</td></tr>
<tr><td>Upward</td><td>a continuous increase in frequency with a range >6.25 kHz</td><td>Upward</td></tr>
<tr><td>Downward</td><td>a continuous decrease in pitch ≥12.5 kHz and >6.25 kHz difference from start to finish</td><td>Downward</td><td>a continuous decrease in frequency with a range >6.25 kHz</td><td>Downward</td></tr>
<tr><td>Chevron</td><td>an ‘inverted-U’; a continuous increase in pitch ≥12.5 kHz followed by a decrease that was ≥6.25 kHz</td><td>Modulated</td><td>frequency modulations in more than one direction with a range >6.25 kHz</td><td>Chevron</td></tr>
</table>

		Complex	one syllable containing more than one directional change in pitch, each ≥6.25 kHz			**Complex**
Quasi-constant (QC)	continuous with a bandwidth ≤10 KHz	**Flat**	a constant beginning and the ending of the pitch frequency ≤3 kHz	**Flat**	>5 ms and ≤6.25 kHz	**Flat**
Short (S)	punctuated and <10ms	**Short**	punctuated and <5 ms	**Short**	<5 ms and ≤6.25 kHz	
				Unstructured	noise with no pure tone component	

As characterized in this study and others [66-68, 83], the utility of emitting different call types hinges on the existence of differential vocal meaning or categories as perceived by the dam and/or the pup; however, it has yet to be shown that dams can distinguish one vocal type from another. It is known that mice have a categorical perception boundary with respect to signal duration in a choice situation; longer calls (>30 ms) are more attracttive than shorter (<25 ms) calls [84]. As yet, there have been no studies demonstrating that mice distinguish different spectral patterns. Recently, Grimsley et al. advocated using cluster analyses based on syllable types and characterized by frequency bands and call continuity rather than discrete USV categories chosen by human observers arguing that statistically-based analyses are more effective for categorizing signals until there is strong evidence for perceptual categories [85]. Of importance to our study is understanding the biological signifance of emitting FJ calls. Future studies should conduct a systematical analysis on the perceptual meaning of calls with different spectral properties through acoustic playback experiments.

4.7 Effect of Age on Temporal Calling Patterns

In our study we distinguished different temporal patterns of vocalizations—calling in bouts *vs* single calls—and observed that appearance of distinct bout calls was developmentally regulated (Fig 5). In addition, the USVs emitted in the bouts and in single isolated calls were comprised of different call types. Our observations are in agreement with previous reports showing more frequent bouts of calling in the first week of life compared to single calls [56]. This is important because the first week of life is a window for the development of the brainstem respiratory generator [86], as well as maturation of key parts of the striatal [87] and stress [88] circuitry. In addition, a recent study by Young et al. has reported increases in the bout duration, rate and number of calls per bout in *Tcs2+/-* compared to WT mice at P10; a difference that was further potentiated when the pup was isolated for a second 5 min period [67]. Altogether, these suggest that temporal patterns convey different messages and could be impacted by both

genotype and age. Playback experiments with calling bouts will allow us to understand maternal behaviour in response to these differences.

4.8 Effect of Strain and Age on USVs

To date, several studies have shown how some temporal and spectral parameters of mouse pup USVs vary with age and strain [55, 56, 68, 83]. In our study, WT and *fmr1 KO* mice emitted USVs of longer duration at P7 than at P4 or P10. Moreover, the WT call numbers did not change over time in the FVB/N strain. Developmental trajectories of call number and signal duration are highly strain dependent. For example, one study reports that BTBR T+tf/J mice show a peak in call number at P6, where FVB/N call numbers peak at P4-6 [83]. In this study we do not observe a peak in call number in WT mice in the first week of life; in contrast, another study reported a WT developmental trajectory in call number to peak at P8 in C57/B6 mice [53]. Moreover, mixed strains can have their own developmental trajectories. Balb/c.129.B6 mice showed a flat trajectory of call number from P3-P7 [52]. In addition, a 129SvEv.B6 chimera was reported to have no difference due to age in WT mice [54]. In a mixed strain of C57BL/10J, DBA/2J,BALB/cJ, and SJL/J, call number and duration peaked at P4-6 [55]. Therefore, considering genetic difference in background strains is necessary to compare and assess the variability in mouse studies of USVs.

To bring together different studies, it is notable that similar phenotypes emerge at different times in different genetic models. For example, the neuroligin 3 mutant (Nlgn3-R451C) shows a trajectory similar to what we report with a decreased number of calls at P8 but not at P4, 6, or 11 [71]. The 15q12 deletion model has a different trajectory, with a reduced number of calls at P6, an increased number at P12, and no difference at P8 or P10 [54]. Pups with a paternal duplication of 15q11-13 emitted more calls on P7 and P14, but not on P5 [48]. Also, gene-dose could possibly interact in certain models and affect phenotype. One study showed a possible gene-dose effect, reporting less of a decrease in call number in the fibroblast growth factor 17-/- (*Fgf17*) mouse where heterozygous compared to the homozygous knockout [89].

4.9 Potential Limitations: maternal behaviour and sex

In our study, we did not control for maternal behaviour. We cannot rule out that possibility that maternal behaviour may be different in the WT and *fmr1* KO mice. Maternal treatment of the pups, and even prenatal factors such as cytokines, can affect offspring phenotype [90]. However, specific to USVs, call numbers and frequency were not affected by maternal genotype in an embryo-transfer study [91]. Furthermore, a study of *TSC2*+/- mice, there were no maternal effects on call number or temporal characteristics of the calls [67]. Specific to the FMR1 genotype, maternal behaviour has been shown to contribute to the increased locomotor activity and has an additive effect on activity along with the pup genotype [92] through a dopaminergic striatal circuit. However, other phenotypes such as startle response, prepulse inhibition and audiogenic seizures were not affected by maternal genotype. Therefore, the possibility that maternal behaviour has an influence on USVs cannot be excluded, however, based on the evidence, the outcomes of USV call number and frequency are likely to be valid.

The majority of USV studies in the literature have not examined the effect of sex (e.g. [48, 89]; some studies of sex-linked disorders have report differences [50, 52], where others collapsed the data after there were no differences detected [51]. Vieira et al (2002) examined USVs in males and females over the postnatal period in California mice and reported that sex differences were only present at P3, when USV call number peaked in that strain [93]. Hammerschmidt et al (2012) reported minor sex differences in USVs in response to stranger mouse [94]. Thus, we would predict that sex differences may exist due to the chromosomal differences, especially since our gene of interest is on the X chromosome and there would be potential gene dosage effects. Studies targeted at determined sex differences in USVs are an important next step.

4.10 Conclusions and Future Directions

Because early postnatal life is a window during which multiple neural systems activate and become established [83-85], mapping out the trajectory of USV development in different strains is crucial to understanding mechanism of action of different genes on development. In particular, knowing the time of development when certain phenotypes present can be associated with the maturation of potential neurobiological mechanisms that may shed light on how variation in genetics influences behaviour. The current data shows that loss of the *fmr1* gene resulted in less changes to USVs than reported for other genetic models of autism suggesting a minor or limited role for FMRP in the development of USVs.

With the number of studies reporting USVs during the first few weeks of life, it is clear that genotype influences the trajectory of USV call number, duration, and call types. The long-term usefulness of USV analysis to understanding brain development and how changes in brain function occur in animal models of autism is limited by the lack of data demonstrating the function of USVs to pups and to maternal behaviour. It is timely to consider experiments that utilize playback of recorded USVs to determine the functional role of these calls in maternal responsiveness. In addition, examination of the predictive value of USVs in the first week of life on the emergence of autistic-like behaviours later in development and associated brain changes will provide new insights into mechanisms of disease.

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Chapter 4: Early Life Differences in Behaviour and Neuroanatomy in Fragile X Knockout Mice

Chapter Link

In the previous chapter, it was demonstrated that there were subtle differences in USV outcomes in *FMR1-KO* mice. It is not known how differences in USVs in the early life period relate to other behavioural changes. Furthermore, the previous study did not account for sex differences which have been shown to influence behaviour outcomes. Given this, the following study was performed to examine the relationship between various behaviours in the pre-pubertal period and examine the influence of sex as well as postnatal adverse exposures. This study was done as part of a larger project (part of POND – Province of Ontario Neurodevelopmental Disorders) in collaboration with Dr. Jacob Ellegood and Dr. Jason P. Lerch to address the relationship between behavioural differences in various strains of mice and genetic mutants related to neurodevelopment in the early life period and regional brain volume changes that may associate with these differences. Overall, these data will help uncover the relationship between brain and behavioural changes reported in ASD mouse models in the early life period.

Early Life Differences in Behaviour and Neuroanatomy in Fragile X Knockout Mice

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Abstract

Fragile X Syndrome (FXS) is the most common single gene cause of ASD. Children with FXS have different developmental trajectories of behaviour and neuroanatomical changes. *FMR1-KO* mice mimic the etiology and phenotypic manifestations of FXS. However, the majority of studies have examined behaviours in adult mice. The early life developmental period is important and adverse challenges may influence the phenotypic trajectory and developmental outcomes. To date, there have been no studies examining early life developmental milestones, social and anxiety behaviours in *FMR1-KO* mice on a FVB background. Here, we examined behavioural outcomes in the first four weeks of life in *FMR1-KO* mice challenged with LPS or SAL at P3 and with or without overnight maternal separation at P9. Testing includes measures of growth and development, ultrasonic vocalizations, open field, sociability, self-grooming and social interaction. Results show early life differences in various outcomes, including pre-pubertal sexually dimorphic changes in *FMR1-KO* mice as well as interactions between genotype, sex, and treatment in behavioural outcomes. This research contributes a developmental perspective in identifying treatments for neurodevelopmental disorders, as well as understanding the relationship between various outcomes used ASD models and the impact of environmental challenges in the early life period.

Introduction

Uncovering the trajectory of core traits during early postnatal life is necessary to better understand the pathophysiology of neurodevelopmental disorders (Richler et al., 2010, Fountain et al., 2012, Anagnostou et al., 2014). Both neuroanatomical and behavioural profiles have identified distinct trajectories in populations on the autism spectrum (Hodapp et al., 1991, Hernandez et al., 2009, Szatmari et al., 2009, Travers et al., 2012, Dennis and Thompson, 2013, Doyle-Thomas et al., 2013, Mak-Fan et al., 2013), including populations with Fragile X Syndrome (FXS), the most common single gene cause of ASD (Budimirovic and Kaufmann, 2011). In children with FXS, core behavioural traits are detected early in life; including autistic traits (Hernandez et al., 2009, Roberts et al., 2009) and deficits in sensory processing (Scerif et al., 2005, Baranek et al., 2008). Other traits, such as IQ, have been reported to have sex-specific trajectories in children with FXS (Fisch et al., 2010). These studies demonstrate that research examining traits in the early life period is needed to advance our understanding of neurodevelopmental disorders.

Developmental milestones and the emergence of behavioural phenotypes in early life have been studied in wild type mice (Fox, 1965, Kodama, 1993) as well as various genetic (Tremml et al., 1998, Scattoni et al., 2008, Wohr et al., 2011a, Wohr et al., 2011b, Molenhuis et al., 2014) and environmental models of neurodevelopmental disorders (Rodier et al., 1979, Chomiak et al., 2010). Only a few reports (Scattoni et al., 2008, Wohr et al., 2011a, Wohr et al., 2011b, Roy et al., 2012, Lai et al., 2014) have examined behaviour during the postnatal period in models of neurodevelopmental disorders. Therefore, here we employ a behavioural battery consisting of established developmental milestones and behavioural tests for core symptoms of neurodevelopmental disorders including communication deficits, social behaviour, and repetitive behaviours in the first four postnatal weeks.

Early life stressors in rodents alter the developmental trajectory of behavioural phenotypes, including changes in activity (Nilsson et al., 2002), anxiety-like behaviour (Breivik et al., 2002, Walker et al., 2004), and social behaviour (Hood et al., 2003, Benner et al., 2014,

O'Leary et al., 2014). Particularly, both postnatal challenge with lipopolysaccharide (LPS) (Lucchina et al., 2010, Sidor et al., 2010, Sidor et al., 2014) and maternal separation treatment (Sachs et al., 2013) results in changes in anxiety-like behaviour, stress responsivity, and gene expression in the brain (Liu et al., 1997, Roceri et al., 2002, MacQueen et al., 2003, Roceri et al., 2004, Plotsky et al., 2005). Moreover, these postnatal exposures interact with sex and genetic background in both neuroanatomical and behavioural outcomes (Ognibene et al., 2007, Laviola et al., 2009, Sidor et al., 2014). Understanding the influence of environmental factors and its interactions with genetics and sex on these outcomes will enhance our appreciation of importance of the early life period. Recent work from our lab has revealed sex differences and different developmental trajectories in exploratory and anxiety-related behaviour in male and female mice following postnatal LPS challenge (Sidor et al., 2014). The current study aims to further examine gene-environment interactions to determine if early life behaviour and brain structure in *FMR1-KO* mice are influenced by early life stressors.

FMR1 knockout (*FMR1-KO*) mice have been used to study the pathophysiology and developmental milestones for FXS (Belmonte and Bourgeron, 2006, Krueger and Bear, 2011, Rousseau et al., 2011, Meredith et al., 2012). Behavioural differences in *FMR1-KO* mice have been reviewed thoroughly (Bernardet and Crusio, 2006, Bagni and Oostra, 2013). Studies to date have identified behavioural deficits in *FMR1-KO* mice including acoustic startle and prepulse inhibition (Chen and Toth, 2001, Nielsen et al., 2002, Pietropaolo et al., 2011), activity in the open field (Peier et al., 2000, Nielsen et al., 2002, Ventura et al., 2004, Restivo et al., 2005), social outcomes (Spencer et al., 2005, Mineur et al., 2006, McNaughton et al., 2008, Spencer et al., 2008, Mines et al., 2010, Pietropaolo et al., 2011, Spencer et al., 2011, Gantois et al., 2013, Heitzer et al., 2013), and anxiety-like measures (Peier et al., 2000, Spencer et al., 2005, Heulens et al., 2012). While a few studies have examined behaviour in *FMR1-KO* mice prior to puberty (Bilousova et al., 2009, Roy et al., 2012, Lai et al., 2014), the majority of studies have examined the adult phenotype only. The objective of this study was to examine early life behaviours in male and female, WT and *FMR1-KO* mice, and to determine the influence of early-life stressors on

behaviour. Postnatal stressors included exposure to lipopolysaccharide (LPS) at postnatal day 3 (P3) and overnight maternal separation at P9. Our design included growth and development milestones, vocalizations in response to maternal separation, exploratory behaviour and activity, social behaviour, and repetitive behaviours. This analysis was completed over the first 28 days of life (Fig. 1).

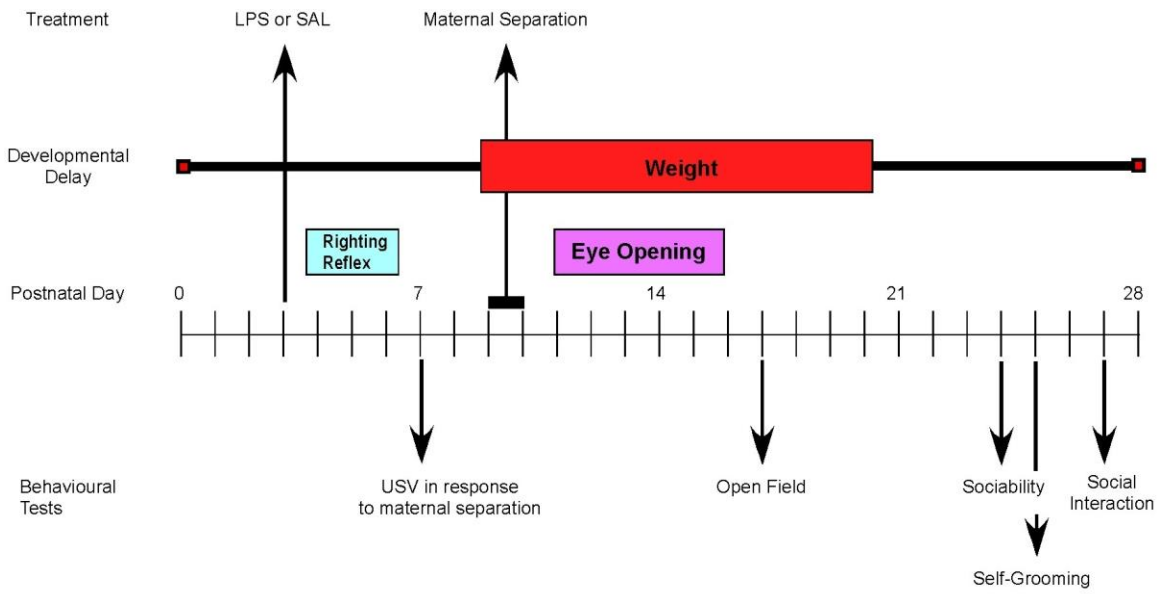

Figure 4-1. Experimental design showing postnatal challenges, developmental outcomes and behavioural tests used in the first 4 weeks of postnatal life.

Methods

Animals

Wild type (FVBCrl) and *FMR1-KO* (FVB.129P2.*Fmr1tm1Cgr*/J, stock #004624) mice were housed at the animal facility at St. Joseph's Healthcare with food and water available *ad libitum.* Mice were housed under a 12h:12h light dark cycle with lights on at 5 AM and lights off at 5 PM. Birth was set as postnatal day 0 (P0). On P2, litters were culled to 10 pups and pups were uniquely tattooed on their paw for identification. Pups were weaned on P21 and caged by sex with up to 4 littermates per cage. In the WT groups, there were: 11 SAL/CON females, 11 LPS/CON females, 10 SAL/CON males, 10 LPS/CON males, 10 SAL/MS females, 10 MS/LPS females, 10 SAL/MS males and 10 LPS/MS males. In the FMR1-KO groups, there were: 10 SAL/CON females, 11 LPS/CON females, 14 SAL/CON males, 10 LPS/CON males, 10 SAL/MS females, 11 MS/LPS females, 10 SAL/MS males and 10 LPS/MS males. All experimental measures were obtained by an individual blinded to treatment groups. All experimental procedures were approved by the Animal Research Ethics Board, McMaster University in accordance with the guidelines of the Canadian Council on Animal Care.

Postnatal challenges

On P3, pups were administered lipopolysaccharide (LPS) (0.1 mg/kg; E. coli LPS; Sigma, St. Louis, MO) or saline (SAL) i.p. at 50 µl/g. Injections were done at 4 PM. On P9, maternal separation (MS) or control (CON) treatment was administered. For MS litters, pups were weighed at 5 PM and the dam was removed from the home cage. The home cage was then placed on a heating pad at 37°C until 9 AM the next day, when the pups were weighed and the dam returned to the cage. Pups were again weighed at 4 PM. For control litters, pups were weighed at 5 PM on P9, 9 AM on P10 and 4 PM on P10; dams were removed briefly during weights and then returned to the home cage.

Righting Reflex

On P4-6, pups were tested for motor development by timing their ability to right themselves after being placed on their backs. Testing was done at 4 PM. A completed righting was defined by all four paws on the ground simultaneously. Time was kept with a stopwatch. The maximum score was set at 30 s at which time the pup was manually righted.

USV Recordings

On P7, pups were consecutively maternally separated from the dam and littermates and placed in a custom-made sound-attenuating chamber as previously described (Lai et al., 2014). Testing took place during the first half of the active period; at least one hour after the active cycle began. Ultrasonic vocalizations (USVs) were recorded for 3 min and then each pup was transferred to a separate holding cage. After all pups were tested, the pups were returned to the dam. Vocalizations were digitized using an Avisoft UltraSoundGate 116-200 recording device and USG CM116/CMPA microphone. The microphone was clamped to a retort stand and situated 17.5 cm above the center of the recording chamber. Calls were digitized in real-time and subsequently analyzed with Avisoft SAS Lab Pro.

Eye Opening

From P10 to P16, eye opening was scored daily: a score of 0, 1, or 2 was assigned per mouse reflecting the number of eyes open.

Open Field

At P17, pups were tested in the open field. Behavioural testing was conducted in a non-colony room after a 30 min habituation to the room. Testing took place in low light during the first half of the active period. Behaviours were automatically recorded for 15 min using the Kinder Scientific Smart Rack System consisting of a 24 cm wide x 45 cm long x 24 cm high cage rack system, with 22 infrared beams (7 X & 15 Y) and a rearing option (22 additional beams). A Plexiglas® box was placed at one end of the chamber to reduce the testing chamber size to 24 x 23 cm. Data were collected using MotorMonitor® software (Kinder Scientific, Poway, CA). A

maximum of 6 pups were tested at a time. After all pups had undergone testing, they were returned to the dam; maternal separation did not exceed 30 min.

Stranger Mice

Age- and sex- matched naïve mice (FVB) that did not undergo behavioural testing were used as stranger mice for sociability testing on P24 and social interaction testing on P27.
Both experimental and stranger mice were handled for 2 min each on the two days prior to the sociability test (P22 and P23). Both experimental and stranger mice were handled 2 min and habituated for 5 min each to the social interaction chamber the day before testing (P26).

Sociability

At P24, sociability was tested using a 3-chamber apparatus (Moy et al., 2004, Nadler et al., 2004). Behavioural testing was conducted in a non-colony room after a 20 min habituation to the room. Testing took place during the first half of the active period. Mice were placed in the centre zone of the chamber with no access to the other chambers for 5 minutes. Subsequently, an age- and sex- matched stranger mouse was placed in an inverted cup in one of the side chambers, the doors from the centre chamber to the outer chambers opened, and behaviour was recorded for 10 minutes. Live-tracking and automated videotape analysis was done using EthoVision® software.

Self-grooming

At P25, mice were observed for 10 min in a standard housing cage without bedding and scored for time spent grooming (Silverman et al., 2010). Mice were habituated to the testing cage for 10 min prior to grooming test. Live-tracking and manual videotape analysis was done using EthoVision® software.

Social Interaction

At P27, mice were placed with a sex- and age-matched stranger in a chamber (20cm x 20cm x 20cm) and videotaped using EthoVision® for 10 min. Mice habituated to the non-colony

room for 5 minutes before test. Live-tracking and videotape analysis was done using EthoVision® software.

Statistics and Analysis

Graphs for behavioural tests were made with GraphPad Prism. Data are expressed as mean +/-SEM. Data were analysed using SPSS software (version 22, IBM). Univariate analyses were performed with genotype, treatment and sex as factors, followed by post-hoc comparisons using Student's test. When the initial analysis showed no main effects, two-way ANOVAs were conducted followed by post-hoc comparisons using Student's t-test. Repeated measures ANOVAs were used in longitudinal datasets, followed by Student's t-tests for within time point comparisons. Statistical significance was set at $p<0.05$.

Results

Weight

There was no effect of genotype on postnatal weight throughout postnatal development up to P28. Similar to what we have previously observed (Sidor et al., 2014), there was no effect of P3 LPS treatment on weight. In contrast, there was an effect of P9 MS treatment on weight revealed as an interaction between genotype-sex and treatment on weight ($F[9,258]=5.180, p<0.001$ - Fig.2A). Post-hoc tests revealed a decrease in weight due to MS treatment in all groups ($p<0.001$).

Righting Reflex

Righting reflex time of all mice improved over development from P4-6 (main effect of age, $F[1.936,517.021]=10.469, p<0.001$ - Fig.2B). In addition, there was a main effect of genotype ($F[1,267]=29.623, p<0.001$) and sex ($F[1,267]=4.662, p=0.032$) on time to right. Specifically, in males, we observed that *FMR1-KO* mice righted in less time compared to WT mice at P4 and P5 in both SAL- and LPS-treated groups (P4: SAL, $p=0.043$; LPS, $p<0.001$; P5: SAL, $p=0.009$; LPS, $p=0.008$). In females, *FMR1-KO* LPS-treated mice righted in less time compared to WT LPS-treated mice at P6 ($p=0.011$). In addition, a sex difference was observed where WT LPS-treated females righted in less time than WT LPS-treated males at P5 ($p=0.033$).

Eye Opening

We examined eye opening scores from P10 to P16. An interaction between genotype and P9 treatment ($F[1,253]=8.936, p=0.003$) was observed for eye opening score. Since there was no effect of P3 treatment ($F[1,253]=0.004, p=0.950$), additional analysis was completed separately for males and females with SAL and LPS-treatment groups combined (Fig. 2C, D). In the CON group, male *FMR1-KO* mice opened their eyes earlier than WT mice (higher EO score at P14, $p<0.001$). MS treatment accelerated eye opening in WT males (higher EO score at P13 and P14, $p=0.026$ and $p=0.007$ respectively) while delaying eye opening in *FMR1-KO* males

(lower EO score at P14 and P15, p=0.008 and p=0.026 respectively). Similar to CON males, *FMR1-KO* females opened their eyes earlier than WT females (higher EO score at P14, p=0.013). MS treatment accelerated eye opening in WT females (higher EO score at P14, p=0.01) but did not have an effect in *FMR1-KO* females. Overall, these data show accelerated EO in *FMR1-KO* mice. Separately, MS treatment accelerated EO in WT mice but delayed EO in *FMR1-KO* males.

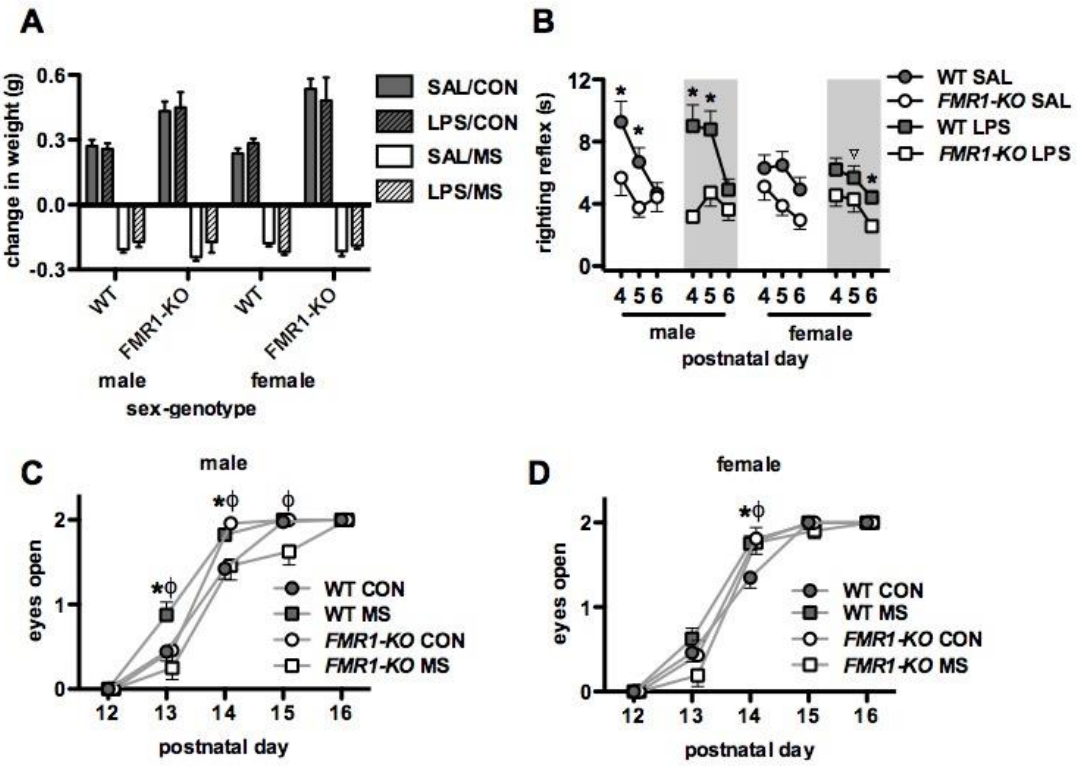

Figure 4-2. Weight change due to overnight maternal separation, righting reflex times and eye opening (EO) scores in male and female WT and *FMR1-KO* mice that were treated with a saline (SAL) or lipopolysaccharide (LPS) injection at postnatal day (P)3 and with or without overnight maternal separation (MS) at P9. Shown are the mean ± SEM of the difference in weight pre- and post- 16 hour maternal separation at P9 in panel A, the amount of time to right from P4 to P6 (B), and the number of eyes opened each day from P12 to P16 in (B) males, and (D) females. There were main effects of age, genotype and sex on righting reflex time and an interaction between genotype and MS treatment on EO score. * indicates genotype-related differences, ∇ indicates sex-related differences, and ϕ indicates treatment-related differences at the $p<0.05$ level.

Ultrasonic Vocalizations

At P7, we recorded USVs in response to acute maternal separation and analyzed the total number and average duration of calls (Fig. 3) as well as the call types based on temporal and spectral characteristics (Lai et al., 2014)(see Figures 4 and 5). Call type are characterized by frequency range, modulation and patterning as well as temporal characteristics. Calls were categorized as composite (C), frequency jump (FJ), frequency jump plus composite (FJ+C), frequency modulated (FM), quasi-constant (QC), and short calls (S). Composite (C) calls are pure tone calls with overtones (harmonics) at integer multiples of the fundamental frequency. Frequency jump (FJ) calls are characterized by an instantaneous discontinuity in frequency that occurs within <0.1 ms. Frequency jump plus composite (FJ+C) are a combination of C and FJ calls with overlapping frequencies and a frequency gap <0.1 ms. Frequency modulated (FM) are continuous calls with a change in spectral bandwidth >10 kHz. Quasi-constant frequency (QC) calls are defined as continuous calls with a change in spectral bandwidth ≤10 kHz. Short (S) calls are punctuated vocalizations with a duration <10 ms. There was no main effect of sex on the number of USV calls. Males and females were analyzed separately. In males, there were no differences due to either genotype ($F[1,129]=0.295, p=0.588$) or P3 treatment ($F[1,129]=0.006, p=0.936$) on the total number of calls (Fig. 3A). In females, there were an increased number of calls in *FMR1-KO* LPS females compared to WT LPS females ($p=0.04$) and compared to *FMR1-KO* SAL females ($p=0.004$) demonstrating a treatment by genotype interaction ($F[1,138]=5.602, p=0.019$ - Fig.3A).

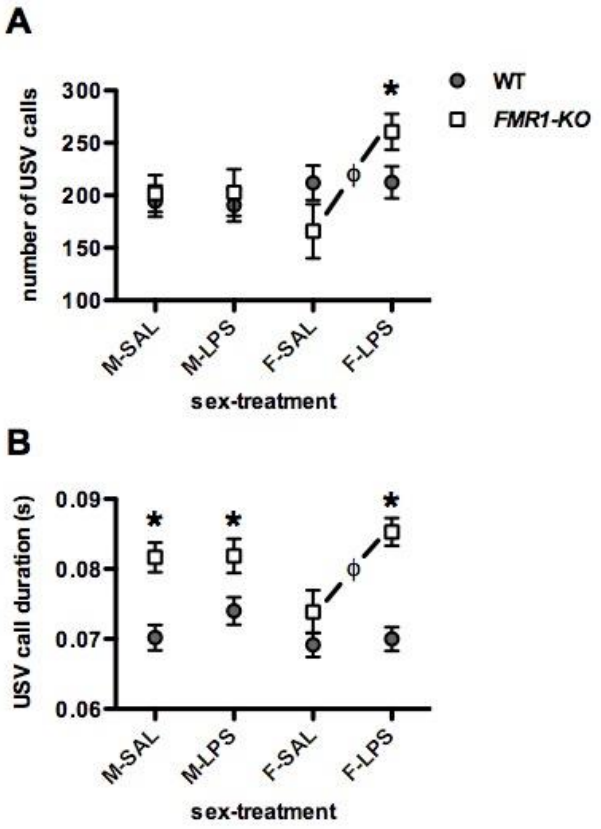

Figure 4-3. Ultrasonic vocalization (USV) call production analysis in SAL- and LPS- treated, male and female WT and *FMR1-KO* mice at P7. Shown are the mean ± SEM of (A) the total number of calls and (B) the duration of calls emitted during a 3-minute acute maternal separation session. In females, there was an interaction between genotype and LPS-treatment on the number of calls. There was a 3-way interaction between genotype, sex, and treatment on USV call duration. * indicates genotype-related differences and ϕ indicates treatment-related differences.

Our analysis of different types of USV calls showed differences in call number for FJ, FJ+C and FM calls (Fig. 4). In FJ calls, *FMR1-KO* SAL females had a decreased call number compared to both *FMR1-KO* LPS females (p=0.05) and WT SAL females (p=0.001)(effect of genotype, F[1,267]=9.952,p=0.002 - Fig. 4B). In FJ+C calls, there was an effect of genotype (F[1,267]=9.972,p=0.002 - Fig. 4C). Specifically, an increase in FJ+C call numbers was observed in *FMR1-KO* SAL males compared to WT SAL males (p=0.03). In addition, an increase in FJ+C calls was observed between *FMR1-KO* LPS females compared to *FMR1-KO* SAL (p=0.01) and WT LPS (p=0.004) females. Further, there was a sex difference (F[1,267]=4.513,p=0.035) and a sex by P3 treatment interaction (F[1,267]=5.18,p=0.024) where the *FMR1-KO* LPS females had an increased number of calls compared to *FMR1-KO* LPS males (p=0.004). In FM calls, female *FMR1-KO* SAL mice showed reduced FM calls compared to female WT SAL mice (p=0.028) and male *FMR1-KO* SAL mice (p=0.01)(effect of sex, F[1,267]=6.501,p=0.011 - Fig. 4D).

A 3-way interaction between genotype, sex and treatment (F[1,266]=5.342,p=0.022) was observed for call duration (Fig. 3B). *FMR1-KO* males had an increased call duration compared to WT males in both treatment groups (SAL, p<0.001; LPS, p=0.016). In females, *FMR1-KO* FMR1 LPS mice showed an increased call duration compared to *FMR1-KO* SAL (p=0.003) and to WT LPS mice (p<0.001). In addition, there was a sex difference where *FMR1-KO* SAL female mice had decreased call duration compared to *FMR1-KO* SAL male mice (p=0.037).

Our analysis of different types of USV calls showed differences in call duration for C, FJ, FJ+C, FM and QC calls (Fig. 5). In C calls, there was an increased call duration in *FMR1-KO* LPS mice compared to *FMR1-KO* SAL and WT LPS mice in both sexes (male: *FMR1-KO* SAL, p=0.03; WT LPS, p=0.001; female: *FMR1-KO* SAL, p=0.035; WT LPS, p=0.013)(effect of sex, F[1,226]=6.707,p=0.01; effect of genotype, F[1,226]=16.344,p<0.001; effect of P3 treatment, F[1,226]=10.358,p=0.001 - Fig. 5A). In FJ calls, there was an increased call duration in *FMR1-KO* males compared to WT males in both treatment groups (SAL, p=0.003; LPS, p=0.006; sex by genotype by P3 treatment interaction, F[1,262]=4.847,p=0.029 - Fig. 5B). In addition, *FMR1-KO*

LPS females had increased call duration compared to *FMR1-KO* SAL (p=0.003) and WT LPS (p=0.001) females. Also, there was a sex difference where *FMR1-KO* SAL females had decreased call duration compared to *FMR1-KO* SAL males (p=0.002). In FJ+C calls, an increased duration was observed in *FMR1-KO* males compared to WT males in both treatment groups (SAL, p<0.001; LPS, p=0.013) and in *FMR1-KO* LPS females compared to WT LPS females (p<0.001; effect of genotype, F[1,262]=50.082,p<0.001 - Fig.5C). *FMR1-KO* LPS females also had increased call duration compared to *FMR1-KO* SAL females (p=0.01; effect of P3 treatment, F[1,262]=4.445,p=0.036). In addition, there were sex differences where *FMR1-KO* SAL females had decreased call duration compared to *FMR1-KO* SAL males (p=0.018) and similarly, WT LPS females had decreased call duration compared to WT LPS males (p=0.038; effect of sex, F[1,262]=6.711,p=0.01). In FM calls, there was an increased call duration in *FMR1-KO* males compared to WT males of both treatment groups (SAL, p=0.002; LPS, p=0.002) and in *FMR1-KO* LPS females compared to WT LPS females (p=0.017; effect of genotype, F[1,264]=21.879,p<0.001 - Fig. 5D). In addition, *FMR1-KO* SAL males had increased FM call duration compared to *FMR1-KO* SAL females (p=0.071; effect of sex, F[1,135]=6.605,p=0.011). There was no difference due to genotype in FM duration in female SAL mice. In QC calls, there was there was an increase in call duration in *FMR1-KO* LPS males compared to WT LPS males (p=0.049; effect of genotype, F[1,128]=7.615,p=0.007 - Fig. 5E).

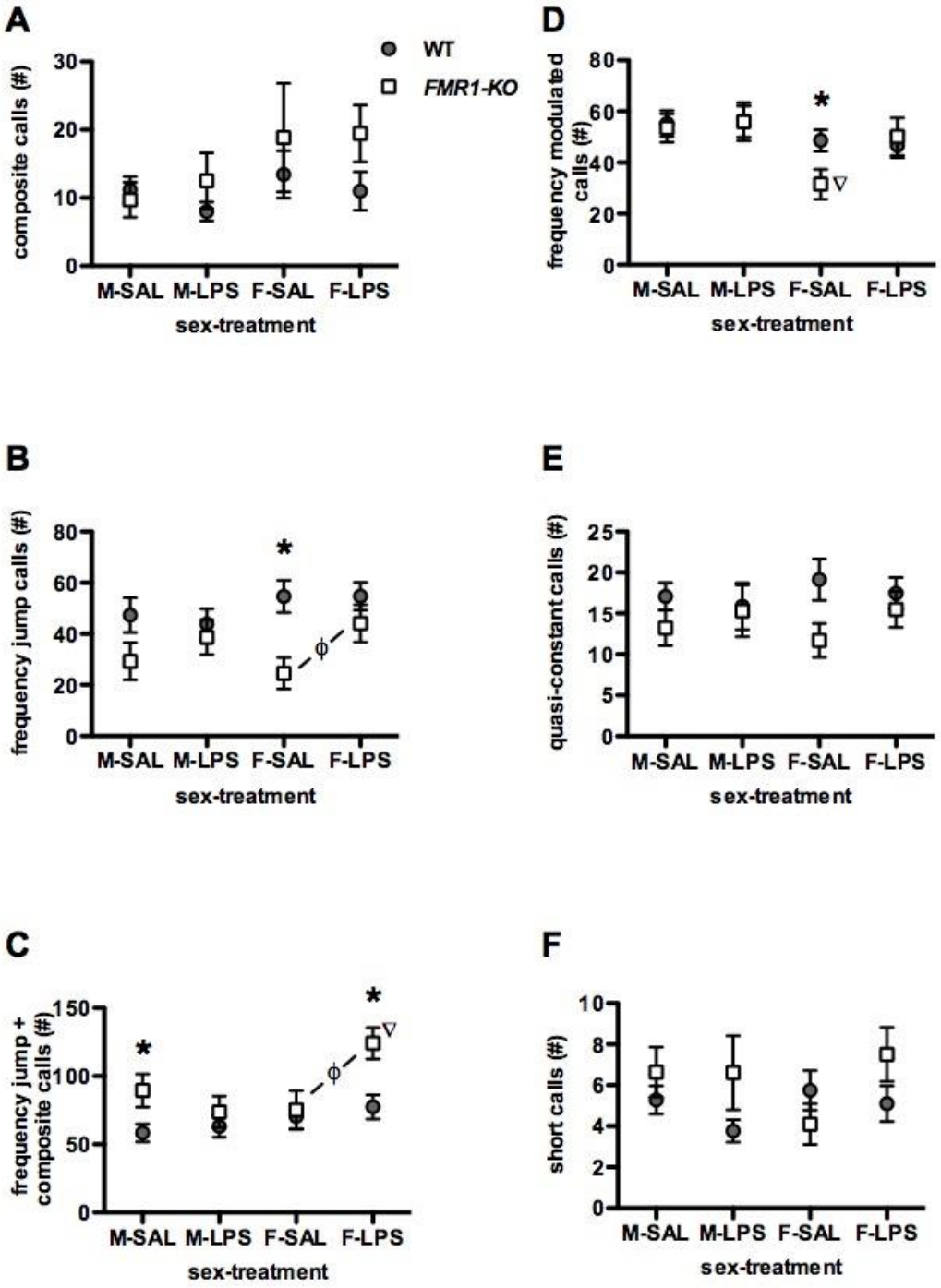

Figure 4-4. Number of USV calls categorized by call type in SAL- and LPS- treated, male and female WT and *FMR1-KO* mice at P7. Shown are representative traces of each call type and the

mean ± SEM of the number of calls defined by frequency modulation range, pattern and temporal duration as (A) composite [C] – pure tone calls with overtones (harmonics) at integer multiples of the fundamental frequency; (B) frequency jump [FJ] – calls with an instantaneous discontinuity in frequency (i.e. a gap) that occurs within <0.1 ms; (C) frequency jump plus composite [FJ+C] – a combination of C and FJ calls with overlapping frequencies and a frequency gap <0.1 ms, (D) frequency modulated [FM] – continuous calls with a change in spectral bandwidth >10 kHz; (E) quasi-constant frequency [QC] – continuous calls with a change in spectral bandwidth ≤10 kHz; and (F) short [S] – punctuated vocalizations with a duration <10 ms. There was a main effect of sex on the number of C calls, an effect of genotype on the number of FJ calls, an effect of genotype as well as an interaction between sex and LPS-treatment on the number of FJ+C calls, an effect of sex on the number of FM calls, and no differences in the number of QC and S calls. * indicates genotype-related differences, ∇ indicates sex-related differences, and ϕ indicates treatment-related differences.

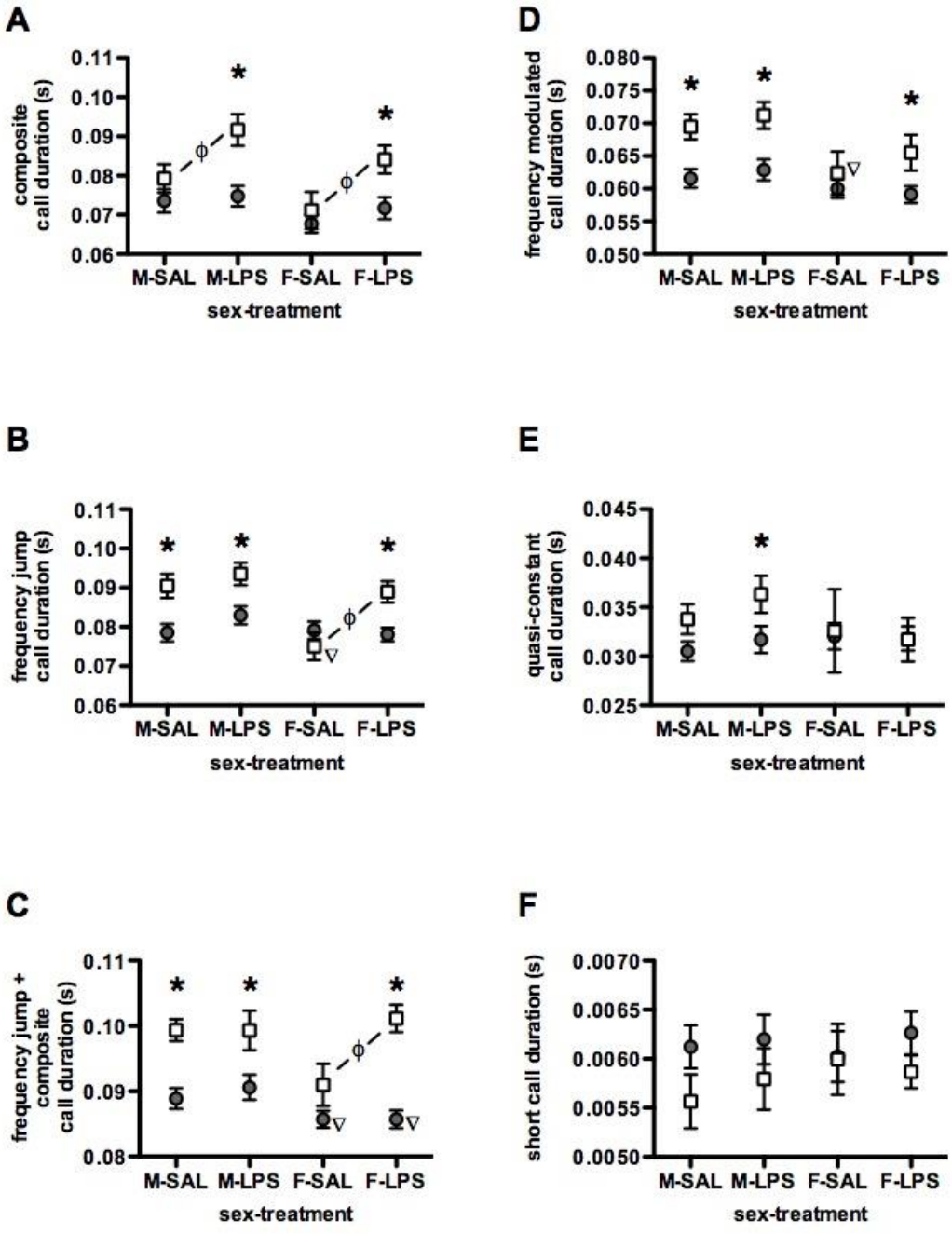

Figure 4-5. Call duration of different USV call types defined by frequency modulation range, pattern and temporal duration in SAL- and LPS- treated, male and female WT and *FMR1-KO* mice at P7. Shown are the mean ± SEM of the call duration of calls categorized as (A) composite, (B) frequency jump, (C) frequency jump plus composite, (D) frequency modulated, (E) quasi-

constant frequency, and (F) short calls. There was a main effect of sex on the duration of C calls, a 3-way interaction between sex, genotype, and LPS-treatment on the duration of FJ calls, an effect of sex, genotype and treatment on the duration of FJ+C calls, an effect of sex and genotype on the duration of FM calls, an effect of genotype on the duration of QC calls, and no differences on the duration of S calls. * indicates genotype-related differences, ∇ indicates sex-related differences, and ϕ indicates treatment-related differences.

Open Field

At P17, total distance, rearing and centre time in the open field were analyzed (Fig. 6). *FMR1-KO* LPS/CON males had increased total distance compared to WT treatment-matched controls ($p<0.001$) and compared to *FMR1-KO* LPS/MS males ($p<0.001$; effect of P9 treatment, $F[1,258]=22.651, p<0.001$; genotype by P9 treatment interaction, $F[1,258]=9.394, p=0.002$). A genotype by sex interaction was observed in total distance ($F[1,258]=12.739, p<0.001$) where WT LPS/CON females had increased total distance compared to WT LPS/CON males ($p=0.02$) and WT LPS/MS females had increased total distance compared to WT LPS/MS males ($p=0.018$). *FMR1-KO* LPS/CON females showed decreased total distance compared to *FMR1-KO* LPS/CON males ($p=0.043$).

No treatment differences in rearing were observed in WT male or female mice. In contrast, there were treatment effects in *FMR1-KO* mice (Fig. 6B; P9 treatment, $F[1,258]=16.507, p<0.001$). MS treatment resulted in decreased rearing in *FMR1-KO* males (SAL: $p=0.046$; LPS: $p=0.006$). Genotype differences were observed in female mice (effect of genotype, $F[1,258]=7.708, p=0.006$; sex by genotype interaction, $F[1,258]=9.399, p=0.002$) with reduced rearing in female *FMR1-KO* mice following MS treatment (both SAL/MS and LPS/MS groups). In addition, increased rearing was observed in female WT mice treated with LPS in comparison to WT males (CON, $p=0.046$; MS, $p=0.043$).

A robust effect of genotype was observed in male mice related to centre time in the open field (genotype, $F[1,258]=17.913, p<0.001$; genotype by sex interaction, $F[1,258]=9.658, p=0.002$). *FMR1-KO* males had increased amount of time in the centre compared to WT treatment-matched controls in all treatment groups (SAL/CON, $p=0.024$; SAL/MS, $p=0.008$; LPS/CON, $p=0.001$; LPS/MS, $p=0.013$). Several sex differences were observed. WT females showed increased centre time compared to WT males in the SAL/MS ($p=0.047$), LPS/CON ($p=0.008$), and LPS/MS ($p=0.008$) treatment groups. In female mice, in *FMR1-KO* SAL/MS mice decreased centre time compared to *FMR1-KO* SAL/CON mice ($p=0.047$).

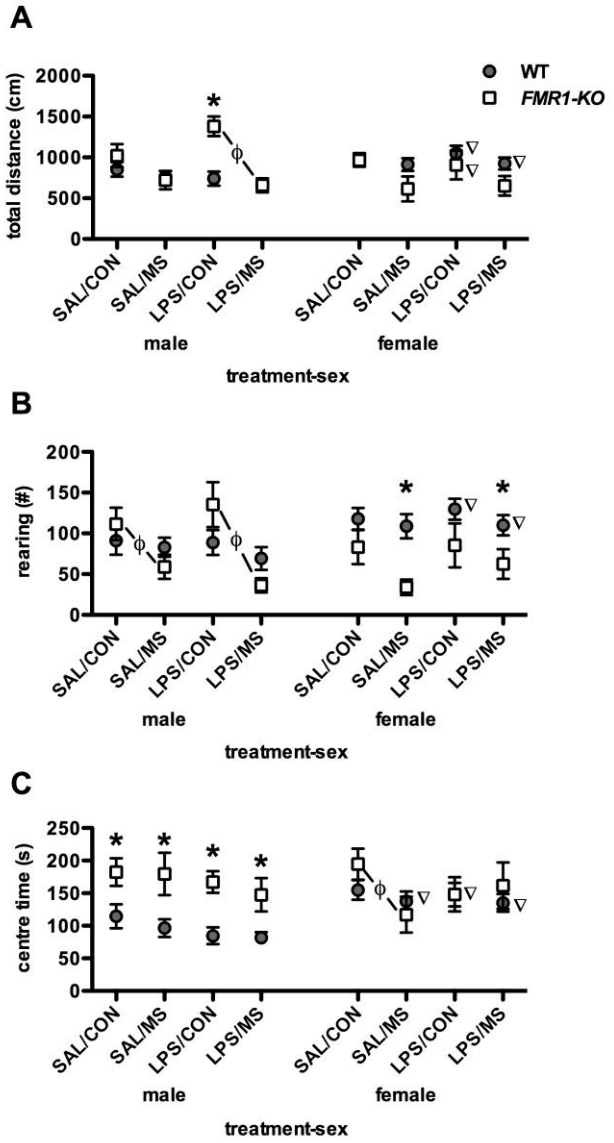

Figure 4-6. Activity measures in an open field in male and female WT and *FMR1-KO* mice in treatment groups of SAL/CON, LPS/CON, SAL/MS, and LPS/MS at P17. Shown are the mean ± SEM of (A) the total distance travelled, (B) the number of rears and (C) time spent in the centre of the chamber. There were interactions between genotype and MS-treatment as well as genotype and sex on the total distance travelled and number of rears, and there was an interaction between genotype and sex on the centre time. * indicates genotype-related differences, ∇ indicates sex-related differences, and ϕ indicates treatment-related differences.

Sociability

We examined sociability in the 3-chambered social apparatus at P24 (Fig. 7). Social preference was determined by comparing the time spent in the chamber containing the stranger mouse and chamber containing the empty cup. Social preference varied by genotype, sex and treatment. Specifically, the following groups showed normal social preference for the stranger compared to the empty chamber: male WT SAL/MS ($p<0.001$), male WT LPS/CON ($p<0.001$), male *FMR1-KO* SAL/CON ($p=0.004$), male *FMR1-KO* SAL/MS ($p=0.004$), male *FMR1-KO* LPS/MS ($p=0.008$), female WT SAL/CON ($p=0.047$), female WT SAL/MS ($p<0.001$), female WT LPS/CON ($p=0.001$), female WT LPS/MS ($p=0.007$), female *FMR1-KO* SAL/MS ($p<0.001$), female *FMR1-KO* LPS/CON ($p<0.001$), and female *FMR1-KO* LPS/MS ($p=0.018$). The following groups lacked a social preference: male WT SAL/CON ($p=0.338$), male WT LPS/MS ($p=0.801$), male *FMR1-KO* LPS/CON ($p=0.769$), female *FMR1-KO* SAL/CON ($p=0.076$).

Comparison of the time spent in the stranger chamber and the centre chamber, showed that all mice spent more time exploring the stranger chamber compared to the centre ($p<0.001$). Lastly, we performed univariate analyses with genotype, sex, P3 and P9 treatments as factors. There were no main effects on the total distance travelled, the ratio between the time spent in the stranger versus the empty chamber, and the time spent in the centre chamber – a measure of task avoidance. Overall, these data suggest no differences in sociability as measured with the 3-chambered apparatus at P24 in these mice.

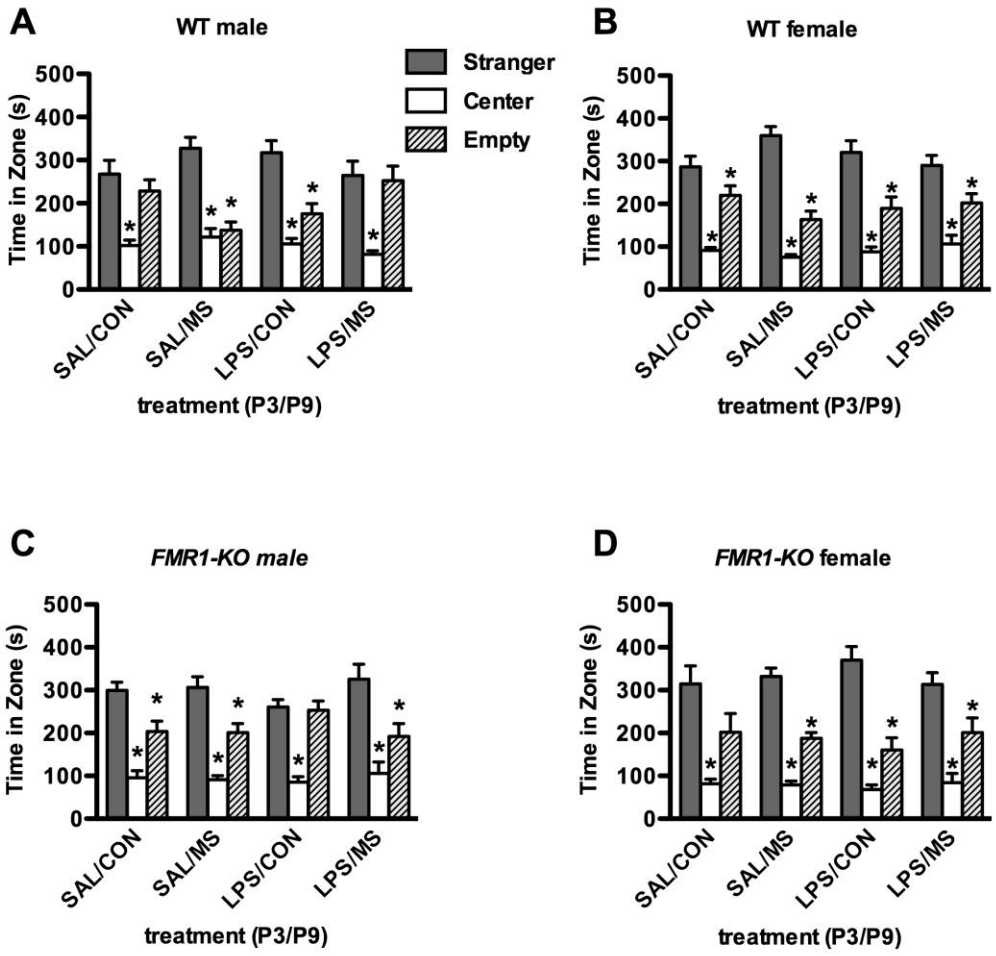

Figure 4-7. Sociability in the 3-chambered social apparatus in male and female WT and *FMR1-KO* mice in treatment groups of SAL/CON, LPS/CON, SAL/MS, and LPS/MS at P24. Shown are the mean ± SEM of the amount of time spent in each chamber in (A) WT males, (B) WT females, (C) *FMR1-KO* males, and (D) FMR1-females. There were no differences in the total distance travelled, the ratio between the time spent in the stranger versus the empty chamber, or in the time spent in the centre chamber between genotypes, sex or treatment groups. * indicates a difference in time spent in the empty or centre compared to the stranger chamber.

Self-Grooming Behaviour

At P25, we analyzed self-grooming behaviour using total grooming duration, frequency of grooms and latency to groom as outcome measures (Fig. 8). There were no differences in grooming duration (Fig. 8A).

In contrast, grooming frequency was affected by genotype, sex and postnatal treatments (effect of P9 treatment, $F[1,258]=6.163, p=0.014$; genotype by P9 treatment interaction, $F[1,258]=6.601, p=0.011$; sex by P3 treatment interaction, $F[1,258]=5.609, p=0.019$ - Fig. 8B). *FMR1-KO* LPS/MS males had decreased grooming frequency compared to *FMR1-KO* LPS/CON males ($p=0.044$). In addition, *FMR1-KO* LPS/CON females had a higher grooming frequency compared to *FMR1-KO* SAL/CON females ($p=0.043$). Furthermore, *FMR1-KO* SAL/CON females had a decreased frequency of grooms compared to *FMR1-KO* SAL/CON males ($p=0.044$).

In the latency to groom, there were no main effects of genotype, treatment, or sex, but in SAL/CON mice, there was a genotype by sex interaction ($F[1,70]=4.908, p=0.03$). This was due to an increased latency to groom in *FMR1-KO* SAL/CON females compared to *FMR1-KO* SAL/CON males ($p=0.025$).

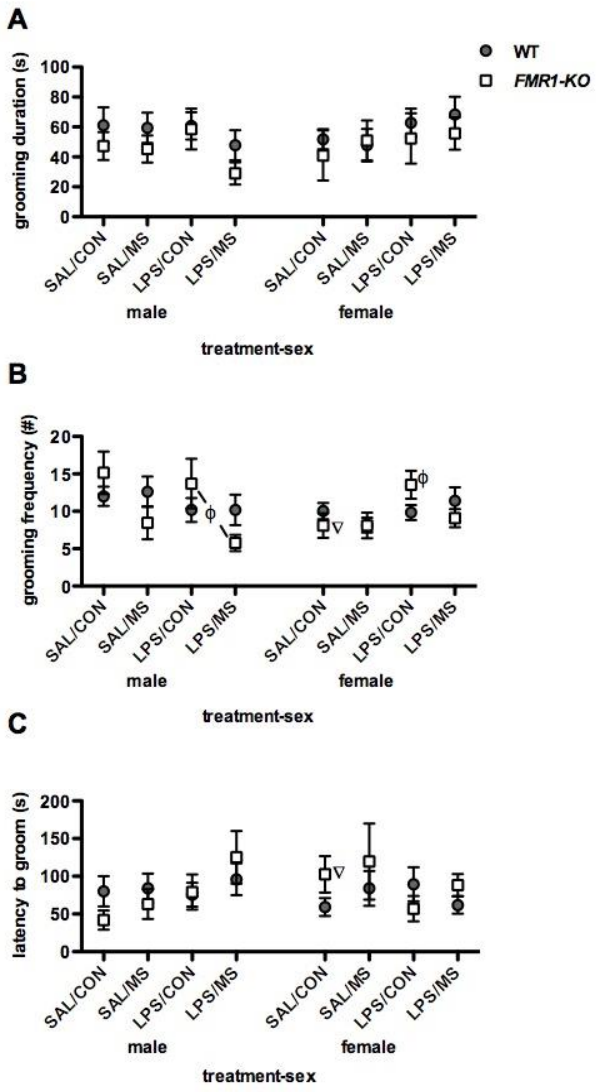

Figure 4-8. Self-grooming in male and female WT and *FMR1-KO* mice in treatment groups of SAL/CON, LPS/CON, SAL/MS, and LPS/MS at P25. Shown are the mean ± SEM of (A) the total grooming duration, (B) grooming frequency, and (C) the latency to groom. There were no differences in grooming duration, but interactions between genotype and MS-treatment and between sex and LPS-treatment on grooming frequency, and an interaction between sex and genotype on the latency to groom in SAL/CON mice. ∇ indicates sex-related differences, and ϕ indicates treatment-related differences.

Social Interaction

At P27, we allowed the mice to undergo free social interaction with a stranger mouse and used the total interaction duration and frequency as outcome measures (Fig. 9). *FMR1-KO* mice had increased social interaction duration compared to WT controls in all groups except in the female SAL/CON group (male SAL/CON, $p=0.004$; male SAL/MS, $p=0.018$; male LPS/CON, $p=0.002$; male LPS/MS, $p<0.001$; female SAL/MS, $p<0.001$; female LPS/CON, $p=0.011$, female LPS/MS, $p<0.001$; effect of genotype, $F[1,259]=87.835, p<0.001$ - Fig. 9A). The frequency of social interactions was increased in *FMR1-KO* LPS/CON females compared to WT controls ($p=0.026$; effect of genotype ($F[1,259]=5.35, p=0.022$).

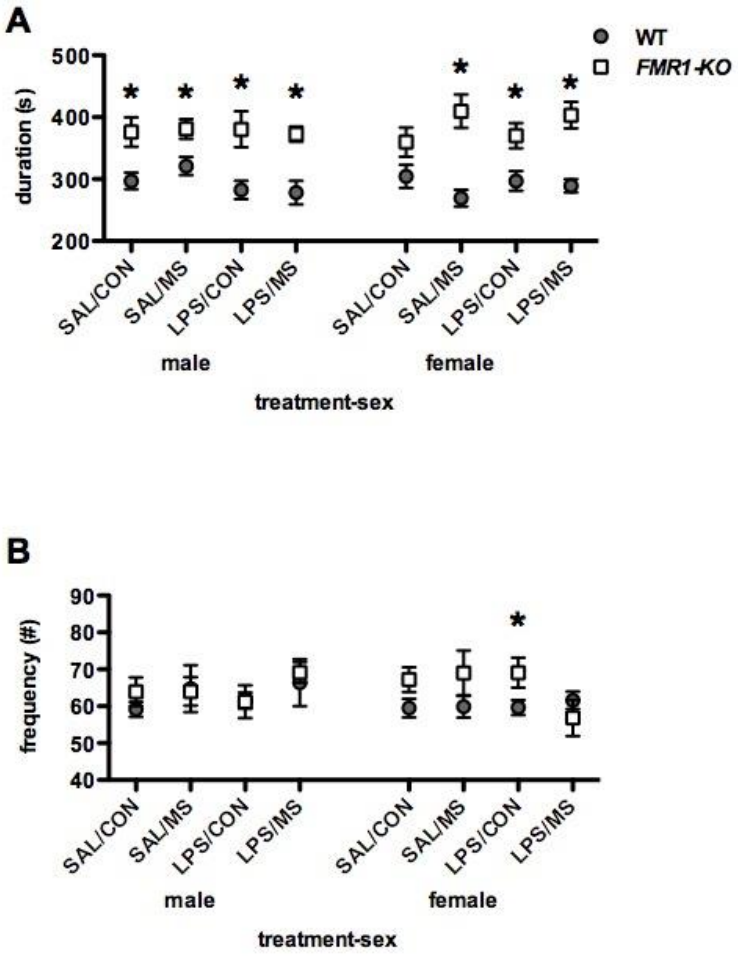

Figure 4-9. Social interaction in male and female WT and *FMR1-KO* mice in treatment groups of SAL/CON, LPS/CON, SAL/MS, and LPS/MS at P27. Shown are the mean ± SEM of (A) the total duration of social interaction with a stranger mouse and (B) the frequency of interactions. There were main effects of genotype on social interaction duration and frequency. * indicates genotype-related differences.

Discussion

In this study, for the first time, we show early life behavioural changes in *FMR1-KO* mice. Importantly, we observe sex differences in *FMR1-KO* mice on an FVB background that were previously not observed in *FMR1-KO* mice on a C57Bl/6 background. These sexually dimorphic outcomes were not present in WT mice. Furthermore, we show that postnatal exposures to LPS and maternal separation affect male and female, WT and *FMR1-KO* mice differently on various behavioural outcomes.

Behavioural Changes in FMR1-KO Mice are Present in the Early Life Period

Behavioural differences in adult *FMR1-KO* mice in both FVB and B6 strains are well reported in the domains of locomotor activity (Peier et al., 2000, Nielsen et al., 2002, Ventura et al., 2004, Restivo et al., 2005), anxiety-like behaviour (Peier et al., 2000, Spencer et al., 2005, Heulens et al., 2012), and sociability (Spencer et al., 2005, Mineur et al., 2006, McNaughton et al., 2008, Spencer et al., 2008, Mines et al., 2010, Pietropaolo et al., 2011, Spencer et al., 2011, Gantois et al., 2013, Heitzer et al., 2013). Here, we report differences in the trajectory of developmental milestones and behavioural differences in pre-pubertal male and female *FMR1-KO* mice on an FVB background. Specifically, in *FMR1-KO* mice compared to WT controls, we observe accelerated righting reflex in male *FMR1-KO* mice, accelerated eye opening in male and female *FMR1-KO* mice, increased USV call duration in male *FMR1-KO* mice as well as changes in the call profiles in both male and female *FMR1-KO* mice, increased centre time in the open field in male *FMR1-KO* mice, and increased social interaction time in male *FMR1-KO* mice. Thus, for the first time, we report some impairments in early life not observed in adult mice and other deficits reported in the adult that were not seen in here in the early postnatal period. For example, there were no differences in activity at P17, but increases are reported in adult *FMR1-KO* mice (Ventura et al., 2004) of similar background strain. On the other hand, increases in social interactions in male *FMR1-KO* mice observed at P27 in this study are not seen in adult *FMR1-KO* mice (Pietropaolo et al., 2011) of similar background strain.

Delays in reaching developmental milestones are commonly reported in children with neurodevelopmental disorders, including motor delays in ASD (Kau et al., 2000, Geschwind and Levitt, 2007), Angelman Syndrome (Clayton-Smith and Laan, 2003), and FXS (Kau et al., 2000, Kau et al., 2002) as well as in mouse models of ASD (Ey et al., 2012, Wohr et al., 2013, Greene-Colozzi et al., 2014, Ju et al., 2014). Changes in achieving these milestones are indicators of which processes are affected in these disorders and shed light on windows of opportunity for treatment (Heyser, 2004, Meredith, 2014). In our study, we observe an accelerated phenotype in reaching developmental milestones and notably, a male-specific genotype difference in righting reflex. The only other ASD model that has been observed to have acceleration in righting reflex is the BTBR mouse compared to the C57Bl/6 mouse (Scattoni et al., 2008), in both males and females. In contrast, reeler heterozygous (RELN+/-) mutant mice have slight delays in righting reflex at P2 that normalized by P4 (Romano et al., 2013). Notably, immune challenge using in utero exposure to poly(I:C) resulted in delay in milestones including righting reflex (Arsenault et al., 2014). Further molecular analysis showed that increased mGluR5 protein levels at P10 was associated with longer total righting time in poly(I:C)-treated pups. Since reducing mGluR5 levels and activity respectively rescues ocular dominance plasticity (Dolen et al., 2007) and auditory plasticity (Kim et al., 2013) in *FMR1-KO* mice during development, one might speculate that these results collectively suggest that mGluR5 signalling may be a convergent point related to both righting reflex and eye opening outcomes. Furthermore, mGLuR5 activity is regulated by estrogen (Mermelstein, 2009), which is differentially regulated in the second and third week of life in males and females (Brock et al., 2011). Overall, changes in these milestones warrant targeting these pathways as an earlier window of intervention to influence the developmental trajectory.

In early life social communication, we previously reported that *FMR1-KO* mice on an FVB background have an increased call number maintained after 10 minutes of maternal separation compared to WT FVB pups (Lai et al., 2014). In that study, the increase in call number was driven by increased FJ calls in *FMR1-KO* mice with no changes in call duration. Here, immediately after

maternal separation, we observed increased call duration with no change in call number and additionally, a different shift in the profile of emitted calls in *FMR1-KO* mice. The increase in call duration was driven by increases in complex call types with multiple elements or fluctuations in frequency (i.e. FM, FJ, and FJ+C type calls). Our previous study was likely a conservative underestimation of differences; previously, to reach a steady state in call emissions, we waited 10 minutes after maternal separation before data collection whereas in this study, we recorded calls immediately following separation. The results of this study are similar to Roy et al. (2012), who reported no changes in the total number of USV calls in *FMR1-KO* on a C57Bl/6 background (Roy et al., 2012). In addition, WT FVB pups are reported to have longer and more USVs calls with a different profile of call types compared to WT C57Bl/6 pups throughout the postnatal period (Scattoni et al., 2008). Collectively, these data show that early life social communication in *FMR1-KO* mice is not strongly affected by background strain immediately after maternal separation.

Notably, in the open field, we observe increased centre time in *FMR1-KO* males at P17. This time point is shortly after mice transition from crawling to walking and, importantly, is in the postnatal window in which serotonergic circuits related to anxiety-like behaviour are developed (Gross et al., 2002, Leonardo and Hen, 2008). Thus, it is possible that differences in the serotonergic wiring in *FMR1-KO* males are influencing this outcome. Few studies have examined the serotonergic system in FXS; however, increased levels of serotonin have been observed in the drosophila model (Zhang et al., 2005) as well as region- and age-dependent changes in the mouse model (Gruss and Braun, 2004). In the mouse, HPLC analysis in *FMR1-KO* females on an FVB background showed decreased serotonin levels in the hippocampus at 4 weeks of age but not in older mice (Gruss and Braun, 2004). Furthermore, treatment with fluoxetine, a serotonin reuptake inhibitor, rescues hyperactivity and increase centre time in male *FMR1-KO* mice on a B6 background compared to treatment in WT mice at 3-4 months old (Uutela et al., 2014). In the same study, SERT mRNA levels were reduced in *FMR1-KO* mice at P7. Altogether, these data suggest that decreases in serotonergic signalling are present early in development. Later in life,

differences in modulating plasticity (both LTP and LTD) through activation of various serotonin receptors has been reported in *FMR1-KO* mice (Costa et al., 2012, Xu et al., 2012), supporting the notion that dysfunction in the serotonergic wiring occurred in development and has raised the possibility of serotonergic receptors as a treatment target in FXS and ASD (Ciranna and Catania, 2014).

In addition, sex differences in the serotonergic system are well-established (Jones and Lucki, 2005, Dawson et al., 2009, Goel and Bale, 2010, Asghari et al., 2011, Gunther et al., 2011, Wu et al., 2012, Clarke et al., 2013) and may contribute to the male-specific phenotype we observed in the open field and to the male-specific accelerated righting reflex in *FMR1-KO* mice. This is supported by a finding that tryptophan hydroxlase-2 (*TPH2-/-)* knockout mice, which have reduced levels of brain serotonin, have delayed righting reflex and eye opening (Kane et al., 2012). Thus, our results, along with others, suggest that male-specific differences in various outcomes may be mediated through differences in serotonergic tone in *FMR1-KO* mice.

Consistent with other studies of *FMR1-KO* mice on an FVB background (Liu and Smith, 2009, Moy et al., 2009, Pietropaolo et al., 2011), we observed that sociability in the 3-chambered apparatus was normal. However, we also observed increased social interaction in *FMR1-KO* males, which was not previously reported in adult *FMR1-KO* males interacting with juvenile strangers (Pietropaolo et al., 2011). Thus, in addition to gene modifiers in different strains interacting with the *FMR1* gene (Dobkin et al., 1999, Panksepp et al., 2007, Moy et al., 2009, Pietropaolo et al., 2011), it is possible that age may be a confounding factor in social interaction. Overall, our data is consistent with the previous reports regarding sociability but is the first to show genotype differences in social interaction on an FVB background and suggest that age and background strain are factors that affect these outcomes.

Sexually Dimorphic Phenotype in FMR1-KO Mice on an FVB Background

Sex differences are found in many brain disorders (Rutter et al., 2003). In FXS, sex differences are reported in clinical profiles (Rinehart et al., 2011), regional brain volume (Reiss (Reiss and Freund, 1990, Gothelf et al., 2008, Bray et al., 2011), cognitive outcomes (Lightbody et al., 2006), behaviours and comorbid conditions (Symons et al., 2010); which is, in part, due to X-activation in females affecting phenotypic variability (Bennetto et al., 2001, Lightbody et al., 2006). Some of these differences are not static, rather, they follow developmental trajectory. For instance, from childhood to adolescence, the trajectory of IQ in boys with FXS declines more than in girls (Fisch et al., 2010). In ASD, sex differences are observed in specific traits; for instance, repetitive behaviour being more severe in males whereas social reciprocity is similar (Szatmari et al., 2012). The significance of an X or Y chromosome and their mechanisms on brain development is not completely understood (Carrel and Willard, 2005, Davies et al., 2006) – although protective factors and risk factors for neurodevelopment disorders and other psychiatric disorders are known to be associated with both (Kopsida et al., 2011).

In our data, sex-specific changes in behavioural outcomes were observed that were associated with genotype. For instance, compared to WT mice, male, but not female, *FMR1-KO* mice had accelerated righting reflex, delayed EO due to maternal separation, increased USV call duration, and increased social interaction. Further, gene-sex interactions were present in both number and duration of USV call types and in grooming frequency. Since there were not differences in the total grooming duration, the average grooming bout was longer in the females. These sex differences emerge only in *FMR1-KO* mice, suggesting that there are epistatic interactions with genes related to sex that affect behavioural profiles.

Furthermore, the behavioural differences are in stark contrast to what has been previously seen in *FMR1-KO* mice on a C57Bl/6 background (Ding et al., 2014) and on a C57Bl/6.albino cross background (Baker et al., 2010), in which were a lack of sex differences in various behavioural assays related to activity, exploratory behaviour, anxiety-like behaviour, sensorimotor gating, passive avoidance and contextual fear were reported at 2-3 months of age.

Here, the sex differences observed in early life communication and exploratory behaviour corroborates with other studies using the adult *FMR1-KO* mouse on an FVB background. Specifically, in OF, male *FMR1-KO* on FVB have increased activity and increased centre time whereas females have increased activity but no difference in centre time (Qin et al., 2005, Liu et al., 2011). Thus, these data collectively suggests that the FMR1 gene confers a different phenotype in males and females based on strain.

These studies suggest that gene modifiers may be housed on the sex chromosomes that affect specific outcomes. For instance, sexually dimorphic presentation of ASD in males and females, both at the neuroanatomical (Lai et al., 2013) and behavioural levels (Lai et al., 2011), has been reported. Sex differences are thought to modulate empathetic behaviour (Christov-Moore et al., 2014). In *FMR1-KO* mice, molecular differences have been shown to be sex-specific in *FMR1-KO* mice (FVB.219) with a male-specific reduction in calbindin-positive neurons in the thalamus (Giraldez-Perez et al., 2013). We also have data showing sex differences in gene expression of the neuroligins and neurexins, ASD risk genes, in *FMR1-KO* mice (Lai et al., 2015, submitted). There is a lack of studies examining the role of sex in preclinical models of ASD. The sex chromosomes house genes important for cognition (Zechner et al., 2001) and others are uniquely expressed in the brain (Kopsida et al., 2009, Kopsida et al., 2011), which may be protective against neurodevelopmental disorders. Understanding outcomes in both sexes will give further insight into the mechanisms that correspond to sexually dimorphic behaviours in response to genetic perturbations.

Postnatal Challenges, LPS and MS, Interacted with Genotype and Sex to Influence Developmental Milestones and Behaviour Phenotype

Postnatal challenges are known to alter behavioural and neuroanatomical outcomes across development (MacQueen et al., 2003, Huot et al., 2004, de Kloet et al., 2005, Levine, 2005, Schmidt et al., 2005, Ognibene et al., 2007, Sidor et al., 2010, Sachs et al., 2013, Gapp et

al., 2014). Both postnatal LPS and MS affected the timing of obtaining developmental milestones and various behavioural outcomes in a manner that interacted with genotype and sex.

Both developmental milestones measured were affected by the early life stressors. Previous work in our lab showed that LPS challenge resulted in sex differences by the third week of life (Sidor et al., 2014) and here, we extend that timeline by reporting faster righting reflex in LPS-treated WT females compared to LPS-treated WT males in the first few days of life. However, there was no effect of LPS treatment compared to SAL treatment. In addition, we see that maternal separation treatment accelerated eye opening in WT mice while delaying eye opening in *FMR1-KO* males, but not females. Eye opening is affected by GABA receptor subunit composition changes in the superior colliculus which is controlled by NMDA activity (Henneberger et al., 2005). Thus, the treatment-sex-genotype interaction on outcome may be due to a combination of changes in the HPA axis and an increased NMDA/AMPA ratio, which has been reported at thalamocortical synapses in the somatosensory cortex of *FMR1-KO* mice (Harlow et al., 2010). Increased HPA responsivity has been reported as a result of lacking the FMR1 gene (Lauterborn et al., 2007, Roberts et al., 2009) and MS treatment (Wigger and Neumann, 1999, Shanks et al., 2000, Kalinichev et al., 2002, Nilsson et al., 2002, Huot et al., 2004, Levine, 2005, Schmidt et al., 2005, Kundakovic et al., 2013, Gapp et al., 2014).

Changes in behaviour due to postnatal adversity are known to be strain-specific (Kundakovic et al., 2013, Own et al., 2013) and sexually dimorphic (Ognibene et al., 2007, Laviola et al., 2009, Kundakovic et al., 2013, Sidor et al., 2014). In this study, treatment effects on USVs, open field behaviour, and self-grooming were observed. There was sex-dependent effect in *FMR1-KO* mice where females were more sensitive to treatment with LPS than WT mice in both call number and duration USV outcomes. In the open field, LPS treatment interacted with genotype to increase activity in *FMR1-KO* males and interacted with sex to increase centre time in WT females compared to WT males. Maternal separation treatment reduced activity in LPS-treated *FMR1-KO* males, exploratory rearing activity in *FMR1-KO* males and centre time in SAL-

treated females in the open field. Furthermore, in a cross-fostering study, differences in maternal behaviour by *FMR1-KO* dams have been shown to affect locomotor behaviour but no other outcomes in the open field, PPI or seizure likelihood in males (Zupan and Toth, 2008). These changes were mediated through dopaminergic changes in the striatum. In contrast, others have reported that in C57Bl/6 mice, males but not females have increased activity after MS (Kundakovic et al., 2013).

In addition, postnatal treatments resulted in sexually dimorphic outcomes in the open field. For instances, WT LPS-treated females had increased overall activity and exploratory behaviour compared to WT LPS-treated males in both CON and MS group. This is contrasting to previous findings from our lab, where we observed decreased activity due to LPS in both sexes and a male-specific decrease in exploratory (rearing) behaviour in the prepubertal period, resulting in the loss of sexually dimorphic behaviour (Sidor et al., 2014). LPS modifies serotonin gene expression in circuits involved in anxiety and emotional regulation (Sidor et al., 2010) and male mice have increased sensitivity to SSRIs after in utero stress (Mueller and Bale, 2008), support the notion that serotonergic differences due to early life influences may mediate behavioural changes (Booij et al., 2014). Furthermore, increased anxiety-like behaviour in adult mice is observed after LPS treatment (Lucchina et al., 2010). Also, exposure to MS after immune change is known to increase the effect of pro-inflammatory cytokines and lead to sexually dimorphic changes in weight and food consumption (Avitsur et al., 2013).

Overall, adverse postnatal exposures modified the phenotype in a genotype- and sex-dependent manner in all these behaviours. The MS treatment affected more outcomes and was of larger effect than LPS treatment. LPS effects were more prevalent in *FMR1-KO* mice and in female mice overall. Early life stress can be adaptive or maladaptive depending on the time window and has been shown to result in sexually dimorphic differences in the inhibitory control network in fMRI studies of maltreatment (Elton et al., 2014).

Summary and Future Directions

In the study, we demonstrate changes in behavioural phenotype and developmental milestones (both righting reflex and eye opening) in the early life period of *FMR1-KO* mice. We observe differences in various outcomes that are not reported in adult mice. Furthermore, we have shown, for the first time, sexual dimorphic early life difference in *FMR1-KO* mice that are modified by postnatal adverse challenges. These results support the notion that epistatic interaction occur due to strain and sex. We show that the influence of postnatal environment greatly influences specific behavioural outcomes. Further studies of environmental factors may lead to precision regarding the therapies and windows of treatment for neurodevelopmental disorders. Lastly, understanding the relationship between these behaviour outcomes and their potential associations with other assays, such as regional brain volume changes and immune profile will greatly increase our ability to identify convergent endophenotypes in the neurodevelopmental disorders (Anagnostou, 2012, Ashwood et al., 2014).

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Chapter 5: Developmental Expression of the Neuroligins and Neurexins in Fragile X Mice

Chapter Link

The work in the following chapter was submitted to the Journal of Comparative Neurology (Ms. No.: JCN-15-0001) and is currently under review.

In the previous two chapters, it was demonstrated that early life behavioural differences were present in *FMR1-KO* mice and that there were sexually dimorphic outcomes in multiple outcomes. The following study was performed to examine molecular changes that may underlie the behavioural differences. At the same time, Dr. Peter Szatmari had suggested to look at a novel family of rare genetic copy number variants that were susceptibility genes for ASD. Therefore, this following study was performed to explore this as a possible shared neurobiological mechanism between FXS and ASD.

Developmental Expression of the Neuroligins and Neurexins in Fragile X Mice

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Abstract

Neuroligins and neurexins are trans-synaptic proteins involved in the maturation of both glutamatergic and GABAergic synapses. Recent research has identified players in synapse structure and function as primary contributors to the development of Fragile X Syndrome and autism spectrum disorders. FMRP, the protein that is lacking in Fragile X Syndrome, binds neuroligin-1 and -3 mRNA. Here, we employed in situ hybridization and examined the temporal and spatial expression patterns of neuroligin (NLGN) and neurexin (NRXN) mRNAs in the mouse somatosensory (S1) cortex and hippocampus in wild type (WT) and FMR1-KO mice during the first 5 weeks of postnatal life. Transient changes in gene expression were observed. Genotype-based differences in expression included increased NLGN1 and decreased NLGN2 mRNA in the CA1 and dentate gyrus regions of the hippocampus between female WT and FMR1-KO mice; decreased NRXN1 and increased NRXN3 mRNA in S1 cortex between female WT and FMR1-KO mice; and multiple differences in NRXN3 mRNA in hippocampus in males and females. Sex differences in hippocampal expression of NLGN2, NRXN1, NRXN2, and NRXN3 mRNAs and in S1 cortex expression of NRXN3 mRNAs were observed WT mice. In contrast, sex differences in NLGN3, NRXN1, NRXN2, and NRXN3 mRNA expression in the hippocampus and in NLGN1, NRXN2 and NRXN3 mRNA expression in S1 cortex were detected in FMR1-KO mice. These results provide a neuroanatomical map of NLGN and NRXN expression patterns over postnatal development in WT and FMR1-KO mice. The differences observed in the developmental trajectory of these synaptic proteins could contribute to long-term differences in CNS wiring and synaptic function.

Introduction

Neuroligins and neurexins are trans-synaptic cell adhesion molecules that are important for synapse maturation (Ichtchenko et al., 1995; Ichtchenko et al., 1996; Varoqueaux et al., 2006, reviewed in Craig and Kang, 2007; Krueger et al., 2012). The expression of neuroligins and neurexins is sufficient to induce presynaptic and postsynaptic specializations (Dean et al., 2003; Graf et al., 2004; Nam and Chen, 2005; Scheiffele et al., 2000) of both inhibitory and excitatory synapses respectively (Budreck and Scheiffele, 2007; Chih et al., 2005; Chubykin et al., 2007). Neuroligin-1 (NLGN1) is predominantly expressed at glutamatergic synapses where neuroligin-2 (NLGN2) is primarily localized at GABAergic synapses (Chubykin et al., 2007). Neuroligin-3 (NLGN3) is present at both glutamatergic and GABAergic synapses and forms heterodimers with NLGN1 or NLGN2 at those synapses respectively (Budreck and Scheiffele, 2007) and is expressed in non-neuronal cells (Kolozsi et al., 2009). The neurexins (NRXNs) are composed of three genes, neurexin-1, -2 and -3, each with α and β transcripts, making up six isoforms and thousands of splice variants (Missler and Sudhof, 1998; Tanaka et al., 2011; Ullrich et al., 1995). α-NRXNs have more extracellular domains than the β- NRXNs, but share the same C-terminus (Missler and Sudhof, 1998), which is the site that binds the NLGNs (Siddiqui et al., 2010). NRXN expression on specific cell types and resulting interaction with the NLGNs determines the synapse type that is formed (Futai et al., 2013; Mishina et al., 2012; Pregno et al., 2013; Zhang et al., 2010).

The expression of the NLGNs and NRXNs during development coincides with periods of functional synapse maturation. For instance, NLGN1 is present in embryonic rat brains, increases at birth to peak at postnatal day (P) 5-8 and peaks again in adulthood (Song et al., 1999). In contrast, NLGN2 expression in rat is detectable at E16 and peaks at P15-21 and maintained in adulthood (Varoqueaux et al., 2004). NLGN3 protein expression is present in utero, peaks at P14, and is maintained in adult rat (Budreck and Scheiffele, 2007). To date, the developmental expression trajectory of neurexin mRNAs has not been reported.

Fragile X Syndrome is a neurodevelopmental disorder, in which the FMR1 gene is silenced and thus, the gene product, FMRP, is not present. FMRP binds mRNA and regulates translation of many genes (Darnell et al., 2011). In *FMR1-KO* mice, transient synaptic changes have been observed during postnatal development (Cruz-Martin et al., 2010; Frankland et al., 2004; Meredith et al., 2012), suggesting that FMRP may play a regulatory role in both synaptic maturation and function. Recently, FMRP has been shown to directly bind to NLGN1, NRXN1, and NRXN3 (Darnell et al., 2011). Previous work in our lab has shown decreased NLGN3 mRNA expression in the hippocampal subregions and somatosensory cortex of mice exposed to valproic acid *in utero* (Kolozsi et al., 2009), an environment-induced model of ASD (Roullet et al., 2013). In the current study, we examined the expression profile of the neuroligin and neurexin mRNAs during postnatal development in *FMR1-KO* mice.

Materials and Methods

Animals

FVB/N-*fmr1* knock out (*FMR1-KO*; RRID:IMSR_JAX:003024) and FVB/N wild type (WT) mice were housed and bred at the McMaster University Central Animal Facility. All experiments were completed in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the McMaster Animal Research Ethics Board. Brain tissue was collected from male and female wild WT and *FMR1-KO* mice (n=4-6) at postnatal day 7 (P7), P14, P21, P28, and P35 from a total of 11 WT litters and 8 *FMR1-KO* litters, only 1 M and 1 F per time point per litter was included in the study.

Genotyping

Mice were genotyped using PCR. Primer sequences for the *fmr1* gene were 5`-CACGAGACTAGTGAGACGTG-3` (WT and *fmr1*-null) and 5`-TGTGATAGAATATGCAGCATGTGA-3` (WT) and 5`-CTTCTGGCACCTCCAGCTT-3` (*fmr1*-null). These yielded a *fmr1*-null band at 400 bp and a wild type band at 131 bp. Conditions were as

follows using the AmpliTaq Gold kit: 95ºC for 7 min, then a cycle of 95ºC for 30s, 62ºC for 30s and 72ºC for 1 min repeated 35 times, followed by an elongation period of 2 min at 72ºC. For confirmation of sex of P7 and P14 pups, detection of the SRY (sex-determining region on Y chromosome) gene was used with a MYO (myogenin) gene control (McClive and Sinclair, 2001). Primer sequences for the *sry* gene were 5`-TCATGAGACTGCCAACCACAG-3` and 5`-CATGACCACCACCACCACCAA-3` and sequences for the *myo* gene were 5`-TTACGTCCATCGTGGACAGC-3` and 5`-TGGGCTGGGTGTTAGTCTTA-3`. These yielded a sry band at 441bp and a *myo* band at 245bp. Conditions were as follows using the AmpliTaq Gold kit: 95ºC for 7 min, then a cycle of 95ºC for 30s, 63.5ºC for 21s and 72ºC for 21s repeated 40 times, followed by an elongation period of 7 min at 72ºC.

Tissue Sections

Brains were removed from the skull and rapidly frozen by immersion in 2-methylbutane at –70°C. Cryostat-cut 12 µm sagittal sections were thaw mounted onto gelatin-coated slides, dried, and stored at –35°C. Sagittal sections were collected from levels corresponding to the dorsal hippocampus and somatosensory cortex in the same plane (lateral 2.40-2.64 mm) (Paxinos and Franklin, 2001).

Riboprobes

Riboprobes for NLGN1-3 and NRXN1-3 were generated in our laboratory (Kolozsi et al., 2009). Antisense and sense riboprobes were transcribed from linearized plasmid DNA by *in vitro* transcription with ^{35}S-UTP (specific activity 1000 Ci/mmol; PerkinElmer, Boston, MA, USA) using appropriate RNA polymerases. No signal was detected using any of the sense riboprobes.

In Situ Hybridization

Tissue sections were pretreated with 4% formaldehyde, rinsed with PBS, rinsed with 0.1 M triethanolamine–HCl (TEA-pH 8.0) and acetylated with fresh 0.25% acetic acid in TEA. Tissue was dehydrated through an ethanol series, delipidated in chloroform, and air dried. Labeled

probes were diluted in hybridization buffer (0.6 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 10% dextran sulfate, 0.01% sheared salmon sperm DNA, 0.05% total yeast RNA, type XI, 0.01% yeast tRNA, and 1X Denhardt's solution) and applied to tissue sections, approximately 500,000 counts per minute (CPM) per tissue section. Tissue sections were hybridized under coverslips overnight at 55ºC for 16 to 18 h. To reduce non-specific binding, tissue sections were washed at room temperature with 20 µg/ml RNase solution (ribonuclease A from bovine pancreas, Sigma R4875, Oakville, ON, CA). One hour high stringency washes of 2X SSC at 50°C, 0.2X SSC at 55°C, and 0.2X SSC at 60°C were completed. The tissue was then dehydrated using ethanol containing 0.3 M ammonium acetate and air dried.

Autoradiography

Slides and ^{14}C plastic standards (American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) containing known radioactivity values were placed in X-ray cassettes and developed in an automatic film developer (Kodak Medical X-ray Processor, Richmond Hill, ON, CA). All sections for one riboprobe were processed at the same time and film was set up together for the same length of time. Developing time varied depending on the riboprobe: NLGN1 – 5 days, NLGN2 – 3 days, NLGN3, NRXN1 – 24 hours, NRXN2, NRXN3 – 2 days.

Data Analysis

Two sections per animal were analyzed for each riboprobe. Film images were captured using Quorum Technologies Qiacam digital camera (QImaging, Surrey, B.C.). mRNA expression in the somatosensory cortex , hippocampal cornu ammonis (CA)1, CA3 and dentate gyrus (DG) was quantified with NIH Image analysis software (http://rsb.info.nih.gov/nih-image). Light transmittance through the film was measured by outlining the structure on the monitor. Transmittance was converted to radioactivity levels (DPM, disintegrations per minute) using the Rodbard curve applied to the standards.

Statistical Analysis

Gene expression data was analyzed in SPSS for main effects of genotype, sex, and age by univariate analysis followed by Fisher's LSD post-hoc test. A p-value of <0.05 was considered statistically significant. All values are expressed as mean ± SEM.

Results

Neuroligin-1 mRNA Expression

The developmental temporal and spatial patterns of expression for NLGN1 mRNA were examined in WT and *FMR1-KO* mice (Table 1). Representative film images of NLGN1 mRNA expression are provided in Figure 1. In the CA1, NLGN1 mRNA expression levels decreased across development in both WT and *FMR1-KO* mice (main effect of age, $F[4,85]=6.91$, $p<0.0001$). In WT males, a decrease from postnatal day (P) 7 to P28 and P35; P14 to P28 and P35; P21 to P28 and P35 was observed ($p<0.05$). In *FMR1-KO* males, there was a similar pattern of decreasing expression throughout postnatal development. Observed decreases included a difference from P7 to P28 and P35; P14 to P35; P21 to P28 and P35 ($p<0.05$). Expression levels in WT females were highest at P7 in the CA1 and decreased gradually over the first five weeks of postnatal development. A decrease from P7 to P28 and P35 was observed ($p<0.05$). In contrast, the expression level of NLGN1 mRNA over postnatal development was steady in *FMR1-KO* females. A difference in NLGN1 mRNA levels in the CA1 regions was observed between WT and *FMR1-KO* females at P28 ($p<0.0.5$).

In the CA3, NLGN1 mRNA expression levels decreased over postnatal development in both WT and *FMR1-KO* mice (main effect of age, $F[4,86]=20.4$, $p<0.0001$). In WT males, expression levels decreased from P7 to P28 and P35, from P14 to P35, and P21 to P35 ($p<0.05$). In *FMR1-KO* male mice, the decreases in expression levels across development were similar to the WT males and were observed between P7 to P21, P28, and P35 and P14 to P35 ($p<0.05$). In female WT mice NLGN1 mRNA expression decreased between P7 and P21, P28 and P35 and from P14 to P28 and P35 ($p<0.05$). Similarly, in *FMR1-KO* females, mRNA expression levels were highest at P7 compared to all other time points ($p<0.05$). There were no differences related to genotype or sex on the expression of NLGN1 mRNA in CA3.

In the dentate gyrus (DG), expression levels for NLGN1 mRNA levels did not change over development in WT and *FMR1-KO* mice. While there was no main effect of age

(F[1,86]=1.12, p=0.35), genotype (F[1,86]=2.68, p=0.11), or sex (F[1,86]=1.52, p=0.22) in NLGN1 mRNA expression in the DG; pairwise comparisons revealed a significant increase in NLGN1 mRNA in female *FMR1-KO* mice compared to female WT mice ($p<0.05$).

In the somatosensory cortex (S1), there was a decrease in NLGN1 mRNA expression over development in WT and *FMR1-KO* mice (main effect of age, F[1,78]=29.2, $p<0.0001$). In WT males, there was decrease in expression between P7 and all later time points and from P14 to P28 and P35 ($p<0.05$). In *FMR1-KO* males, NLGN1 mRNA expression levels decreased between P7 and P28and P35 and P14 and P35 ($p<0.05$). In WT females, NLGN1 mRNA expression levels were highest at P7 compared to all other time points and decreased from P21 and P35 ($p<0.05$). In *FMR1-KO* females, NLGN1 mRNA expression levels were highest at P7 compared to all other time points ($p<0.05$). Increased expression of NLGN1 mRNA in S1 was observed in female *FMR1-KO* mice compared to female WT, and compared to male *FMR1-KO* mice ($p<0.05$).

Table 5-1. Neuroligin-1 (NLGN1) mRNA expression in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NLGN1 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	586.7 ± 48.1	582.6 ± 50.2	646 ± 65.8	565.3 ± 27.6
	14	575.5 ± 67.4	519.2 ± 76.2	597.9 ± 65.6	521.7 ± 28.5
	21	556.5 ± 61.6	540.9 ± 33	563.3 ± 28.5	455 ± 28.8
	28	404.7 ± 20.7	**414.4 ± 47.7**[a]	468.9 ± 49.2	**573.2 ± 68.9**[a]
	35	417.4 ± 40	410 ± 31.9	426.2 ± 26.1	516.5 ± 38.3
CA3	7	911.8 ± 85.9	1008.6 ± 116.1	1065.7 ± 125.1	1080.5 ± 39.4
	14	790 ± 63.1	782.4 ± 125.6	851 ± 130.4	740.7 ± 42.3
	21	785.9 ± 104.5	725.5 ± 46.7	785.1 ± 32.2	611 ± 61.2
	28	583.1 ± 56.3	507.5 ± 42.3	628.8 ± 93.7	721.7 ± 107
	35	521.5 ± 42.7	539.2 ± 34.6	556.1 ± 23.7	613.8 ± 53.8
DG	7	437.2 ± 65.9	420.5 ± 19.4	491.1 ± 52	411.4 ± 26.1
	14	464.7 ± 20.3	472.4 ± 67.1	537.7 ± 66	423.9 ± 20.4
	21	536.3 ± 72.5	461.2 ± 48.3	517.8 ± 38.8	422.2 ± 20.4
	28	378.9 ± 32.4	**376.1 ± 16.8**[a]	467.5 ± 53.3	**519 ± 104.7**[a]
	35	402.8 ± 41	397 ± 24.4	424.4 ± 20.1	487.1 ± 46.2
S1	7	436.9 ± 66.3	**417.2 ± 59.6**[a]	**358.8 ± 24.3**[b]	**506.1 ± 58.6**[a,b]
	14	331.3 ± 4.7	254.3 ± 39.5	324.4 ± 31	283.7 ± 13.9
	21	276 ± 20.2	289.4 ± 34.2	283 ± 13.7	313.7 ± 25
	28	208.9 ± 16.7	216.9 ± 11.3	254.7 ± 45.9	225.2 ± 25.1
	35	223.9 ± 11.2	198.9 ± 16.3	198.2 ± 10	244.7 ± 29.4

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1, CA3 and dentate granule layer (DG). mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype
[b] statistically significant effect of sex

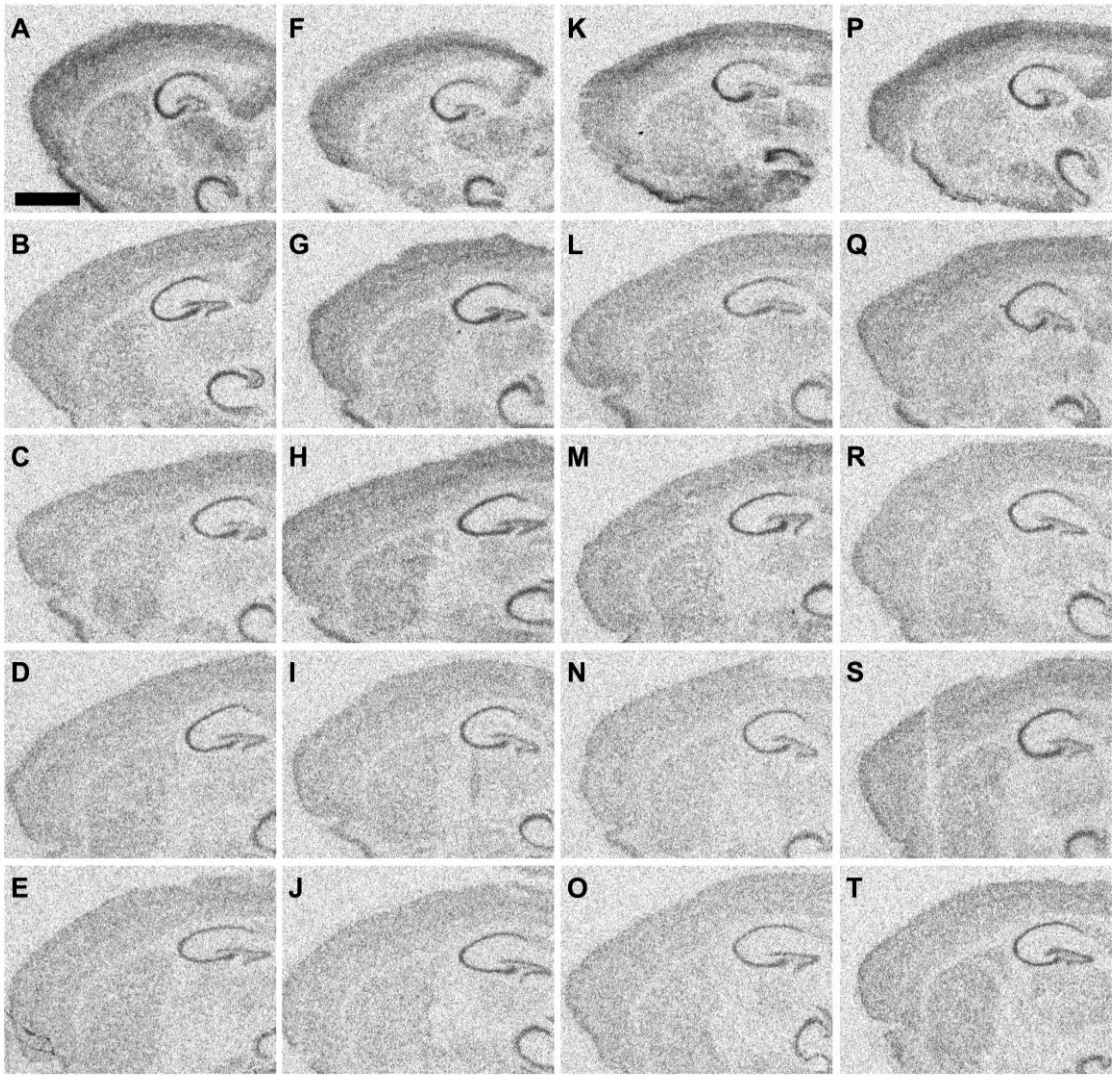

Figure 5-1 – Neuroligin-1 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Neuroligin-2 mRNA Expression

NLGN2 mRNA expression increased over postnatal development in WT and *FMR1-KO* mice (Table 2). Representative film images of NLGN2 mRNA expression are provided in Figure 2. In the CA1, NLGN2 mRNA expression levels increased throughout the first three weeks ($F[4,92]=7.74$, $p<0.0001$). In addition, there was a genotype by age interaction ($F[4,92]=2.6$, $p=0.04$). In male WT mice an increase in expression was observed between P7 and P14, P21, P28, and P35 ($p<0.05$). A similar temporal expression patterns was observed in *FMR1-KO* males. An increase between P7 and all other time points was observed ($p<0.05$). WT females showed an increase in expression levels between P7 and P21 at which point expression levels peaked before decreasing at P28 and P35 ($p<0.05$). The trajectory of expression for NLGN2 mRNA was altered in *FMR1-KO* female mice compared to WT female WT mice. Qualitatively, expression levels of NLGN2 mRNA in *FMR1-KO* female mice showed a steady increase over postnatal development and the peak of expression at P21 observed in WT female mice was not present. An increase in expression levels was observed from P7 and P35; from P17 and P35 in *FMR1-KO* females ($p<0.05$). Genotype differences included reduced NLGN2 mRNA expression at P21 in *FMR1-KO* female mice compared to WT female mice.

In the CA3, there was a main effect of age in NLGN2 mRNA expression over development ($F[4,91]=4.83$, $p=0.001$). In WT males, there was increased expression from P7 to P21and P28 ($p<0.05$). In *FMR1-KO* male mice, there was an increase in NLGN2 mRNA from P7 to P28 ($p<0.05$). In WT females, NLGN2 mRNA expression levels increased from P14 to P21 and decreased from P21 to P28 ($p<0.05$). In *FMR1-KO* females, an increase in NLGN2 mRNA levels was observed between P14 and P35 ($p<0.05$).

In the DG, expression levels in NLGN2 mRNA increased over postnatal development (main effect of age: $F[4,92]=11.2$, $p<0.001$). In addition there was an effect of sex ($F[4,92]=6.08$,$p=0.016$) and an interaction between genotype and age ($F[4,92]=2.70$, $p=0.036$) in NLGN2 mRNA expression in the DG. In WT males, increased expression was observed from P7

to P21, P28, and P35 and from P14 to P21 and P28 ($p<0.05$). In *FMR1-KO* males NLGN2 mRNA expression levels in DG increased from P7 to P14, P21, P28, and P35. In WT females, expression levels were highest at P21 with an increase from P7 and P14 to P21 followed by a decrease from P21 to P28 and P35. In *FMR1-KO* females NLGN2mRNA showed a steady increase over development with an increase in NLGN2 mRNA expression from P7 to P28 and P35 ($p<0.05$). A decrease in NLGN2 mRNA expression was observed between WT female mice in DG at P21 and *FMR1-KO* female mice ($p<0.05$). In addition, a sex difference was observed in WT mice in which NLGN2 mRNA was increased in WT male mice at P28 compared to WT female mice ($p<0.05$).

In the S1, there was an effect of age on NLGN2 mRNA expression during postnatal development ($F[4,88]=3.14$, $p=0.018$). In WT male mice, there was an increase in NLGN2 expression between P7 and P14, P21, and P28 ($p<0.05$). In *FMRI-KO* male mice, there was an increase between P7 and P35 ($p<0.05$). In WT female mice, there was an increase from P21 to P28 and P35 ($p<0.05$), whereas no differences in NLGN2 mRNA expression were observed in *FMR1-KO* female mice.

Table 5-2. Neuroligin-2 (NLGN2) mRNA expression in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NLGN2 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	209.2 ± 52.5	271.5 ± 36.9	245.3 ± 23	290.9 ± 54.9
	14	348 ± 39.5	249 ± 23.3	405.4 ± 28.6	294.2 ± 34.7
	21	460.8 ± 93.5	**505.5 ± 43**[a]	388.7 ± 32.2	**325.8 ± 54.6**[a]
	28	448.8 ± 71.4	294.4 ± 14.9	382 ± 49.8	412.5 ± 48.3
	35	407.39 ± 42.8	331.8 ± 43.2	422.7 ± 32.4	441.3 ± 48.7
CA3	7	231.3 ± 51.6	274 ± 46.5	263.3 ± 27.1	292.6 ± 53.8
	14	271.9 ± 29.1	239.4 ± 15.7	288.7 ± 33.6	255.7 ± 29.6
	21	385.7 ± 69.2	391 ± 27	332.1 ± 25.3	324.7 ± 39.6
	28	379.4 ± 54.7	257.7 ± 14.3	376.8 ± 45.9	343.9 ± 42.9
	35	335.5 ± 37.1	289.1 ± 37.6	358.9 ± 38.6	390.4 ± 51.2
DG	7	209.9 ± 43.7	227 ± 40.5	211.4 ± 24.4	232.6 ± 33.7
	14	295.6 ± 37.2	226 ± 8.4	370.7 ± 39.2	263.1 ± 38.4
	21	443.8 ± 80.8	**447.1 ± 29.2**[a]	376.4 ± 32.4	**309.3 ± 48**[a]
	28	**417.6 ± 61.4**[b]	**282.3 ± 18.1**[b]	379.8 ± 39.8	378.7 ± 49.3
	35	367.9 ± 31.6	291.2 ± 27	416.3 ± 38.5	372.2 ± 36
S1	7	204.2 ± 19.1	272.1 ± 34.4	277.7 ± 33.3	291 ± 39.6
	14	318.7 ± 25.6	275 ± 27.7	327.2 ± 4.5	321.5 ± 27.6
	21	357.8 ± 51.9	368.8 ± 44	319.3 ± 25.7	319.3 ± 38.1
	28	319.2 ± 37.1	255.6 ± 12.3	299.9 ± 21.3	271.5 ± 26.2
	35	280.1 ± 20.8	261.8 ± 38.6	367.7 ± 36.8	347.3 ± 45.1

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1, CA3 and dentate granule layer (DG). mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype
[b] statistically significant effect of sex

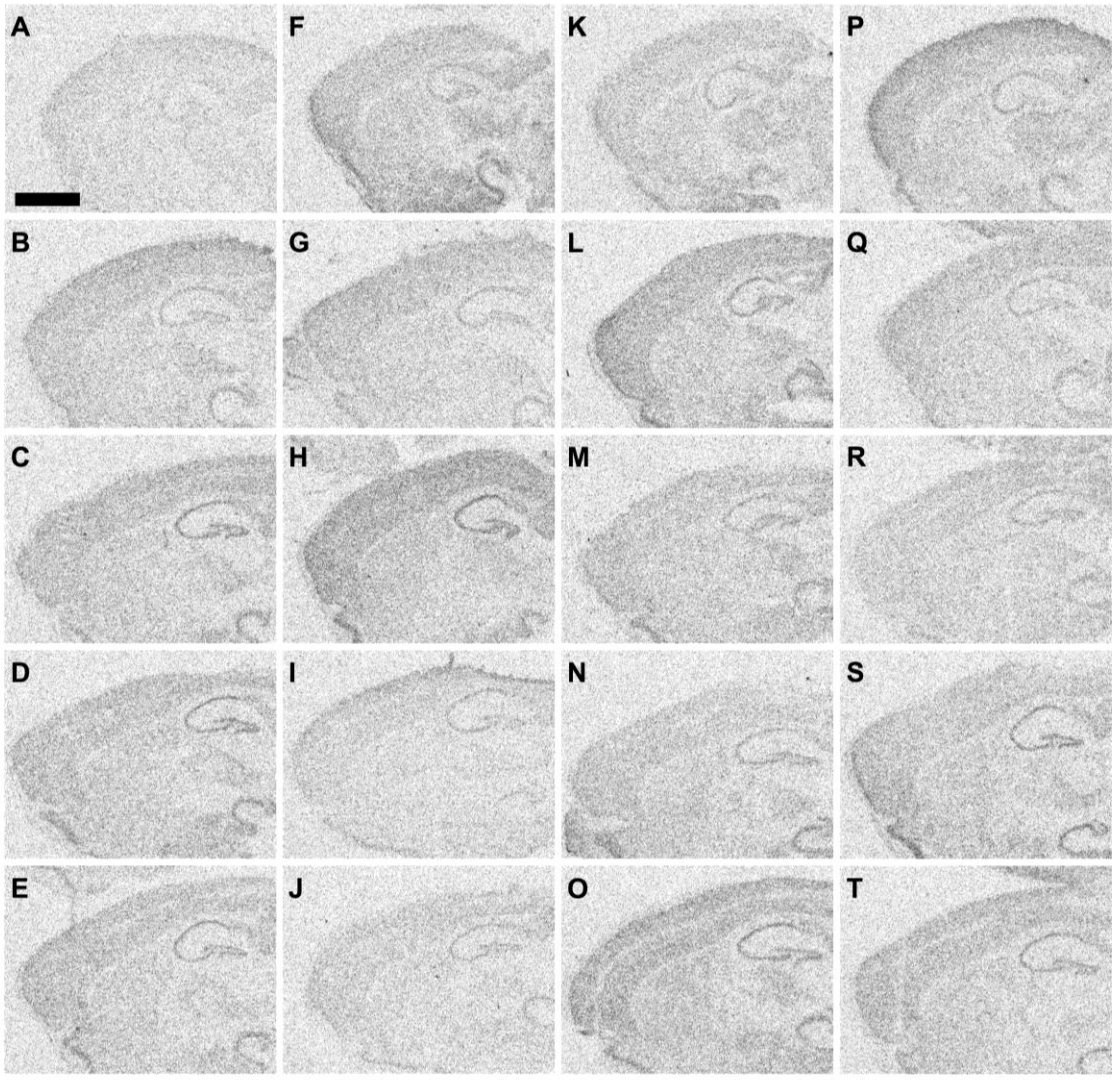

Figure 5-2 – Neuroligin-2 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Neuroligin-3 mRNA Expression

The trajectory of NLGN3 mRNA expression differed from NLGN1 and NLGN2, generally peaking earlier on in development (Table 3). Representative film images of NLGN3 mRNA expression are provided in Figure 3. In the CA1, expression levels peaked at P14 across both genotypes and sexes (main effect of age; $F[4,93]=16.5$, $p<0.0001$). In WT males, a decrease in NLGN3 mRNA was observed from P14 to P28 and P35 ($p<0.05$). *FMR1-KO* males had a similar trajectory; there was a decrease in NLGN3 mRNA expression from P7 to P28 and P35 and from P14 to P21, P28, and P35. In WT females, a decrease in NLGN3 mRNA expression levels was seen from P14 to P21, P28, and P35 ($p<0.05$). *FMR1-KO* females showed an increase in NLGN3 mRNA expression between P17 and P17 followed by a decrease in levels from P14 to P21, P28, and P35 ($p<005$). No differences were observed for genotype or sex.

In the CA3, NLGN3 mRNA expression levels showed higher levels of expression at P7 and P14 followed by a gradual decrease in expression levels (main effect of age: $F[4,93]=13.9$, $p<0.0001$). Specifically, in WT males showed a decrease from P6 to P35 and from P14 to P35 ($p<0.05$). Similarly, in *FMR1-KO* males a decrease in NLGN3 mRNA was observed from P7 to P28, and P35 and from P17 to P21, P28, and P35 ($p<0.05$). In WT females, expression levels decreased from P14 to P21 and P35. In *FMR1-KO* females, the expression level at P14 was higher than P21, P28, and P35 ($p<0.05$). No differences were observed for genotype or sex.

In the DG, NLGN3 mRNA expression peaked at P14 in both males and females in WT and *FMR1-KO* mice (main effect of age: $F[4,93]=12.3$; $p<0.0001$). In WT males, NLGN3 mRNA levels increased from P7 to P14 and then decreased from P14 to P21 and P35 ($p<0.05$). In *FMR1-KO* males, there was an increase in expression from P7 to P14, and a decrease from P14 to P21, P28, and P35 ($p<0.05$). In females, WT mice had increased expression of NLGN3 mRNA from P7 to P14, and a decrease from P14 to P21 and P35 ($p<0.05$). In *FMR1-KO* females, there was a similar pattern of increased expression from P7 to P14 and then a decrease from P14 to P21 and P35 ($p<0.05$). There was an effect of sex ($F[4,93]=3.8$, $p=0.05$). Post-hoc comparisons

showed an increase in NLGN3 mRNA expression in the DG of female *FMR1-KO* mice compared to male *FMR1-KO* mice ($p<0.05$).

In the S1, NLGN3 mRNA expression levels were highest in the first two weeks and decreased over development in both genotypes and sexes (main effect of age: $F[4,93]=28.6$, $p<0.0001$) . WT males had a decrease in NLGN3 mRNA expression from P7 to P35, and from P14 to P21, P28, and P35 ($p<0.05$). In *FMR1-KO* males, NLGN3 mRNA levels were similarly decreased from P7 to P21, P28 and P35 and from P14 to P21, P28, and P35 ($p<0.05$). In WT females, NLGN3 mRNA expression levels decreased from P7 to P21 and P35 and from P14 to P21, P28 and P35 ($p<0.05$). In *FMR1-KO* female mice, NLGN3 mRNA expression levels at P7 and P14 were higher compared to all other time points ($p<0.05$). There were no differences in NLGN3 mRNA expression due to genotype or due to sex in the S1.

Table 5-3. Neuroligin-3 (NLGN3) mRNA expression in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NLGN3 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	2370.2 ± 443.6	2631.7 ± 714.2	2627.7 ± 528	2957.8 ± 271.3
	14	3256.3 ± 520	3612.5 ± 475.3	3882.4 ± 484.9	4328.6 ± 606.2
	21	2139.4 ± 372.5	1449.5 ± 63.6	2076.4 ± 410.3	2123.2 ± 434.9
	28	1959.1 ± 400.2	2318.7 ± 567.7	1430.4 ± 129.6	2412.6 ± 542.4
	35	1293.1 ± 200.5	1712.2 ± 347.8	1104.4 ± 159.5	1741.3 ± 260.4
CA3	7	2690 ± 565.5	2635.6 ± 407.6	2981.6 ± 453.9	3360.2 ± 275.5
	14	3274.9 ± 620.5	3885 ± 584.6	3922.1 ± 758.4	4322.6 ± 558.1
	21	2224.5 ± 403.1	1437.4 ± 106.4	2070.7 ± 409.9	2333.9 ± 425.8
	28	2220.7 ± 625.1	2637.1 ± 699.9	1414.3 ± 171.2	2621.5 ± 509.1
	35	1340.1 ± 247.1	1921.8 ± 439	1235.9 ± 141	1953.1 ± 297.6
DG	7	1934.7 ± 336.1	2175.5 ± 489.4	2306.7 ± 522.9	2482.3 ± 184.7
	14	3661.9 ± 503.4	4391.2 ± 621.2	4130.7 ± 499.8	4596.3 ± 567.3
	21	2733.1 ± 481.3	1923.3 ± 117	2640.9 ± 517.3	2999.1 ± 552.7
	28	2680 ± 526.9	3040.8 ± 635.8	**1957.7 ± 248.6[b]**	**3482.6 ± 905.3[b]**
	35	1802 ± 249.4	2239.5 ± 478.8	1628.2 ± 169.9	2364.4 ± 336.7
S1	7	1537.5 ± 342.3	1510.3 ± 337.6	1657.5 ± 294.6	1898.5 ± 121.6
	14	1704.3 ± 252.9	2120.6 ± 327.1	2149.3 ± 252.1	2317.4 ± 307.2
	21	1023.2 ± 132.9	649.5 ± 48.8	1021.1 ± 228	1123.3 ± 211.2
	28	926.4 ± 176.5	957.0 ± 215.5	677.5 ± 97.6	997.6 ± 233.2
	35	540.9 ± 95.5	810.2 ± 183.3	529.9 ± 80.2	772.6 ± 117

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1, CA3 and dentate granule layer (DG). mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype – none
[b] statistically significant effect of sex

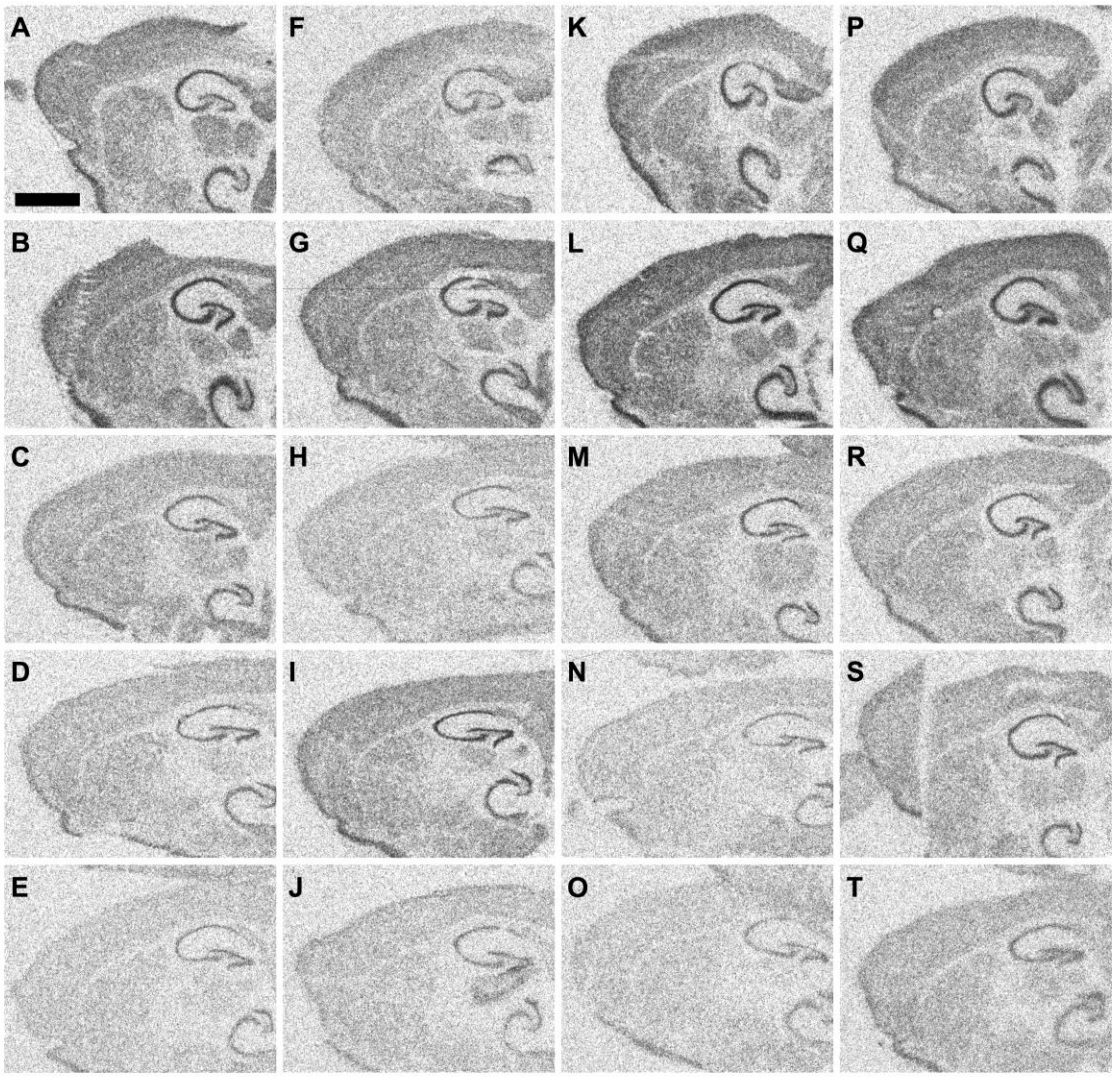

Figure 5-3 – Neuroligin-3 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Neurexin-1 mRNA Expression

Comparison of NRXN1 mRNA expression levels in WT and *FMR1-KO* mice over development is summarized in Table 4. Representative film images of NRXN1 mRNA expression are provided in Figure 4. Across brain regions, high levels of NRXN1 mRNA were observed during the first and second week of postnatal development. In the CA1, mRNA expression levels were quite variable within each time point and a decrease was observed across postnatal development (main effect of age: $F[4,89]=6.39$, $p<0.0001$). In WT males, qualitatively, NRXN1 mRNA levels were relatively low at P35 in comparison to earlier time points. In *FMR1-KO* males, NRXN1 mRNA expression levels decreased from P7 to P35, from P14 to P21 and P35, and from P21 to P35 ($p<0.05$). In WT females, there was a decrease in expression across postnatal development with decreases from P7 to P28 and P35; as well as P14 compared to P21, P28 and P35 ($p<0.05$). In *FMR1-KO* females, a decrease in NRX1 mRNA was observed from P14 to P21, P28 and P35 ($p<0.05$).

In the CA3, expression levels of NRXN1 mRNA decreased in both male and female WT and *FMR1-KO* mice over development ($F[4,89]=11.4$, $p<0.001$). There was also an effect of sex ($F[4,89]=5.33$, $p=0.023$). There was a qualitative decrease in NRXN1 mRNA expression across development in WT males with lowest levels at P35 and a decrease between P7 and P35 ($p<0.05$). In *FMR1-KO* males, expression levels decreased from P7 to P21 and P35. Expression levels in WT females were highest at P7 and decreased across development with statistically discernable decreases between P7 and P14, P21, P28, and P35. In *FMR1-KO* females, a decrease in NRXN1 mRNA expression over development was present but more gradual. Post-hoc comparisons showed decreases between P7 and P21, P28 and P35. Furthermore, there were decreases from P14 to P28 and P35 ($p<0.05$). In addition, there was a sex difference in WT mice at P7 ($F[1,44]=16.15$, $p=0.0002$), where WT males had increased NRXN1 mRNA expression compared to females in CA3 at P28 ($p<0.05$).

In the DG, there was a main effect of age on NRXN1 mRNA expression levels over development ($F[4,89]=6.38$, $p<0.001$). Expression levels were stable across time points were in WT males; however, in *FMR1-KO* males, mRNA levels decreased from P7 and P14 to P35 ($p<0.05$). Female WT mice had highest NRXN1 mRNA expression levels at P7, and showed a decrease between P7 to P28 and P35 ($p<0.05$). In addition, there were decreases from P14 to P28 and P35 ($p<0.05$). In *FMR1-KO* females, there was a peak in NRXN1 mRNA expression at P14 that was not observed in the WT trajectory. Corresponding to that, post-hoc comparisons indicated a decrease in NRXN1 mRNA expression from P14 to P28 and P35 ($p<0.05$).

In the S1, NRXN1 mRNA expression levels decreased across development ($F[4,86]=15.7$, $p<0.001$). In WT males, levels were highest at P7 and a decrease in NRXN1 mRNA expression was observed from P7 to P35 and from P14 to P35 ($p<0.05$). In *FMR1-KO* males, levels decreased gradually across development, but had a transient increase at P28. Post-hoc comparisons across ages showed NRXN1 mRNA expression levels decreasing from P7 to P21 and P35, between P14 and P35 as well as from P28 to P35 ($p<0.05$). In WT females, NRXN1 mRNA expression levels decreased from P7 onward. Post-hoc comparisons reveal differences between P7 and all other time points and from P14 to P21, P28, and P35 ($p<0.05$). The pattern of NRXN1 mRNA expression was different in *FMR1-KO* females, which had lower levels of expression compared to WT females at P7 ($p<0.05$). Expression of NRXN1 mRNA in *FMR1-KO* females decreased from P7 to P28 and P35; from P14 to P28 and P35 ($p<0.05$). In addition, NRXN1 mRNA expression was decreased in female *FMR1-KO* S1 at P7 compared to WT female S1 ($p<0.05$).

Table 5-4. Neurexin-1 mRNA (NRXN1) in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NRXN1 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	2380.1 ± 746.4	2596.5 ± 592	2545.1 ± 397.9	2098.6 ± 281
	14	2437.7 ± 885.6	2810.5 ± 1255	3527.3 ± 694.7	3415.1 ± 580
	21	2147.9 ± 801.5	845.8 ± 179	1501.1 ± 420.5	1441.2 ± 484.2
	28	**2254.6 ± 643**[b]	**322.5 ± 49.2**[b]	3137 ± 1009.3	1249.5 ± 722.2
	35	1396.5 ± 1005	371.3 ± 18.7	740.7 ± 195.6	1209.5 ± 530.2
CA3	7	7014.5 ± 2753	7546.9 ± 1409.6	7381.2 ± 1113	6656.5 ± 925.5
	14	4896.5 ± 1773	3236.8 ± 909.8	4980.6 ± 917.2	5625.4 ± 940.9
	21	4015.9 ± 1581	1200.6 ± 212.5	2873.8 ± 1108	2633.5 ± 927.6
	28	**3913.1 ± 1097**[b]	**552.2 ± 76.4**[b]	4961.6 ± 1461	2111 ± 1240.1
	35	2149.4 ± 1448	561.1 ± 67.7	2856.2 ± 1044	2213 ± 1226.2
DG	7	2509.7 ± 813.6	2874.5 ± 601.7	3027.7 ± 601.0	2508.1 ± 329
	14	2477.7 ± 895.8	2800.5 ± 1360.9	3631.4 ± 741.6	3299.4 ± 647.7
	21	2360.9 ± 958	907.3 ± 199	1674.5 ± 556.2	1575 ± 474.1
	28	2105.4 ± 532	373.3 ± 60	**3303.2 ± 1014**[b]	**1317.3 ± 722.7**[b]
	35	1158.3 ± 749	346.7 ± 49.5	864.5 ± 186.6	1396.1 ± 618.3
S1	7	2400.7 ± 567.6	**3712.6 ± 92.6** [a]	3314.4 ± 605.8	**2297.3 ± 329** [a]
	14	1840.6 ± 834.6	1828.5 ± 921.5	2200.5 ± 353.8	2332.3 ± 584.5
	21	1582.6 ± 624.4	449.1 ± 93.1	961.2 ± 313.8	1025.8 ± 389.1
	28	1100.4 ± 335	208.6 ± 34.3	1869.3 ± 630.4	613.7 ± 368.16
	35	578.4 ± 327.1	178 ± 13.4	420.1 ± 105.9	762.3 ± 432.2

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1, CA3 and dentate granule layer (DG). mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype
[b] statistically significant effect of sex

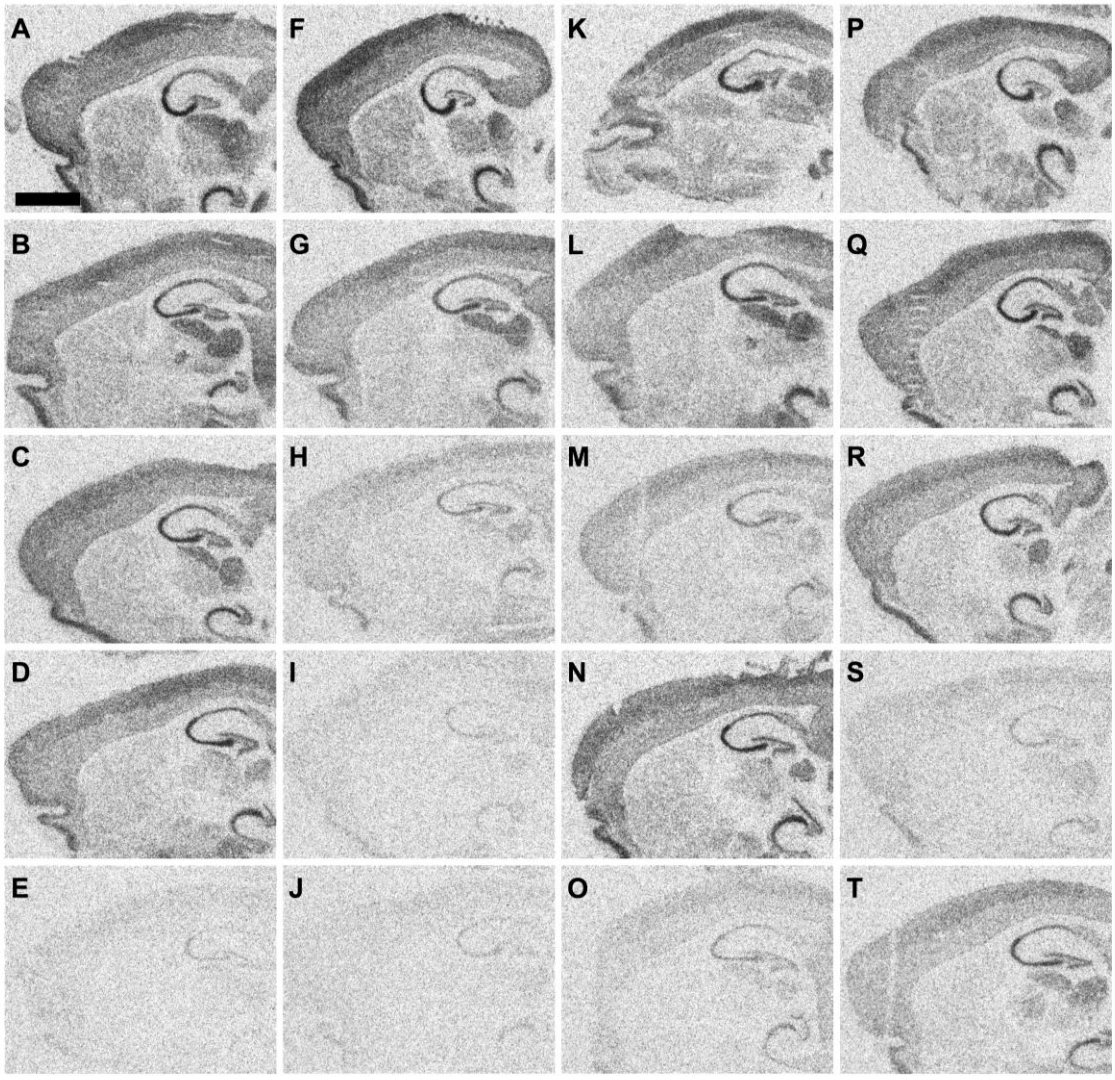

Figure 5-4 – Neurexin-1 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Neurexin-2 mRNA Expression

The developmental temporal and spatial patterns of expression for NRXN2 mRNA were examined in WT and *FMR1-KO* mice (Table 4). Representative film images of NRXN2 mRNA expression are provided in Figure 4. While there was no difference in NRXN2 mRNA expression between genotypes in any of the regions examined, there were age-effects in developmental trajectory in NRXN2 mRNA expression.

In the CA1, NRXN2 mRNA expression levels decreased across development ($F[4,93]=24.3$, $p<0.05$). In WT males, NRXN2 mRNA levels decreased from P7 to P21, P28 and P35 as well as from P14 to P21 and P35 ($p<0.05$). In *FMR1-KO* males, there was a decrease in NRXN2 mRNA expression levels from P7 to all the other ages ($p<0.05$). In addition, NRXN2 mRNA expression levels decreased between P14 and P35 ($p<0.05$). In WT females, NRXN2 mRNA levels decreased between P7 and P14, P21, P28, and P35 and from P14 to P28 and P35 ($p<0.05$).. In *FMR1-KO* females, there were decreases in NRXN2 mRNA expression from P7 to all later time points as well as from P14 to P21, P28 and P35 ($p<0.05$).

In the CA3, NRXN2 mRNA expression levels decreased across development ($F[4,93]=64.2$, $p<0.0001$). In WT males, a decrease in NRXN2 mRNA expression was observed between P7 and all other time points as well as from P14 to all later time points ($P<0.05$).. *FMR1-KO* males had a similar trajectory, with decreases in NRXN2 mRNA expression between P7 and all other time points and between P14 and P35 ($p<0.05$). In WT females, NRXN2 mRNA expression levels was higher at P7 compared to all other time points and showed a decrease from P14 to P35 ($p<0.05$). In *FMR1-KO* females, there was a decrease in NRXN2 mRNA expression between P7 to all other time points and from P14 to P28 and P35 ($p<0.05$). A sex difference was observed in NRXN2 mRNA in the CA3 region at P7 between male and female *FMR1-KO* mice ($p<0.05$).

In the DG, the pattern of NRXN2 mRNA expression over development was different than the other hippocampal regions as levels were more stable across the development, and yet there was an effect of age with lower levels expressed later in development ($F[4,93]=5.8$, $p<0.0001$). In WT males, there were no differences in NRXN2 mRNA expression over time. In *FMR1-KO* males, NRXN2 mRNA levels were relatively stable, although a decrease in NRXN2 mRNA expression was observed between P7 and P35 ($p<0.05$). In WT females, NRXN2 mRNA expression levels gradually decreased; post-hoc comparisons showed a decrease in NRXN2 mRNA expression from P7 to P28 and P35 and from P14 to P35 ($p<0.05$). In *FMR1-KO* females, NRXN2 mRNA expression levels peaked at P14 and stayed high before decreasing at P28. Post-hoc comparisons showed an increase in expression from P7 to P14 and a decrease from P14 to P28 and P35 ($p<0.05$).

In the S1, a consistent gradual decrease in NRXN2 mRNA expression levels across postnatal development was observed in all mice ($F[4,89]=18.7$, $p<0.0001$). In WT males, NRXN2 mRNA expression levels decreased between P7 and P21, P28, and P35 ($p<0.05$). In *FMR1-KO* males, NRXN2 mRNA expression levels decreased between P7 and all other ages ($p<0.05$). In WT females, levels decreased across time, however, expression levels at P7 were more variable than at other time points. Post-hoc comparisons revealed NRXN2 mRNA decreases from P7 and P21, P28, and P35 and between P14 and P35 ($p<0.05$). In *FMR1-KO* females, NRXN2 mRNA expression decreased from P7 to P28 and P35 and from P14 to P28 and P35 ($p<0.05$).

Table 5-5. Neurexin-2 (NRXN2) mRNA expression in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NRXN2 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	1623.0 ± 266.8	2001 ± 565.1	1995.9 ± 142.1	1575.1 ± 164
	14	1408.3 ± 172	1332.5 ± 173.2	1313.8 ± 220.4	1562.5 ± 65.6
	21	893.1 ± 87	989.8 ± 127.5	1042.4 ± 83.6	1041.2 ± 142.1
	28	1065.9 ± 78.2	814.2 ± 118.6	931.4 ± 104.7	707.1 ± 85.1
	35	895.1 ± 137.8	800.4 ± 96.4	766.8 ± 106.3	711.6 ± 85.1
CA3	7	2856.7 ± 256	2614.2 ± 573.9	**3114 ± 184.4**[b]	**2580.4 ± 191.3**[b]
	14	1909.1 ± 256	1566 ± 160.9	1633.6 ± 93.6	1815.5 ± 127.7
	21	1010.3 ± 119.2	1130.5 ± 196.3	1211.2 ± 84	1332 ± 125.7
	28	1381.9 ± 132	1051.7 ± 145.2	1086.8 ± 110.7	889 ± 100.5
	35	1000.1 ± 127.4	977.2 ± 125	873.4 ± 117.5	855.3 ± 126.4
DG	7	1472.4 ± 169.6	1618.1 ± 375.2	1596.1 ± 154.9	1237.3 ± 90.7
	14	1597.7 ± 172.6	1483.3 ± 167.4	1365 ± 127.5	1626.8 ± 78.1
	21	1181.5 ± 93.4	1212.5 ± 134.4	1394.5 ± 117.6	1483.4 ± 160.1
	28	1481.2 ± 98.8	1106.6 ± 111.2	1340.2 ± 116.8	1006.1 ± 84.5
	35	1273.3 ± 162.7	1058.5 ± 99.5	1103.2 ± 120.2	976.3 ± 91.1
S1	7	1530.5 ± 298.7	1748.6 ± 582	**1916.4 ± 171.1**[b]	**1285.2 ± 163.2**[b]
	14	1101.2 ± 135.8	1361.1 ± 240.8	1217.4 ± 225.6	1267.9 ± 88.4
	21	830.7 ± 83.3	897 ± 100.4	1086.6 ± 109.6	948.1 ± 45.6
	28	877.6 ± 94.1	764.1 ± 126.3	735.5 ± 72.9	690.5 ± 31.5
	35	750.5 ± 132.5	662.6 ± 63	706.9 ± 116	565.3 ± 81.3

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1, CA3 and dentate granule layer (DG). mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype – none
[b] statistically significant effect of sex

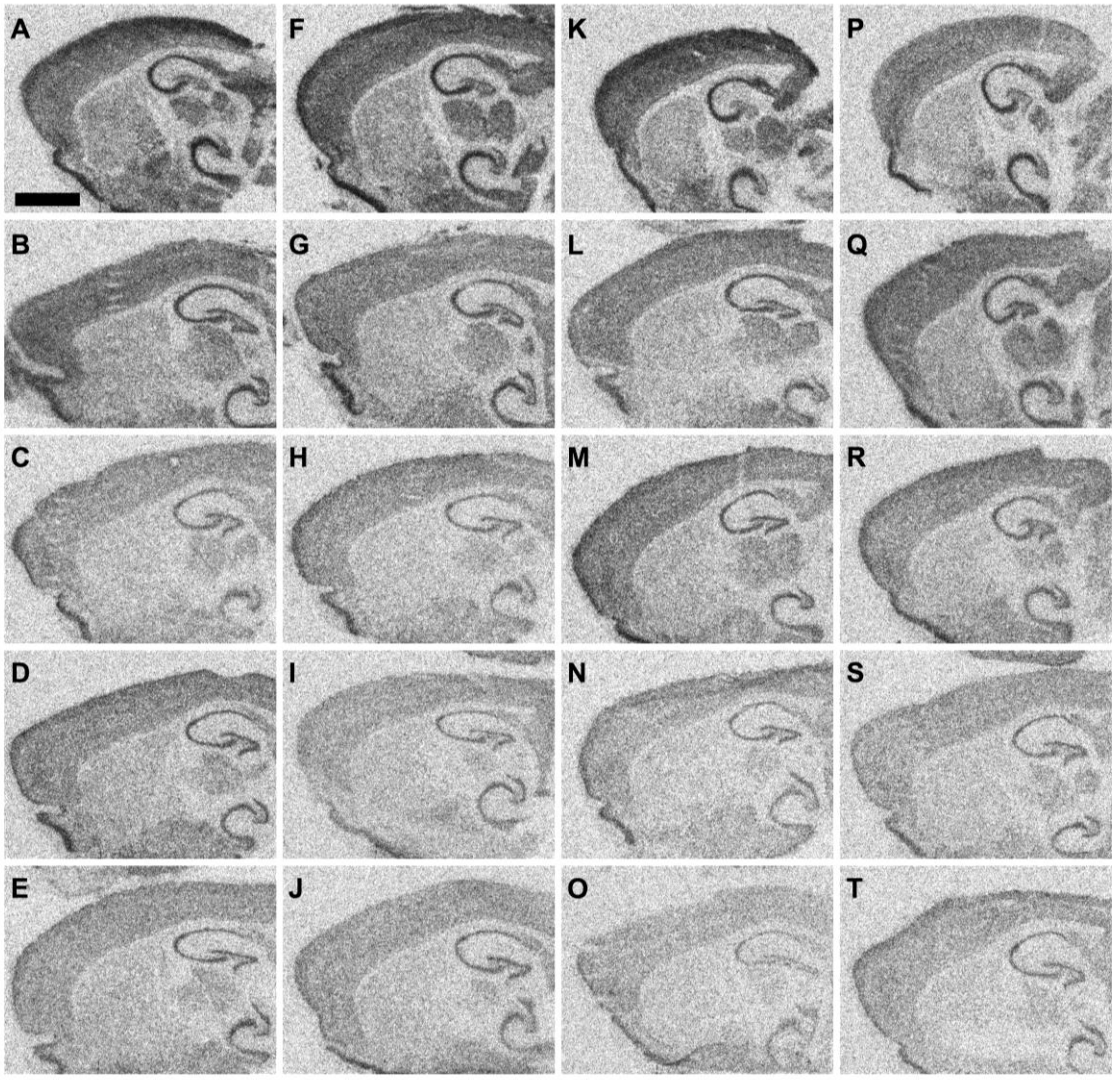

Figure 5-5 – Neurexin-2 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Neurexin-3 mRNA Expression

The developmental temporal and spatial patterns of expression for NRXN3 mRNA were examined in WT and *FMR1-KO* mice (Table 6). Representative film images of NRXN3 mRNA expression are provided in Figure 6. NRXN3 mRNA expression levels generally peaked in the second and third week of life. There was no detectable expression of NRXN3 mRNA in the DG regions in any of the mice throughout development.

In the CA1, there was an effect of age on NRXN3 mRNA expression levels ($F[4,92]=9.49$, $p<0.0001$). In WT males, levels transiently increased in the first 3 weeks before decreasing at P28. Specifically, increased expression was observed from P7 to P14and P21 and there was a decrease from P21 to P35 ($p<0.05$). In *FMR1-KO* males, NRXN3 mRNA expression levels were steady throughout the first 3 weeks. An increase in NRXN3 mRNA was observed from P7 to P28 and P35 ($p<0.05$). In WT females, NRXN3 mRNA expression levels increased from P7 to P14 ($p<0.05$) and remained steady after that. This is in contrast to the trajectory in *FMR1-KO* females, which had increased NRXN3 mRNA expression from P7 to P14, P21, P28, and P35, and between P14 to P21, followed by decreases between P21 and P28 and P35 ($p<0.05$). In the CA1 regions, there was an interaction between sex and genotype ($F[4,92]=8.96$, $p=0.004$) and an interaction between sex, age, and genotype ($F[4,92]=2.64$, $p=0.039$). Genotype differences were confirmed by post-hoc testing including a decrease in NRXN3 mRNA expression in the CA1 region of male *FMR1-KO* mice at P14 and P21 compared to male WT mice ($p<0.05$) and a decrease in expression between female *FMR1-KO* mice and female WT mice at P21 ($p<0.05$). In addition, WT male mice showed increased levels of NRXN3 mRNA compared to WT female mice at P21 and P28, whereas *FMR1-KO* male mice showed decreased NRXN3 mRNA compared to *FMR1-KO* female mice at P21 ($p<0.05$).

In the CA3, the trajectory of NRXN3 mRNA expression levels showed an effect of age ($F[4,92]=9.7$, $p<0.0001$) and an interaction between sex and genotype ($F[4,92]=6.85$, $p=0.01$). In WT males, an increase in expression was observed between P14 and P35 and between P21 and

P35 ($p<0.05$), whereas in *FMR1-KO* males, NRXN3 mRNA expression levels were steady across development. In WT female mice, expression increased from P7 to P14 and then decreased between P14 and the older ages ($p<0.05$). In *FMR1-KO* females, the increase in NRXN3 mRNA expression from P7 was observed at P14 and P21, followed by a decrease from both P14 and P21 to P28 and to P35. Genotype differences included increase expression of NRXN3 mRNA in WT male compared to *FMR1-KO* mice in the CA3 region at P14 and a decrease in expression in WT female compared to *FMR1-KO* mice at P21 ($p<0.05$). These differences reflect the different trajectories of expression for NRXN3 mRNA across the groups. In addition, sex differences included increase NRXN3 mRNA expression in CA3 in male WT mice compared to female WT mice at P7 and reduced NRXN3 mRNA expression in CA3 in male *FMR1-KO* mice compared to female *FMR1-KO* mice at P21 ($p<0.05$).

In the S1, NRXN3 expression was highest in the second week of life and then decreased over development ($F[4,94]=14.1$, $p<0.0001$). In WT males a decrease in NRXN3 mRNA expression was observed from P14 to P28 and P35, and from P21 to P35 ($p<0.05$). In contrast, in *FMR1-KO* males, the NRXN3 mRNA expression pattern was steady across development. In WT females, NRXN3 mRNA expression levels decreased between P7 and P35 and between P14 and P21, P28, and P35 ($p<0.05$). In *FMR1-KO* females, NRXN3 mRNA expression levels peaked at P14, remained high at P21 and decreased from these levels at P28 and P35. Post-hoc comparisons showed an increase in NRXN3 mRNA expression from P7 to P14 and P21 and decreases from both P14 and P21 to both P28 and P35. An increase in NRXN3 mRNA expression was observed in female *FMR1-KO* mice in S1 at P21 ($p<0.05$). In addition, sex differences included an increase in female *FRM1-KO* compared to male *FMR1-KO* mice at P14 and an increase in NRXN3 mRNA expression in male WT compared to female WT mice at P21 ($p<0.05$).

Table 5-6. Neurexin-3 (NRXN3) mRNA expression in wild type and *FMR1-KO* mice in the hippocampus and somatosensory cortex

region	age	NRXN3 mRNA expression levels (DPM ± SEM)			
		wild type		*FMR1-KO*	
		males	females	males	females
CA1	7	1168.8 ± 99.4	1012.2 ± 179.8	891.2 ± 77.7	852.1 ± 42
	14	**1635.2 ± 186.7[a]**	1547.3 ± 153.3	**1150 ± 129.2[a]**	1457.5 ± 124.1
	21	**1806.4 ±255.9[ab]**	**1287.6 ±189.7[ab]**	**1247.7 ± 54.8[ab]**	**1888.8 ±199.5[ab]**
	28	**1467.1 ± 182.8[b]**	**1297 ± 142.4[b]**	1370.5 ± 62.4	1265.2 ± 133.6
	35	1358.6 ± 109.1	1197.4 ± 64.8	1321.6 ± 95.9	1299.3 ± 72.1
CA3	7	**2307.4 ± 122.7[b]**	**1681.8 ± 281.2[b]**	1791.2 ± 185.1	1533.8 ± 119
	14	**2748.9 ± 268.7[a]**	2644.2 ± 54	**2091 ± 221.4[a]**	2495 ± 244.5
	21	2504.1 ± 264.8	**1974.7 ± 267.3[a]**	**1968.3 ± 136.4[b]**	**2768 ± 284.6[ab]**
	28	2281.9 ± 263.2	1963.3 ± 147.3	2009.4 ± 140.7	1822.9 ± 185
	35	1804.7 ± 181.3	1715.3 ± 74.2	1814.7 ± 129.2	1726.8 ± 122.1
S1	7	853.6 ± 60	819.5 ± 70.4	698 ± 20.1	666.5 ± 21
	14	971.5 ± 100.9	947.3 ± 31.8	**804.8 ± 80.3[b]**	**1040.7 ± 113.6[b]**
	21	**919.8 ± 78.8[b]**	**683.7 ± 79.4[ab]**	806 ± 21.5	**939.4 ± 86[a]**
	28	732.8 ± 54.7	690.8 ± 39.9	701.6 ± 71.9	658.5 ± 58.7
	35	640.7 ± 48.3	543.6 ± 19.8	656.7 ± 46.6	650.6 ± 42.2

Brain regions examined included the somatosensory cortex (S1) and subregions of the hippocampus, CA1 and CA3. There was no expression in the dentate granule layer. mRNA expression levels were determined at postnatal ages 7, 14, 21, 28 and 35 days.
[a] statistically significant effect of genotype
[b] statistically significant effect of sex

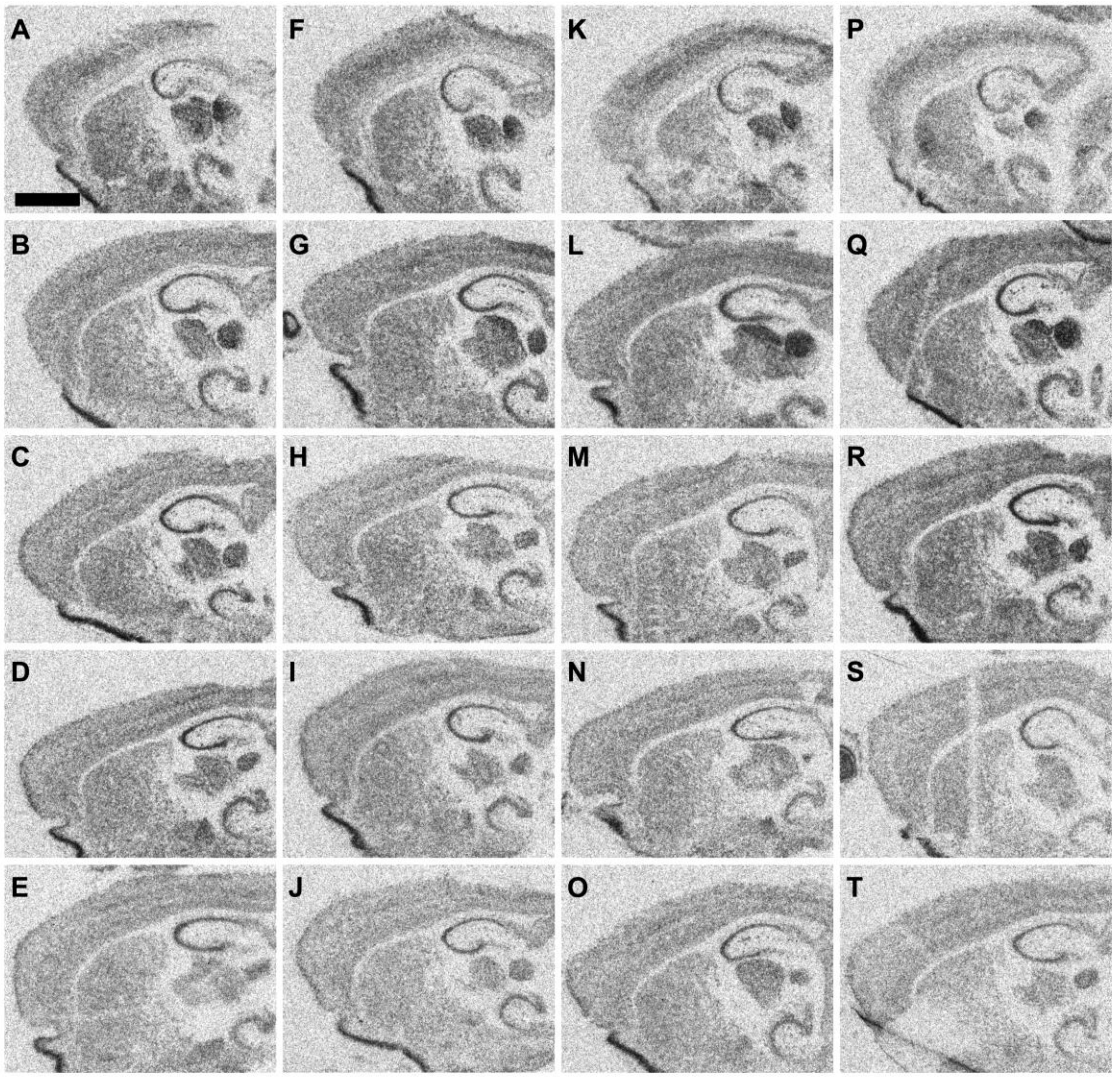

Figure 5-6 – Neurexin-3 mRNA expression in sagittal sections of regions of the hippocampus and somatosensory cortex of (A) male WT at P7, (B) male WT at P14, (C) male WT at P21, (D) male WT at P28 (E) male WT at P35; (F) male *FMR1-KO* at P7, (G) male *FMR1-KO* at P14, (H) male *FMR1-KO* at P21, (I) male *FMR1-KO* at P28, (J) male *FMR1-KO* at P35; (K) female WT at P7, (L) female WT at P14, (M) female WT at P21, (N) female WT at P28 (O) female WT at P35; (P) female *FMR1-KO* at P7, (Q) female *FMR1-KO* at P14, (R) female *FMR1-KO* at P21, (S) female *FMR1-KO* at P28, and (T) female *FMR1-KO* at P35. Tracings used in densitometry comparisons are shown. Scale bar is 160 µm.

Discussion

In this study, our results show that gene expression patterns of the neuroligin and neurexin family were affected by gene, age, sex, and brain region. Each gene had a unique trajectory and changes in expression were present but transient in *FMR1-KO* mice, with the majority of differences occurring after the known window of synaptogenesis, in the third and fourth weeks of life, and also mostly in females. Changes in gene expression are summarized in Figure 7 and superimposed with corresponding events in structural connectivity, glutamatergic and GABAergic synaptic maturation in mouse brain development and discussed below.

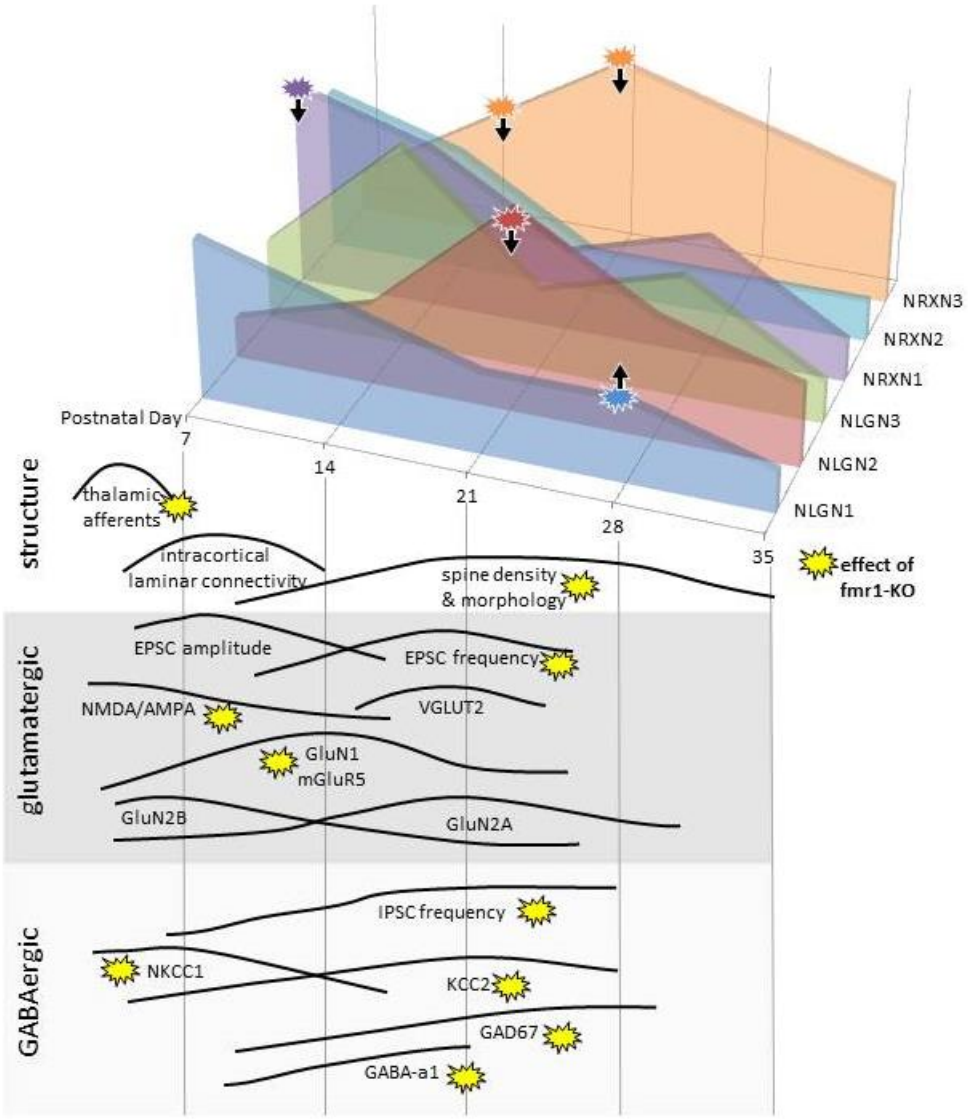

Figure 5-7 – Summary diagram illustrating the transient regional gene and sex specific changes in mRNA expression of the neuroligins and neurexins in WT and *FMR1-KO* mice corresponding with other events associated with synaptic maturation across postnatal development. The top of the schematic shows the relative mRNA levels of the each gene across postnatal development in various regions found in this study. The age and direction of mRNA expression changes reported in this study are indicated by the “star bursts” and arrow respectively. The bottom of the schematic maps out the relative activity of processes and levels of specific molecules that affect

the development of the synapse during the same time period in mice; these are separated in structural/morphologic events, processes related to glutamatergic maturation and GABAergic maturation (from top to bottom). Trajectories known to be dysregulated in *FMR1-KO* mice are indicated by the “star burst”. References are listed here (italicized references report perturbations due to *FMR1-KO*) and discussed throughout the text: (Structure: Molnar et al., 2003; *Harlow et al., 2010*; Pangratz-Fuehrer and Hestrin, 2011; Cohen-Cory, 2002; Micheva and Beaulieu, 1997; Desai et al., 2002; Frick et al., 2007; Oswald and Reyes, 2008. Glutamatergic: Kumar et al., 2002; Stubblefield and Benke, 2010; Ritter et al., 2002; Sheng et al., 1994; Watanabe et al., 1992; Baude et al., 1995; Tyzio et al., 1999; Lendvai et al., 2000; Wijetunge et al., 2008; *Pilpel et al., 2009; Dolen and Bear, 2008.* GABAergic: Leinekugel et al., 1995; Murguia-Castillo et al., 2013; Yamada et al., 2004; Takayama and Inoue, 2006; Zhang et al., 2011; Kobayashi et al., 2008; *Micheva and Beaulieu, 1997; He et al., 2014; Adusei et al., 2010; Paluszkiewicz et al., 2011; Gibson et al., 2008; Hong et al., 2012; El Idrissi et al., 2005; Le Magueresse and Monyer, 2013*)

Each gene has a different trajectory

The temporal pattern of neuroligin mRNAs in postnatal hippocampus and S1 cortex observed in the current manuscript is similar to what has been previously reported in wild type rat (Budreck and Scheiffele, 2007; Song et al., 1999; Varoqueaux et al., 2004); there are gene-specific transient changes in the developmental trajectory of mRNA expression in wild type mice. NLGN1 mRNA levels decreased gradually throughout postnatal development whereas NLGN2 and NLGN3 mRNA expression peaked at P21 and P14 respectively.

Since these genes are involved in refining and maturing synapses during early development (Bang and Owczarek, 2013; Krueger et al., 2012) and their function is not completely redundant (Varoqueaux et al., 2006), it follows that each gene has a specific and different role in this process. For instance, the expression profile of NLGN1 we observed coincides with the timing of localization and stabilization of glutamatergic receptors. NLGN1, but not NLGN2 or NLGN3, interacts with PSD95 and NMDA receptors directly (Barrow et al., 2009; Budreck et al., 2013), regulates postsynaptic NMDA-dependent pathways (Chen et al., 2010; Chubykin et al., 2007; Hoy et al., 2013) and affects AMPAR localization (Ko et al., 2009; Mondin et al., 2011). It follows that expression levels of NLGN1 specifically would be increased during the functional maturation period of glutamatergic synapses, although compensatory mechanisms do exist (Soler-Llavina et al., 2011). The pattern of expression in the neurexins is more varied, with NRXN1 and NRXN2 mRNAs generally decreasing through development and NRXN3 mRNA peaking at P14 and 21.

Interestingly, we observe a sex-specific trajectory in NLGN2, NRXN1, NRXN2, and NRXN3 mRNA expression in the hippocampus and in S1 cortex expression of NRXN3 mRNA in WT mice. Pre-pubertal differences in gene expression between the male and female brain have been shown by others (Armoskus et al., 2014; Ingleby et al., 2014; Xu et al., 2012) and may be related to behavioural differences (Davies, 2013; Davies et al., 2006; Fairless et al., 2012; Frick and Gresack, 2003). Given the role of these genes at synapses and differences in brain

development between males and females (Bian et al., 2012; Cahill, 2006; Ingalhalikar et al., 2014; Wierenga et al., 2014), it is not surprising that gene expression may be different as well. Overall, the changes in expression of these genes suggest their role in specific stages of circuit maturation which may be sex-specific, although the specific function of individual genes are not well established.

Lack of FMRP affects mRNA expression levels

In our data, we observed genotype-based decreases in expression in NLGN2 mRNA in the CA1 and dentate gyrus regions of the hippocampus between female WT and *FMR1-KO* mice and decreases in NRXN1 mRNA and increases in NRXN3 mRNA in S1 cortex between female WT and *FMR1-KO* mice. FMRP is involved in the translation process of its mRNA targets by repressing and stalling ribosomes (Darnell et al., 2011; Maurin et al., 2014), inhibiting translation initiation (Napoli et al., 2008) and regulating the stability of other mRNAs (Zalfa et al., 2007; Zhang et al., 2007) necessary for experience-dependent plasticity. It follows that one would expect increased expression of target mRNAs in the *FMR1-KO* mice that were directly regulated. The mRNA targets of FMRP include synaptic scaffolding proteins (Darnell et al., 2011; Zalfa et al., 2007), receptors (Darnell et al., 2011) and voltage gated channels (Brager and Johnston, 2014; Brown et al., 2001; Chen et al., 2002; Darnell et al., 2011; Gross et al., 2012; Lee et al., 2011; Strumbos et al., 2010) and, of direct relevance to this study, NRXN1, NRXN3 and NLGN3 (Darnell et al., 2011; Iossifov et al., 2012). In the brain regions we examined, we observed decreased and increased expression of NRXN1 and NRXN3 mRNAs respectively, and no changes in NLGN3 mRNA, in the *FMR1-KO* mice. In addition, we observe changes in NLGN2 mRNA expression, which has not been reported to directly bind FMRP. The changes in NLGN2 mRNA, discussed below, and the lack of differences in NLGN3 mRNA may be related to compensatory mechanisms in *FMR1-KO* mice to other perturbations (Baudouin et al., 2012; Shipman et al., 2011) based on the known developmental expression of FMRP (discussed below).

In addition, the genotype differences were sex-specific which raises the question of whether other genetic factors influence synaptic gene expression in pre-pubertal mice. Furthermore, in contrast to the sex differences in WT mice, sex differences in NLGN3, NRXN1, NRXN2, and NRXN3 mRNA expression in the hippocampus and in NLGN1, NRXN2 and NRXN3 mRNA expression in S1 cortex were detected in *FMR1-KO* mice. These data demonstrate that sexually dimorphic changes in *FMR1-KO* mice occur at the level of gene expression. Further work is needed to determine the functional outcome of sex differences in gene expression during early postnatal life and the potential interactions between other genetic factors that may mitigate these differences.

Alterations in neuroligin-2 in FMR1-KO mice may be indicative of dysfunctional GABAergic maturation

Specific genotype changes include a delay in the peak of NLGN2 mRNA expression in the *FMR1-KO* female hippocampus. We observe a sustained and delayed peak in NLGN2 mRNA expression from P21 to P28 in female *FMR1-KO* mice. This was prominent in the CA1 and a trend in the same direction was seen in the dentate. NLGN2 is postsynaptically expressed at certain GABAergic synapses (Gibson et al., 2009; Graf et al., 2004), colocalizing with collybistin and gephyrin (Pettem et al., 2013; Poulopoulos et al., 2009). Recently, it was reported that NLGN2 expression precedes and regulates KCC2 expression (Sun et al., 2013), the transporter responsible for GABA-induced chloride currents in vitro (Ben-Ari, 2002; Rivera et al., 1999) and the transition from excitatory GABAergic function to adult inhibitory function (Cancedda et al., 2007). Typically, NKCC1 expression levels decrease in the second week (Leinekugel et al., 1995; Murguia-Castillo et al., 2013; Yamada et al., 2004; see Fig. 7) while KCC2 expression levels increase through the second and third week of life before reaching adult levels (Leinekugel et al., 1995; Takayama and Inoue, 2006; Yamada et al., 2004). This results in the E/I ratio decreasing due to a net increase in inhibitory conductance during the second week (Zhang et al., 2011), concomitant with an increase in GABAergic synapse number (Kobayashi et al., 2008; Micheva

and Beaulieu, 1997; Fig. 7). In *FMR1-KO* mice, the chloride conductance change is delayed in the second week, due to increased NKCC1 expression early in the second week (He et al., 2014). The changes we see in NLGN2 mRNA in the hippocampus of *FMR1-KO* females may be related to this. This is also supported by the observation that, in rat, males have a different trajectory of NKCC1 expression that peaks at P9 and decreases whereas in females, the levels remain constant until P15 (Damborsky and Winzer-Serhan, 2012; Murguia-Castillo et al., 2013). In *FMR1-KO* mice, however, KCC2 protein expression is reported be similar to WT mice at P5, P12 and at 2-3 months (Adusei et al., 2010); however, the developmental window we see changes in NLGN2 mRNA are between those time points and effects on KCC2 and long-term changes in protein levels may be present but not measured in our current experiment.

Additionally, many defects in GABAergic transmission have been reported in *FMR1-KO* mice (Paluszkiewicz et al., 2011; see Fig. 7), including differences in E/I balance by the second postnatal week (Gibson et al., 2008; Harlow et al., 2010), increased GABAa1 receptor expression during the second week (Hong et al., 2012) and an increased GAD65/GAD67 ratio early in the second week (Adusei et al., 2010) and also at the end of the third week (El Idrissi et al., 2005). Thus, it could be possible that NLGN2 expression is necessary for this switch in GABAergic signaling (Huang and Scheiffele, 2008) and in the *FMR1-KO* female mice, the dysregulation of NLGN2 mRNA we observe is related to a shift in the induction of inhibitory GABAergic transmission, altering the timing of developmental milestones in the neural circuit (Le Magueresse and Monyer, 2013).

Alterations in neurexin mRNA in FMR1-KO mice may impact early functional synapse formation

In addition to changes in NLGN2 mRNA expression due to genotype, we observe changes in NRXN1 and NRXN3 mRNAs in the S1, the former only in females and the latter in both sexes. Specifically, there was a decrease in expression of NRXN1 mRNA at P7 and, with NRXN3 mRNA, a female-specific increase that was statistically discernable at P14. Changes in these two specific genes have been reported at P7 and not older ages in *DISC1-KO* mice (Brown

et al., 2011), another risk gene for neurodevelopmental disorders. Both NRXN1 and 3 mRNA expression increases when synaptic activity is blocked (Kitamura et al., 2007), suggesting both genes have a role in establishing functional synapses. Specifically, a splice variant of NRXN3 has been shown to decrease AMPA receptor levels through increase receptor endocytosis (Aoto et al., 2013). Thus, the changes we observe at P7 suggest that differences in neuronal activity occur early on during the first week of postnatal development in *FMR1-KO* mice when, in the cortex, thalamic afferents are known to reach the cortex by that age (Molnar et al., 2003) and, in *FMR1-KO* mice, the maturation of these glutamatergic projections is delayed (Harlow et al., 2010; Fig. 7).

In addition, since these two genes are activity-dependent (Iijima et al., 2011), decreased expression of these two FMRP target mRNAs could be the result of too much activity and excessive connectivity at this age due to a lack of FMRP translational repression. In the cortex, this period is when initial excitatory connections are formed (Groc et al., 2002) and activity is crucial for further development (see Fig. 7). For instance, GluA2-lacking AMPA receptors, which are permeable to calcium, are present only in the first week (Kumar et al., 2002; Stubblefield and Benke, 2010), allowing for increased plasticity (Palmer et al., 2004) and resulting in the consolidation of the network. This corresponds to changes in the NMDA receptor composition in the first week, when GluN2B-containing receptors dominate before GluN2A-containing receptors reach adult levels in the third and fourth weeks (Ritter et al., 2002; Sheng et al., 1994; Watanabe et al., 1992). In the second week, studies report a robust increase in the total number of AMPA receptors at the synapse (Baude et al., 1995; Tyzio et al., 1999) and in the number of dendritic spines (Cohen-Cory, 2002; Micheva and Beaulieu, 1997), corresponding to functional measures including decreased EPSC amplitudes (Desai et al., 2002; Frick et al., 2007; Oswald and Reyes, 2008) while frequency increases (Desai et al., 2002; Oswald and Reyes, 2008), established connectivity within cortical layers (Pangratz-Fuehrer and Hestrin, 2011) and experience-dependent spine remodeling (Lendvai et al., 2000). In *FMR1-KO* mice, there is an increase in

NMDA to AMPA ratio, resulting in NMDA-dependent LTP to low frequency stimulation (Pilpel et al., 2009). Lastly, during this second week, mGluR5 expression in somatosensory cortex peaks (Wijetunge et al., 2008), which is known to be affected in *FMR1-KO* mice (Dolen and Bear, 2008). Furthermore, functional GABAergic connections are beginning to form at the end of the first week of postnatal life (Pangratz-Fuehrer and Hestrin, 2011). In *FMR1-KO* mice, changes in GABA receptor levels have been reported as early as P5 (Adusei et al., 2010). Therefore, early perturbations in the expression of the neurexins would influence the setup of the neural circuit, which various steps are already affected by the lack of FMRP, and alter the possible trajectories of circuit dynamics later on in development.

FMRP expression is developmentally regulated

The age of onset of FMRP expression and subsequent interaction with the synaptic machinery, including the regulation of the neuroligin/neurexin genes themselves, may explain the timing of some of the expression differences in *FMR1-KO* mice. During early postnatal development, FMRP expression itself is regulated (Gaur and Prasad, 2014; Till, 2010). Levels peak at the end of the first week of life at P7, when we see the changes in NRXN1 and NRXN3 mRNAs, and decrease after P21 and P28 in whole brain lysates (Davidovic et al., 2011; Wang et al., 2004), and in the hippocampus (Lu et al., 2004) respectively. In the somatosensory cortex, FMRP expression peaks between P7 and 14 (Harlow et al., 2010). These changes result in circuit dysfunction, as subtle synaptic-level, layer-specific changes occur in *FMR1-KO* mice in this early window (Harlow et al., 2010; Till et al., 2012). In our data, most of the changes we observe occur after the reported onset of FMRP expression. The timing of the differences we observe suggests that they may be directly related to the lack of translation regulation by FMRP. Additionally, there can be indirect interactions between these genes and that defects arise as a consequence of earlier changes. For example, neuroligin expression is regulated by the mTOR pathway through the eIF4E transcription factor (Gkogkas et al., 2013), which is reported to have increased phosphorylation in FXS patients (Hoeffer et al., 2012). Overall, these data suggest that a gene-

gene interaction exists between FMRP and genes in the neuroligin/neurexin family. Based on our data, one could consider FMRP as protective factor during neurodevelopment as mutations of FMRP target genes exist in ASD (Iossifov et al., 2012).

Conclusions

Genetic mutations in neuroligins and neurexins are identified in cases of ASD (Autism Genome Project et al., 2007; Bena et al., 2013; Feng et al., 2006; Gauthier et al., 2011; Glessner et al., 2009; Iossifov et al., 2012; Jamain et al., 2003; Kim et al., 2008; Laumonnier et al., 2004; Pinto et al., 2010; Sanders et al., 2011; Vaags et al., 2012; Yan et al., 2008; Yan et al., 2005; Yu et al., 2011). Previous work in our laboratory showed altered expression of NLGN3 mRNA in the somatosensory cortex in the valproic acid (VPA) mouse model of ASD (Kolozsi et al., 2009). Further, many of the ASD-related genes are FMRP-associated (Darnell et al., 2011), suggesting that synaptic dysfunction may be a shared substrate of ASD and Fragile X Syndrome. Therefore, we hypothesized that the developmental expression patterns of NLGNs and NRXNs would be altered in *FMR1-KO* mice. Our results revealed genotype differences in NLGN2, NRXN1, and NRXN3 mRNAs, with most effects observed in female *FMR1-KO* mice. In addition, sex differences in gene expression were observed in both WT and *FMR1-KO* mice, however, the genes that showed sex differences in mRNA expression in WT mice were distinct from the those showing sex differences in *FMR1-KO* mice reinforcing the importance of including both sexes in animal studies. Overall, these data, along with characterizations of the neuroligin (Blundell et al., 2010; Blundell et al., 2009; Hoy et al., 2013; Jamain et al., 2008; Jaramillo et al., 2014; Tabuchi et al., 2007; Wohr et al., 2013) and neurexin (Etherton et al., 2009; Grayton et al., 2013; Ju et al., 2014; Rabaneda et al., 2014) mutant mouse models, suggest that this gene family may be an important part of the shared biology between FXS and a subset of ASD.

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Conflict of Interest Statement.

None.

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Chapter 6: Regional Brain Volumes Changes in Adult Male FMR1-KO Mouse on the FVB Strain

Chapter Link

The work in the following chapter was submitted to Molecular Autism (Ms. No.: 9936517091671542) and is currently under review.

In chapters three and four (study one and two, respectively), behavioural differences were observed in the early life period in *FMR1-KO* mice. This study was performed to examine brain volume differences in adult male *FMR1-KO* mice compared to WT controls. Previous work by collaborators Dr. Jacob Ellegood and Dr. Jason P. Lerch had shown regional volume differences at the same age in various mouse models of ASD, including the *FMR1-KO* model on a C57Bl/6 background. Therefore, among the first studies in this collaboration, the adult neuroanatomical phenotype was examined in the *FMR1-KO* mouse used in in chapter 4 (study two).

Regional Brain Volumes Changes in Adult Male *FMR1-KO* Mouse on the FVB Strain

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Abstract

Background: Fragile X Syndrome is the most common heritable single gene cause of ASD. FMR1-KO mice mimic the etiology and phenotypic manifestations of Fragile X Syndrome. Neuroanatomical changes in specific brain regions have been reported in clinical settings and in preclinical models. FMR1-KO mice have been generated in different strains including C57Bl/6 and FVB. Mice on different genetic backgrounds have stable yet distinct behavioural phenotypes that may lead to unique gene-strain interactions on brain structure. Previous MRI studies have examined FMR1 knockout male mice on a C57Bl/6 and found few differences compared to wild type mice.

Method: Here, we examine brain volumes in FMR1 knockout male mice on a FVB background using high resolution (multi-channel 7.0 Tesla) MRI.

Results: We observe multiple differences in the neuroanatomy of male FMR1-/y mice on a FVB background. Significantly larger relative volume (% total brain volume) differences were found in major white matter structures throughout the brain. In addition, there were changes in areas associated with fronto-striatal circuitry and other regions.

Conclusion: Functional and structural connectivity differences are often seen in human ASD, and therefore, this increased white matter seen in the FMR1-KO-FVB could be highlighting a structural over-connectivity, which could lead to some of the behavioural abnormalities seen with the FMR1-KO-FVB mice. Furthermore, these results highlight the importance of genetic strain contribution to brain structure.

Keywords: autism spectrum disorder, regional brain volumes, genetic mouse model

1.0 Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder affecting approximately 1 in 7000 males and 1 in 11000 females (Hunter et al., 2014). Fragile X Syndrome is caused by silencing of the Fragile X Mental Retardation 1 (FMR1) gene, due to a trinucleotide repeat expansion mutation that leads to methylation of its promoter (Fu et al., 1991, Oostra and Verkerk, 1992, Sutcliffe et al., 1992, Verheij et al., 1993). The gene product, Fragile X Mental Retardation Protein (FMRP), is involved in translation regulation of mRNA targets (Nakamoto et al., 2007, Zalfa et al., 2007, Napoli et al., 2008, Darnell et al., 2011, Maurin et al., 2014); many that affect plasticity and the dynamics of the brain wiring (Dolen and Bear, 2008, Darnell, 2011). Patients with FXS exhibit a wide-range of symptoms which include: intellectual impairment (ranging from mild to severe), anxiety, hyperactivity, seizures, and autistic-like behaviour (Yu and Berry-Kravis, 2014). In fact, 25-67% of males and 6-23% of females that have FXS can also be classified as having an autism spectrum disorder (ASD) (Hatton et al., 2006, Clifford et al., 2007, Harris et al., 2008).

Several clinical studies have reported neuroanatomical changes in brain volume associated with FXS, particularly those associated with fronto-striatal circuitry (Bray et al., 2011, Hallahan et al., 2011, Dennis and Thompson, 2013a, Peng et al., 2014), a network involved in the integration of information from limbic and motivational states to elicit or inhibit a behavioural response (Bonelli and Cummings, 2006, Fox et al., 2010, Shepherd, 2013). Reports consistently see enlargement of the caudate nuclei (Reiss et al., 1995, Eliez et al., 2001, Hoeft et al., 2008, Bray et al., 2011, Hallahan et al., 2011) and ventricles (Reiss et al., 1991, Reiss et al., 1995, Eliez et al., 2001, Lee et al., 2007) and reductions in volume of the cerebellar vermis (Reiss et al., 1991, Kaufmann et al., 2003, Lee et al., 2007, Gothelf et al., 2008). Furthermore, decreases in the insula have been reported (Cohen et al., 2011), which along with the cerebellar vermis (Baldacara et al., 2008, Sacchetti et al., 2009), is involved with the coordination of the response to emotional stimuli, which is heightened in patients (Hessl et al., 2004, Baranek et al., 2008, Roberts et al., 2009, Cohen et al., 2013). These regional brain volume changes have been correlated with functional

impairments in patients. For example, changes in superior and medial prefrontal gyri volumes correlate with cognitive outcomes in spatial relations and verbal fluency scores in adolescences with FXS (Bray et al., 2011). In addition, increases in caudate volume in patients are associated with repetitive behaviours and cognitive deficits (Peng et al., 2014). Altogether, these studies show regional brain volume differences in FXS patients are directly related to functional impairments.

The FMR1-KO mouse, which was created in 1994, has been useful in advancing our understanding of the pathophysiology of Fragile X Syndrome (Consortium, 1994, Kooy, 2003, Bear et al., 2004, Nakamoto et al., 2007, Dolen and Bear, 2008, Brager and Johnston, 2014). Current research using the FMR1-KO mouse is based on two separate inbred strains with difference genetic backgrounds, the FVB and C57/Bl6 (B6) background. The genetic background strain of the FMR1-KO has differing effects on the behaviour outcomes (Dobkin et al., 1999, Moy et al., 2009, Pietropaolo et al., 2011, Roy et al., 2012, Lai et al., 2014). For instance, one study reports that FMR1-KO B6 mice have increased self-grooming, increased aggressive behaviours with a juvenile mouse, increased prepulse inhibition compared to their WT counterparts whereas FMR1-KO FVB mice do not (Pietropaolo et al., 2011). However, Moy et al (2009) reported no differences in anxiety and sociability in either strain (Moy et al., 2009). In contrast, there are more changes in early life communication in FMR1-KO mice on FVB background (Lai et al., 2014) compared to a B6 background (Roy et al., 2012). Overall, the contrasts in behavioural outcomes suggest the presence of gene-gene interactions and modifiers of the FMR1 gene in the genetic strain background.

Magnetic resonance imaging (MRI) can been used to examine and measure differences in the neuroanatomy in both human studies and mouse models. In the mouse, robust high-throughput anatomical phenotyping has been valuable in detecting several subtle phenotypic differences in mouse models related to human disease, such as Huntington's disease (Lerch et al., 2008), Alzheimer's disease (Lau et al., 2008), dopaminergic dysfunction (Cyr et al., 2005), and prenatal

alcohol exposure (O'Leary-Moore et al., 2010). MRI has been used to examine neurodevelopmental disorders in the mouse, specifically, autism (Horev et al., 2011, Ellegood et al., 2012, Ellegood et al., 2013) and schizophrenia (Ellegood et al., 2014b). Furthermore, a recent study clustered 26 different mouse models related to autism in an effort to subset the models into similar groups based on their neuroanatomy (Ellegood et al., 2014a). The neuroanatomy of FXS in a C57BL6/J mouse model has been previously examined and in that study minimal volumetric differences were found (Ellegood et al., 2010). These differences were limited to two deep cerebellar nuclei and trends in the cortex and striatum. Due to the differences in the behavioural profiles of the FMR1-KO depending on their background (FVB vs. B6), we hypothesize that the neuroanatomical differences will also be different in FMR1-KO mice on FVB background compared to FMR1-KO mice on B6 background (Ellegood et al., 2010). The purpose of this study is to use high resolution MRI analysis to examine the neuroanatomical differences in FMR1-KO males on an FVB background and compare and contrast those findings to the human literature and the previously reported neuroanatomical changes FMR1-KO mice on the B6 background.

2.0 Methods

2.1 Animals

Male FVB/N and FVB/N-*Fmr1tm1Cgr* (*fmr1-/y*) mice were housed and bred at McMaster University Animal Facility. All experiments were completed in accordance with the guidelines set out by the Canadian Council on Animal Care and were approved by the McMaster Animal Research Ethics Board.

2.2 Perfusions

Mice were perfused at P60 (n=10 per group) at McMaster University in Hamilton, Ontario prior to being transferred to the Mouse Imaging Centre in Toronto for imaging and analysis. The perfusion protocol was as follows: Mice were anesthetized with ketamine/xylazine and intracardially perfused with 30 mL of 0.1 M PBS containing 10 U/mL heparin (Sigma) and 2 mM ProHance (a Gadolinium contrast agent) followed by 30 mL of 4% paraformaldehyde (PFA) containing 2 mM ProHance (Spring et al., 2007). Perfusions were performed with a minipump at a rate of approximately 1mL/minute. After perfusion, mice were decapitated and the skin, lower jaw, ears, and the cartilaginous nose tip were removed. The brain and remaining skull structures were incubated in 4% PFA + 2 mM ProHance overnight at 4C then transferred to 0.1M PBS for at least 7 days prior to MRI scanning.

2.3 Magnetic Resonance Imaging

A multi-channel 7.0 Tesla MRI scanner (Agilent, Palo Alto, CA) was used to image the mouse brains within skulls. Sixteen custom-built solenoid coils were used to image the brains in parallel.

2.4 Anatomical Imaging - Volume Changes

Parameters for the MRI scan were optimized for gray/white matter contrast and high efficiency: T2- weighted, 3-D fast spin-echo sequence, with a TR of 200 ms, an echo train length of 6, an effective TE of 42 ms, two averages, field-of-view of 14 x 28 x 25 mm^3 and matrix size = 250 x 504 x 450 giving an image with 0.056 mm isotropic voxels. In the phase encoding direction,

consecutive k-space lines were acquired with alternating echoes to move discontinuity-related ghosting artifacts to the edges of the field-of-view (Thomas et al., 2004). This sequence requires the oversampling of the phase encode direction by a factor of two to avoid interference of these artifacts. The field of view was subsequently cropped to 14 mm after reconstruction. Total imaging time was 11.7 hr (Lerch et al., 2011).

2.5 Registration and Analysis

To visualize and compare any changes in the mouse brains the images were linearly (6 parameter followed by a 12 parameter) and nonlinearly registered towards a pre-existing atlas (Dorr et al., 2008). All scans were then resampled with the appropriate transform and averaged to create a population atlas representing the average anatomy of the study sample. The result of the registration had all scans deformed into exact alignment with each other in an unbiased fashion. This allowed for the analysis of the deformations needed to take each individual mouse's anatomy into this final atlas space, the goal being to model how the deformation fields relate to genotype (Nieman et al., 2006, Lerch et al., 2008). The jacobian determinants of the deformation fields were then calculated as measures of volume at each voxel. Significant volume changes were calculated by warping a pre-existing classified MRI atlas onto the population atlas, which allows for the volume of 62 segmented structures encompassing cortical lobes, large white matter structures (i.e. corpus callosum), ventricles, cerebellum, brain stem, and olfactory bulbs (Dorr et al., 2008) to be assessed in all brains. Further, these measurements were examined on a voxel-wise basis in order to localize the differences found within regions or across the brain. Multiple comparisons in this study were controlled for using the False Discovery Rate (Genovese et al., 2002).

3.0 Results

In this study, we observe multiple differences in the FMR1-KO on a FVB background that were not present in the FMR1-KO on a B6 background (Ellegood et al., 2010). Figure 1 shows coronal, axial and sagittal images of several different slices illustrating the relative volume differences that were detected in FMR1-KO-FVB mice compared to WT FVB control mice.

Overall, there was no difference in total brain volume between the WT FVB and FMR1-KO-FVB mouse ($p=0.39$). In our analysis, comparisons were made with relative volume (% total brain volume) in 62 different brain regions and 6 summary regions (cerebral white, cerebral gray, olfactory, brainstem, ventricles, and cerebellum) based on divisions in Dorr et al. (2008) and also quantified in the Ellegood et al. (2013) (Dorr et al., 2008, Ellegood et al., 2013). There were relative volume differences in multiple regions and a subset of the 62 regions as well as volumes of the 6 summary regions are shown in Table 1. Of note, we observed fewer differences using absolute volume comparisons despite no overall brain differences between the two groups. There was an increased variability in the FMR1-KOs compared to the WT mice (total brain volume: WT FVB = 468.8 +/- 8.2 and FMR1-KO = 475.5 +/- 23.0), therefore, we used relative volumes to account for the variability in brain sizes within groups.

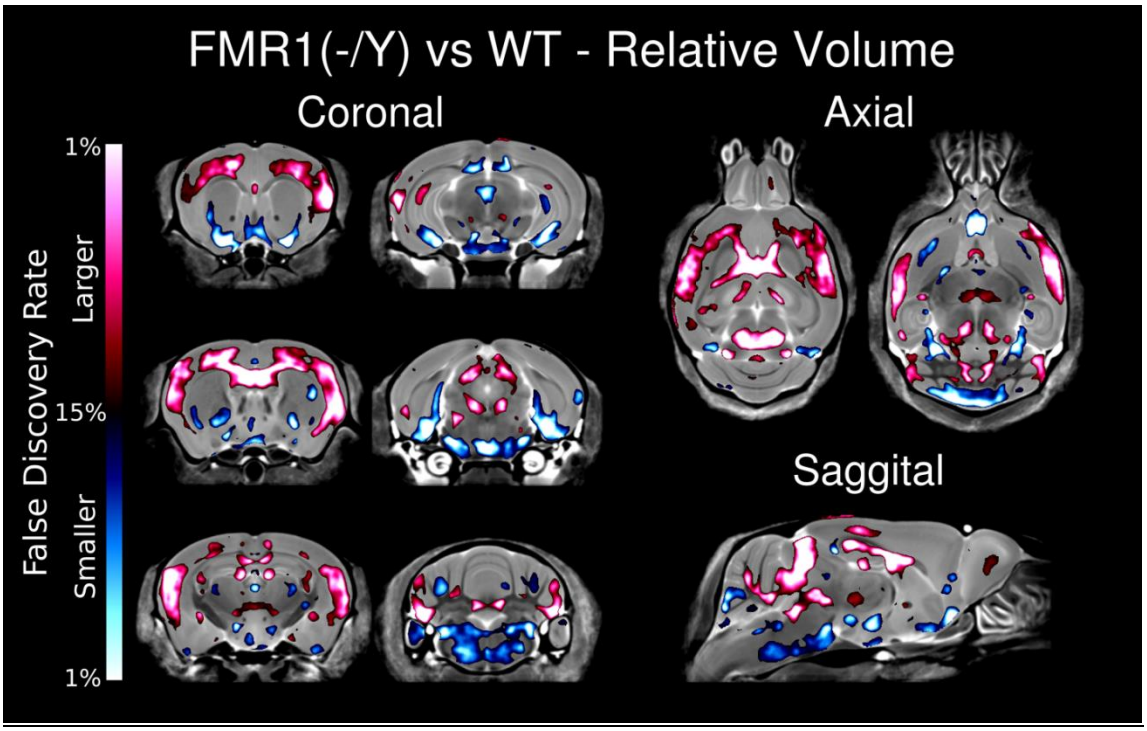

Figure 6-1 - Relative volume differences in *FMR1-KO*-FVB mice compared to WT FVB control mice in coronal, axial and sagittal slices.

Table 6-1 – *FMR1-KO* (-/y) versus WT-FVB Relative Volume Differences. Selected regions highlighting several of the differences in relative volume between the *FMR1-KO* and FVB mouse. Multiple comparisons were controlled by false discovery rate. "-" indicates a q-value of < 0.15, * <0.10, ** < 0.05, and *** < 0.01.

Regions	Relative Volumes (% Total Brain Volume) Mean ± SD		%Diff	q-value	
	FVB	FMR1-KO (-/y)			
amygdala	2.96 ± 0.072	2.97 ± 0.085	0.16	0.81	
anterior commissure: pars anterior	0.25 ± 0.007	0.26 ± 0.004	1.76	0.21	
anterior commissure: pars posterior	0.09 ± 0.003	0.09 ± 0.002	-0.40	0.99	
arbor vita of cerebellum	2.36 ± 0.066	2.37 ± 0.094	1.21	0.78	
basal forebrain	1.18 ± 0.018	1.14 ± 0.018	-3.57	0.01	*
cerebellar cortex	12.46 ± 0.372	12.18 ± 0.384	-2.14	0.21	
cerebellar peduncle: inferior	0.19 ± 0.009	0.17 ± 0.011	-7.58	0.02	*
cerebellar peduncle: middle	0.30 ± 0.012	0.28 ± 0.013	-5.52	0.05	-
cerebellar peduncle: superior	0.22 ± 0.006	0.23 ± 0.006	3.32	0.05	-
cerebral cortex: entorhinal cortex	2.40 ± 0.004	2.36 ± 0.076	-1.52	0.38	
cerebral cortex: frontal lobe	8.70 ± 0.256	8.94 ± 0.248	2.40	0.13	
cerebral cortex: occipital lobe	1.78 ± 0.085	1.72 ± 0.063	-3.28	0.21	
cerebral cortex: parieto-temporal lobe	17.56 ± 0.31	18.17 ± 0.360	3.06	0.02	*
cerebral peduncle	0.45 ± 0.011	0.44 ± 0.014	-1.96	0.16	
colliculus: inferior	1.25 ± 0.041	1.3 ± 0.038	3.84	0.06	-
colliculus: superior	1.85 ± 0.050	1.92 ± 0.074	3.99	0.06	-
corpus callosum	3.28 ± 0.053	3.37 ± 0.098	2.32	0.06	-
dentate gyrus of hippocampus	0.77 ± 0.020	0.78 ± 0.021	0.28	0.81	
fimbria	0.63 ± 0.019	0.67 ± 0.025	6.91	0.02	*
fornix	0.13 ± 0.003	0.13 ± 0.004	1.63	0.50	
globus pallidus	0.57 ± 0.012	0.56 ± 0.006	-2.53	0.02	*
hippocampus	4.53 ± 0.122	4.51 ± 0.085	-0.68	0.71	
hypothalamus	2.15 ± 0.051	2.09 ± 0.055	-2.50	0.06	-
internal capsule	0.55 ± 0.006	0.54 ± 0.007	-1.43	0.05	-
lateral septum	0.63 ± 0.020	0.64 ± 0.011	1.71	0.27	
lateral ventricle	0.67 ± 0.025	0.71 ± 0.021	5.45	0.02	*
medial septum	0.26 ± 0.009	0.25 ± 0.010	-3.00	0.13	
medulla	5.78 ± 0.188	5.45 ± 0.262	-4.93	0.03	*
midbrain	2.85 ± 0.049	2.88 ± 0.087	0.77	0.82	
nucleus accumbens	0.90 ± 0.029	0.88 ± 0.016	-2.27	0.21	
olfactory bulbs	5.16 ± 0.399	5.21 ± 0.158	1.00	0.81	
olfactory tubercle	0.81 ± 0.019	0.77 ± 0.027	-4.27	0.03	*
periaqueductal grey	0.82 ± 0.025	0.85 ± 0.022	3.59	0.06	-
stratum granulosum of hippocampus	0.19 ± 0.008	0.19 ± 0.006	1.21	0.64	
striatum	4.36 ± 0.110	4.27 ± 0.082	-2.20	0.15	
thalamus	3.49 ± 0.061	3.47 ± 0.107	-0.47	0.52	

Summary Regions					
Cerebral Gray	53.88 ± 0.71	54.23 ± 0.52	0.64	0.24	
Cerebral White	6.19 ± 0.06	6.30 ± 0.12	1.69	0.06	-
Olfactory	6.22 ± 0.42	6.24 ± 0.17	0.29	0.93	
Cerebellum	15.52 ± 0.44	15.26 ± 0.50	-1.68	0.29	
Ventricles	1.12 ± 0.03	1.16 ± 0.02	3.52	0.03	*
Brainstem	17.53 ± 0.28	17.27 ± 0.42	-1.48	0.15	

For the summary regions it was found that the FMR1-KO-FVB mouse had significantly larger ventricles (+3.5%, q=0.03), as well as larger cerebral white matter (+1.7%, q=0.06). There was also a noticeable trend in the brainstem structures which were smaller albeit not significantly smaller (-1.5%, q=0.15). There was not a difference in the cerebellum as a whole (0.29), and interestingly there was also no difference found in the cerebral gray matter as a whole (q=0.24).

Changes in white matter clearly showed a pattern of larger inter-hemispheric and smaller intra-hemispheric connection fibers. For instance, the volume of the corpus callosum was larger by 2.32% (q=0.06) and the fimbria was larger by 6.91% (q=0.02) while the internal capsule (-1.43%, q=0.05) was smaller and the cerebral peduncle (-1.96%, q=0.16) was trending in the same direction. In addition, 2 of the 3 peduncles of the cerebellum were smaller (inferior: -7.58%, q=0.02; middle: -5.52%, q=0.05).

The changes in the ventricles were driven by a larger cerebral aqueduct (3.59%, q=0.06) and lateral ventricle (5.45%, q=0.02), whereas the smaller ventricles were decreased or unchanged in volume, namely the third ventricle (-2.77%, q=0.07) and the fourth ventricle (q=0.64).

The majority of brain regions examined (16 regions, q<0.10) were smaller in the FMR1-KO mouse compared to the WT FVB. These include the basal forebrain (-3.57%, q=0.01), globus pallidus (-2.53, q=0.02), medulla (-4.93, q=0.03), olfactory tubercle (-4.27%, q=0.03) and hypothalamus (-2.50%, q=0.06). A few areas were found to be larger (9 regions, q<0.10). The parieto-temporal lobe (3.06%, q=0.02), inferior and superior colliculi (3.99%, q=0.06 and 2.32%, q=0.06, respectively), and periaqueductal grey (3.59%, q=0.06) were all found to be larger in the FMR1-KO. There were no differences in the cerebellar cortex (q=0.21) or arbor vita (q=0.78) nor in the hippocampus (q=0.71), dentate gyrus (q=0.81) or granular layer of the hippocampus (q=0.64).

4.0 Discussion

The results of this study show that the FMR1-KO brain on a FVB background is different compared to WT FVB which was not apparent in the FMR1-KO on a C57Bl/6J (B6) background compared to the WT C57Bl/6J brain. Multiple regions are affected in the FMR1-KO-FVB mice that were unaffected on the B6 background. The most interesting difference found in terms of what is reported in the literature is the smaller striatum which is contrary to what is found in human patients but does match the decrease seen in the B6 mouse albeit more significant here. Furthermore, we observe differences in white matter in the FMR1-KO-FVB mouse that were not present on the B6 background. Functional and structural connectivity differences are often seen in human autism, and therefore, this increased white matter seen in the FMR1-KO-FVB could be highlighting a structural over-connectivity, which could lead to some of the behavioural abnormalities seen with the FMR1-KO-FVB mice.

4.1 Changes in FMR1-FVB compared to FMR1-B6

Previous work in FMR1-KO mice on a B6 background showed only modest differences in the arbor vita of the cerebellum, specifically in two deep cerebellar nuclei (fastigial nucleus and nucleus interpositus) (Ellegood et al., 2010). In comparison, we did not observe any difference in the volume of the arbor vita of the cerebellum ($q=0.78$) in FMR1-KO-FVB mice. It is possible that those changes are not as evident in the FVB mouse since the cerebellum is more developed and has more foliation in this strain compared to the B6 (Ellegood et al., 2013). Thus, the cerebellum may be more resilient to the effect of the FMR1-KO and an epistatic strain-gene interaction may mask the effect on this region. However, we do see volume decreases in the middle and inferior cerebellar peduncle which has not been reported previously. This is in contrast to what is reported clinically where decreases in the vermis are commonly found in FXS patients (Reiss et al., 1991, Kaufmann et al., 2003). Interestingly, the volume of the posterior vermis has been positively correlated with FMRP levels in that region (Gothelf et al., 2008). In ASD, both reductions (Rojas et al., 2006, Spencer et al., 2006) and increases (Abell et al., 1999, Bonilha et

al., 2008, Ke et al., 2008) in cerebellar volume have been reported. Altogether, this suggests that cerebellar changes are more dynamic and variable, possibly due to interactions with genetic modifiers, and in the clinical case, environmental influences (Steinlin, 2008). Other similarities between our study and the previous work are trends toward a larger parieto-temporal lobe and a smaller striatum (-1.93%, q=0.16). Another reason for these differences could be age-related; in the B6 study, the animals were 30 days old whereas in this study, they were 60 days old. The effect of age is not well-studied in these mice. Overall, given these limitations, it is clear that the number of differences found in the FMR1-KO-FVB mouse shown here is substantially more than in the FMR1-KO-B6 model.

4.2 Differences in White Matter

In our study, we observed a larger white matter volume overall in FMR1-KO-FVB compared to WT-FVB, mostly due to the larger corpus callosum, which is also the largest white matter structure found in the mouse brain. This has also been reported in patients with FXS (Hallahan et al., 2011). In that study, the increase in the corpus callosum is specific to the tracts associated with the parieto-temporal lobe (Lee et al., 2007), which had a trend for increased volume in the FMR1-KO-FVB mouse as well. Other ASD mouse models show differences in white matter as well. For instance, the BTBR mouse, has deficits in the core behavioural domains of autism (McFarlane et al., 2008), and has been examined recently in three studies (Dodero et al., 2013, Ellegood et al., 2013, Miller et al., 2013). One of the defining neuroanatomical characteristics is the lack of callosal fibers and several other differences in white matter. Other autism relevant mouse models also show differences in multiple white matter structures, including the neuroligin-3 R451C knock-in (Ellegood et al., 2011) and the integrinβ3 knockout (Ellegood et al., 2012), which is typical in studies of idiopathic ASD (Just et al., 2007, Keary et al., 2009). Interestingly, longitudinal studies have shown that these reductions may normalize over time, especially in the rostral regions of the corpus callosum (Frazier et al., 2012), and therefore it

would be interesting to examine the developmental trajectory of the corpus callosum over time in this model.

In contrast to the corpus callosum, we observe volume decreases of the internal capsule and the middle and inferior cerebellar peduncle. Abnormal connectivity is common in autism patients, with both functional and structural connectivity showing both over-connectivity and under-connectivity. In patients with 22q11.2 deletions, a non-syndromic form of ASD, decreased white matter associated with frontal regions, cerebellum and internal capsule have been reported (Campbell et al., 2006). DTI has shown decreased FA of those fibres as well in ASD (Sivaswamy et al., 2010). These data have led to the hypothesis that white matter maturation is accelerated in ASD (Ben Bashat et al., 2007), and thus, the developmental trajectory of brain volume differences may need to be accounted for in order to compare volume differences. In a recent report of the anatomical clustering of 26 different mouse models related to autism (Ellegood et al., 2014a), the models were clustered into 3 groups, one which had increases in overall white matter, one that had decreases, and the other which showed little differences in white matter. Overall, abnormal white matter volume may be a marker of the long range connectivity issues thought to be relevant in certain subsets of the disorder (Bigler et al., 2010, Anderson et al., 2011, Peters et al., 2012).

4.3 Differences in Corticostriatal Circuitry

Changes in frontostriatal circuitry have been implicated in the FXS (Dennis and Thompson, 2013b) as well as other brain disorders (Qiu et al., 2011, Shepherd, 2013). In FXS patients, the lack of response inhibition and conscious regulation of anxiety are among the phenotypes that relate to the functions of these regions (Bonelli and Cummings, 2006, Eagle et al., 2008, Fox et al., 2010), so it is not surprising that frontostriatal pathways are affected in patients (Barnea-Goraly et al., 2003, Haas et al., 2009). In this study, we see a larger parieto-temporal lobe volume and a trend towards a larger frontal lobe as well as a smaller striatum in the FMR1-KO compared to the WT mouse. In FXS patients, others have reported both a trend to increased volume in all those regions (Lee et al., 2007), although some have observed decreases

specific to the frontal lobe (Hallahan et al. 2010). In an ASD population, temporal, but not parietal, volume changes were seen in infants (Schumann et al., 2010). These frontal and parieto-temporal lobe changes are also consistent with the reported differences in the autism related intergrinβ3 mouse (Ellegood et al., 2012).

Part of the classic brain volume changes in FXS patients is an enlarged caudate, even more so than in ASD (Hazlett et al., 2009) and these changes are negatively associated with FMRP levels (Gothelf et al., 2008, Hoeft et al., 2008) and IQ (Gothelf (Gothelf et al., 2008). In our study, we observe a smaller striatum in the FMR1-KO, which is in contrast to the clinical picture. However, we do not separate out the caudate and the putamen, which may account for differences in findings. In ASD patients, both the volume (Sears et al., 1999, Langen et al., 2007), and the growth rate of the caudate has been reported to be enlarged and accelerated (Langen et al., 2014), the latter found to be associated with repetitive behaviour in the same study, although others report reductions in caudate volume (McAlonan et al., 2005, McAlonan et al., 2008). Further, another ASD study reports increased basal ganglia volumes that were negatively associated with repetitive behaviour (Estes et al., 2011). The variability could be due to the “type of autism”: genetic developmental disorders associated with autism have opposite findings regarding caudate size - enlarged in cases of 22q11.2 deletions (Campbell et al., 2006) and reduced in Rett Syndrome (Reiss et al., 1993).

In other mouse models of ASD, changes in corticostriatal circuitry are observed and have been associated with repetitive behaviours. For example, neuroligin-1 KO mice have increased repetitive behaviour and reduced glutamatergic transmission at corticostriatal synapses (Blundell et al., 2010). Similarly, SHANK3-KO have increased repetitive grooming as well as weaker corticostriatal transmission and increased caudate volume (Peca et al., 2011). In addition, BTBR mice have increased repetitive grooming bouts that is positively correlated with volume decreases in the globus pallidus (-2.5%, q=0.02) (Ellegood et al., 2013). These mice also have a trend

towards a decrease in the striatum (-1.9%,q=0.16). Overall, volumetric and physiological changes in various parts of this circuit seem to be related with repetitive behaviour, a core domain of ASD.

5.0 Conclusions

Strain differences are known to alter behavioural phenotype differences to genetic perturbations, clearly seen in FMR1-KO mice (Dobkin et al., 1999, Moy et al., 2009, Pietropaolo et al., 2011, Roy et al., 2012, Lai et al., 2014) as well as in other ASD models (contrast Tabuchi et al., 2007 and Chadman et al., 2008). Strain differences are known to affect volume differences as well. For example, male C57Bl/6 mice have increased cortical volume compared to CD1 and 129Sv while having less body mass (Chen et al., 2006).

Here, based on these results and contrasted to previous findings in the B6 strain, we show that the FMR1 gene interacts with modifiers in the background strain to influence regional brain volume changes. The changes in FMR1-KO-FVB mouse are more reflective of the clinical FXS neuroanatomical phenotype compared to the FMR1-KO-B6 mouse. These differences have utility for understanding neurodevelopmental disorders since changes in regional brain volumes across ASD models may result in identifying a convergence point of a subset of ASD cases – especially given that genetic and behavioural heterogeneity is high in the disorder. Future studies should identify when neuroanatomical changes appear and if these changes are directly associated with behavioural differences. Clinically, a distinct neuroanatomical phenotype has been shown in toddlers with FXS compared to idiopathic autism (Hazlett et al., 2009) although they have an overlapping symptomatology. Thus, to utilize the FXS model as a representation of an ASD subtype and understand the mechanisms that govern behavioural changes, studies need to examine both brain and behavioural outcomes together. In addition, it would be beneficial to involve both sexes in future studies of brain and behaviour since our understanding of the sex differences in ASD and other neurodevelopmental disorders is lacking and differences have been reported in the brain (Sidor 2014).

Authors' Contributions

JKYL carried out the perfusions and drafted the manuscript. LCD provided the animals. JE performed the imaging and registration, statistical analysis and helped draft the manuscript. JAF and JPL conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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Chapter 7: Discussion

Summary of Findings

This dissertation set out to examine (1) changes in ultrasonic vocalizations in the early life period, (2) behavioural outcomes in the first four weeks of life after exposure to early-life stressors, (3) the gene expression of the neuroligins and neurexins and (4) brain volume differences in *FMR1-KO* mice compared to wild type FVB mice. The central hypothesis tested was that the biology and phenotype of the FXS mouse model is shared across neurodevelopmental disorders and the results of this dissertation support this hypothesis.

At the level of behaviour, study one showed that subtle differences in early life communication are present in *FMR1-KO* mice at postnatal day 7 in contrast to many other mouse models of ASD that have differences of large effect size and consistent alterations across postnatal development. These results highlight the importance of considering age and trajectory since these changes were transient. Further work will need to determine the methodology for measuring USVs that has the most utility.

Subsequently, study two demonstrated that the FMR1 gene product affects multiple aspects of the early life behavioural phenotype and that those changes are highly contextual. In addition, this study sheds light on the relationship between various behavioural assays used to assess early life changes in neurodevelopmental disorders. In relation to study one, I have shown that changes in USVs are sex-specific and that USVs do not have predictive value in relation to sociability and interaction, which is significant to understanding the validity of USVs as an early life behavioural tool.

At the level of gene expression, study three showed that a set of ASD risk genes are developmentally and differentially regulated in both WT and *FMR1-KO* mice in a region and sex-specific manner. Furthermore, these results show interactions between gene expression, sex, and age in *FMR1-KO* mice and shed light on the window of changes in the NLGN-NRXN gene

family induced by lacking the FMRP protein. In particular, the delay in NLGN2 expression from P21 to P28 in FMR1-KO females may indicate changes in the timing of the excitatory-inhibitory GABA switch. These results are significant since they identify a possible neurobiological mechanism that patients with Fragile X Syndrome have in common with a subset of individuals with ASD.

Lastly, in study four, I show that regional brain volume changes are present in the *FMR1-KO* mouse on an FVB background strain, suggesting that this knockout model resembles the clinical neuroanatomical phenotype better than the *FMR1-KO* on a C57BL/6 background. Importantly, this study supports the role of gene-gene interactions between the FMR1 locus and modifiers in background strain to a role in the phenotypic expression of the disease.

Overall, the results of these studies demonstrates that alterations in various early life outcomes in *FMR1-KO* mice recapitulate some of the outcomes reported in other ASD mouse models and that these outcomes are affected by complex interactions. First, this work shows that the phenotypic expression of FMR1 gene silencing is highly dependent on sex and background strain. Second, this work demonstrates that the *FMR1-KO* mouse model is useful in uncovering mechanisms that lead to ASD. Specifically, these studies emphasize the need in translational ASD research to identify relevant outcomes at multiple levels related to ASD mouse models in the early life period and employ rigorous, precise genotype-phenotype associations to classify the heterogeneity that plagues the field. Third, this work extends our knowledge of what is known about the *FMR1-KO* mouse and makes a case for the utility of this model for understanding neurodevelopmental disorders and translation efforts toward new treatments and therapeutics.

The novel findings of this thesis contribute a better understanding of the multifactorial influences on the phenotypic manifestation of lacking this single gene form of neurodevelopmental dysfunction. Specifically, these studies clarify some of the reasons for inconsistencies in the *FMR1-KO* mouse behaviour literature through demonstrating the relevance of age, sex and strain on various outcomes, refine the knowledge of factors that influence brain

and behaviour and provided a more precise understanding of the complexity of the interactions on the early life period. Ultimately, the knowledge gained from these studies will assist in reverse translation efforts for targeted treatments and therapeutics for ASD.

Below, I will discuss the significance of my results in light of the current literature, implications of these studies on key concepts in the field by placing them in the context of changes in this field during the tenure of my doctoral studies, and lastly, state major conclusions of this work and potential future directions for the field.

Interactions of Sex by Strain in the *FMR1-KO* mouse

Strains of mice consistently have different behavioural (Crawley and Paylor, 1997, Holmes et al., 2002, Bothe et al., 2004, Moy et al., 2007) and neuroanatomical phenotypes (Chen et al., 2006, Ellegood et al., 2013). The results from the studies in this thesis support the idea that background strain plays a crucial role in determining phenotype, specifically mediating sexually dimorphic outcomes. One possible mechanism underlying these changes is the FMR1 gene interacting with genetic modifiers in the FVB background strain to produce a different phenotype in males and females. Clinically, these epistatic interactions with other genes modulate phenotype and susceptibility. For example, Rett Syndrome, modifier loci in the form of CNVs that modulate symptom severity have been identified (Artuso et al., 2011). Therefore, gene modifiers may play a key role in the contextual understanding of the management of these disorders.

Epistasis in the FMR1-KO Mouse: the Role of Genetic Background

Behavioural differences have been reported between the FVB and C57Bl/6 strains. For example, FVB mice are more active in the open field and have delayed motor learning compared to C57Bl/6 (Bolivar et al., 2000, Bothe et al., 2004). These inherent strain differences on mouse models may lead to a “ceiling or floor” effect on the outcome depending on the direction of change and interaction the gene in question has with different gene modifiers in the genetic background. In *FMR1-KO* mouse studies, behavioural differences due to background strain have

been reported in several behavioural outcomes (Dobkin et al., 1999, Baker et al., 2010, Pietropaolo et al., 2011, Spencer et al., 2011, Ding et al., 2014). For example, deficits in spatial learning are reported in *FMR1-KO* mice on an FVB.129 background but not on a C57Bl/6J background (Dobkin et al., 1999). C57Bl/6 mice are known to have larger hippocampus field size of mossy fiber terminals (Mineur et al., 2002) and, correlated to that, better spatial learning compared to other strains (Schwegler et al., 1989, Crusio et al., 1993). Thus, overall, inherent genetic differences due to strain may play an additive, antagonistic/masking role or synergistic on different outcomes through epistatic events (Murphy et al., 2003).

In USVs, study one of this dissertation showed that *FMR1-KO* mice on an FVB background have an increased call number maintained after 10 minutes of maternal separation compared to WT FVB pups. Just previous to our related publication, Roy et al. (2012) reported no changes in the total number of USV calls in *FMR1-KO* on a C57Bl/6 background (Roy et al., 2012). At that time, it was proposed that the differences observed in two studies were due to strain as well as methodology. This was supported by the report that WT FVB pups have longer and more USVs calls with a different profile of call types compared to WT C57Bl/6 pups throughout the postnatal period (Scattoni et al., 2008). However, study two shows that immediately after separation, there is no difference in call number but rather, there is an increase in call duration in male *FMR1-KO* on an FVB background. This latter study shows similarities to Roy et al., (2012) in methodology and results, and thus, collectively, these studies show that early life social communication in *FMR1-KO* mice is not strongly affected by background strain immediately after maternal separation.

Studies that have looked at other behaviours in adult *FMR1-KO* mice have reported a strain difference. In addition, age seems to be a factor that leads to behavioural differences due to strain in developmental studies. For instance, nicotine exposure in DBA/2J and C57Bl/6 mice prenatally until weaning at P21 led to brain structural and histological changes to the striatum at P24 although behavioural differences were only detected at P75 (Balsevich et al., 2014), possibly

due to the transcriptomes differences between strains increasing with age. Thus, differences in behaviour may manifest well after neuroanatomical differences are present. This is also supported by differences at the neuroanatomical level that are seen in the two strains of the *FMR1-KO* mouse. In a follow-up to study two, brain imaging at P28 (after behavioural testing was complete) show regional volume differences in *FMR1-KO* males (unpublished to date). Interestingly, a large subset of these regions was different than those reported at P60 in study four. These data are in contrast to findings using the C57Bl/6 background strain (Ellegood et al., 2010), indicating differences in strain at the level of brain imaging. Additionally, at the circuit level, *FMR1-KO* mice on a FVB background have a wider window of audiogenic seizures (Yan et al., 2004) than those on a C57Bl/6 background (Veeraragavan et al., 2011, Goebel-Goody et al., 2012), supporting the notion that circuitry differences in strain are present early on in life.

Other ASD models have reported these differences due to background strain when tested as adults. For instance, the NLGN3-R451C ASD mouse model on a Sv129/C57Bl6 strain from the Sudhof lab has reduced social and spatial learning (Tabuchi et al., 2007, Etherton et al., 2011), but not on the C57Bl/6 strain generated by the Crawley lab (Chadman et al., 2008). Recently, the Crawley lab generated this mutant mouse on a 129S2/SvPasCrl background and reported social deficits, enhanced spatial learning, and increased locomotor activity (Jaramillo et al., 2014). Conversely, mutant mice that are heterozygous for the SHANK3 gene, another ASD risk gene, on the C57Bl/6, 129SVE, and FVB/Ntac strains have similar behavioral phenotypes (Drapeau et al., 2014). Collectively, these demonstrate that the strain-gene interactions are highly specific to the gene and may mask or modulate phenotypes. No other studies have examined these strain interactions in the early life period and the developmental window may play an important role in the epistatic interactions on behaviour with various risk genes.

Sexually Dimorphic Phenotype in the FMR1-KO Mouse on the FVB Background

In study two and three respectively, sexually dimorphic behaviour and gene expression in early life period were observed in *FMR1-KO* mice on the FVB background. In contrast to previous

studies of *FMR1-KO* mice on a C57Bl/6 background (Ding et al., 2014) and a C57Bl/6-albino cross (Baker et al., 2010), study two shows sexually dimorphic behaviour in *FMR1-KO* mice on a FVB background. The manifestation of a sex difference in the *FMR1-KO* FVB is further seen in the sex-specific differences in NLGN and NRXN gene expression in study three where, in many instances, the gene expression levels were not different between WT male and female mice, but different in *FMR1-KO* mice. There have been no studies examining gene expression of the NLGNs and NRXNs in other strains. Lastly, in a follow-up to study two, sex differences in neuroanatomical phenotype were observed in between *FMR1-KO* mice compared to WT-FVB mice (unpublished). Specifically, at P28, 16 brain regions had volume changes due to genotype in males, but only one of those regions was different in females, demonstrating once again that sex differences affects various outcomes. These sex and strain interactions are likely the results of a complex web of various gene modifiers, including genes found on the sex chromosomes. Since many of these genes are not unique to the FVB strain, there must be other autosomal genes whose expression is changed by the presence or absence of those on the sex chromosomes. Further study will be needed to elucidate the specific factors that contribute to these interactions.

Overall, these data show that a sexually dimorphic phenotype is present in multiple outcomes in *FMR1-KO* in the FVB strain but may be masked in the C57Bl/6 strain. This highlights the complexities of gene-gene interactions and is significant because sex differences are reported in FXS patients as well (Reiss and Freund, 1990, Lightbody et al., 2006, Gothelf et al., 2008, Symons et al., 2010, Bray et al., 2011, Rinehart et al., 2011).

Shared Neurobiology between ASD and FXS: the Neuroligins and Neurexins

The studies in this dissertation support the hypothesis that gene-gene interactions exist between the FMR1 gene and the NLGN/NRXN family. The silencing of FMR1 lead to changes in the NLGN and NRXN mRNA expression in both directions at multiple ages. Since the NLGN and NRXN are susceptibility genes for ASD (Jamain et al., 2003, Laumonnier et al., 2004, Yan et al., 2005, Feng et al., 2006, Talebizadeh et al., 2006, Kim et al., 2008, Lawson-Yuen et al., 2008,

Yan et al., 2008, Gauthier et al., 2011, Sanders et al., 2011, Iossifov et al., 2012, Vaags et al., 2012, Bena et al., 2013), there is the possibility that some of the shared phenotypic manifestations of FXS are mediated through the NLGN and NRXN gene family. Study three is the first demonstration that there are transient changes in expression of these ASD risk genes in *FMR1-KO* mice compared to WT mice, and supports the suggestion of a mechanism for an ASD phenotype. FMRP is known to bind a myriad of mRNAs in which some overlap with known ASD risk genes, including NLGN1, NRXN1, and NRXN3 (Darnell et al., 2011). In addition, mutations of FMRP gene targets are increased in ASD populations and seem to be more dose sensitive than other mRNAs (Iossifov et al., 2012). Lastly, FMRP represses the mTOR pathway and when disinhibited, as in the case of FXS, leads to an increase in NLGN1 expression (Gkogkas et al., 2013). Thus, links between the FMR1 and NLGN/NRXN genes are well supported.

In addition to gene expression changes, the behavioural results in this dissertation, along with other studies of *FMR1-KO* mouse behaviour, show alterations in similar domains as those reported in NLGN/NRXN mutant mice (Mackowiak et al., 2014). For instance, in the domain of learning, both NLGN1-KO mice (Blundell et al., 2010) and *FMR1-KO* mice (Dobkin et al., 1999, Baker et al., 2010) show deficits in spatial memory. In contrast, the humanized NLGN3-R451C mutant mouse shows enhanced spatial learning (Tabuchi et al., 2007, Etherton et al., 2011). This is interesting since the results in study three show that NLGN3 mRNA is not differentially expressed in *FMR1-KO* mice. Furthermore, there are increased social interactions reported in NLGN2-KO (Hines et al., 2008) and beta-NRXN1 truncated mutant (Rabaneda et al., 2014), two mRNAs that have different levels of expression in *FMR1-KO* mice in study three. Similarly, increased social interactions were observed in study two using the FVB background strain and also by others in the B6 background strain (Spencer et al., 2005, Spencer et al., 2008, Spencer et al., 2011, Gantois et al., 2013). Also, in the domains of activity, NLGN3-KO mice have hyperactivity (Radyushkin, 2009), which is seen in various reports of adult *FMR1-KO* mice (Peier et al., 2000, Mineur et al., 2002, Ventura et al., 2004, Qin et al., 2005, Restivo et al., 2005,

Spencer et al., 2005). In the domain of anxiety-like behaviour, increases are reported in both NLGN2-KO mice (Blundell et al., 2009) and after overexpression of NLGN2 (Hines et al., 2008) whereas in *FMR1-KO* mice, anxiety-like behaviour is dependent on age and strain, but increases have been reported (Peier et al., 2000, Bilousova et al., 2009, Heulens et al., 2012). Lastly, USV call numbers are reduced and call profiles are different in NLGN2-KO mice (Wohr et al., 2013) and NLGN3-KO (Radyushkin et al., 2009). Similarly, in *FMR1-KO*, study one and two report differences in USVs, and the call type repertoire is modulated in both strains (study one and two, (Roy et al., 2012). Overall, some of the behavioural phenotypes reported in *FMR1-KO* mice are shared with NLGN/NRXN mutant models. However, there are differences in the magnitude and direction of behavioural outcomes between the various NLGN/NRXN mutant mice and the *FMR1-KO* mouse. This is not surprising, since the changes in study three involve multiple genes and are transient over the developmental window whereas most of the studies of the NLGN/NRXN mice utilize a complete continual knockout of gene. The caveat is that the behavioural phenotype after a complete gene knockout is recovery of a physiological system missing components, and thus, the outcomes reflect the functions that remain after the perturbation (Teitelbaum and Pellis, 1992). Since a reduced system cannot do more with less, a knockout may have many indirect, accessory and compensatory effects. In addition, the complexities of strain interactions influencing phenotype in *FMR1-KO* mouse (discussed above) and other models, such as the NLGN 3 R451C mutant mouse (compare Tabuchi et al., 2007, Etherton et al., 2011, and Jaramillo et al., 2014) may explain some of the variability in behaviour.

Furthermore, in addition to the molecular links and behavioural similarities, cluster analysis of neuroanatomical phenotype of genetic ASD mouse models also supports the hypothesis that shared biology of FXS and ASD may be through the NLGN/NRXN gene family. In study four, results show increases in white matter regions, PT lobes and smaller striatum. Using this dataset aggregated with 25 other ASD models imaged by our collaborators, Ellegood et al (2014) found that similar regions varied together in alpha-NRXN1 mutant mice (both

heterozygous and homozygous knockouts) and the *FMR1-KO* mouse (Ellegood et al., 2014a). Interestingly, the NLGN3 R451C mouse did not cluster with the same group in that study. This furthermore supports the notion that the gene-gene interactions observed in study three affect regional brain volume similarly – we did not see change in NLGN3 mRNA expression levels in *FMR1-KO* mice during postnatal development. More recently, the NLGN3 R451C mutant mouse (C57Bl/6.129 background) was reported to have increased volume in the pons and medulla and reduced volumes in the caudate, substantia nigra, various white matter structures, and somatosensory cortex (Kumar et al., 2014), similar to previous results on a C57Bl/6 background (Ellegood et al., 2011), strengthening this assertion.

In summary, both behavioural and neuroanatomical changes after perturbations of the NLGN and NRXN family in mice result in similar changes as those seen in *FMR1-KO* mice and, along with study three of this thesis demonstrating changes in NLGN and NRXN mRNA expression levels in *FMR1-KO* mice, these data suggests that a shared neurobiology of the phenotypic manifestations of FXS and ASD may be through the NLGN/NRXN gene family.

Animal Modelling of Neurodevelopmental Disorders

The Evolving Strategies to Understanding Neurodevelopmental Disorders

The understanding and treatment of neurodevelopmental disorders have greatly evolved over the last decade. Changes in the definitions to classify and approaches to identify underlying biological processes have occurred. The arrival of the DSM 5.0 and proposed single spectrum of ASD will be helpful in studying neurodevelopmental disorders (Mandy et al., 2012). Previously, characterizing behavioural phenotypes of the core domains of autism did not link with the biology well (Szatmari et al., 2007) and categorical distinctions between autism, Asperger's and the PDD-NOS proved to be ineffective in characterizing these disorders (Georgiades et al., 2013, Georgiades et al., 2014) and advancing biological understanding of the disorders (Rapin, 2014), thus, hampering efforts for effective treatment plans. The current model allows for a strategy that

embarks on a collective search for endophenotypes (Liu et al., 2008). By providing a single spectrum with significant individual variability, it is possible to delve into the dimensional frameworks to find subgroups based on endophenotypes, reliable biomarkers, and more targeted treatments. Teasing this group apart will be the next step in the understanding and groupings of neurodevelopmental disorders.

Through genetic population studies over the last decade, the role of de novo CNVs and rare variants has become apparent in the genetic architecture of ASD (Pinto et al., 2010, Gai et al., 2012, Vaags et al., 2012, Pinto et al., 2014). These studies have identified genes that converge on a few cellular pathways (Krumm et al., 2014), mostly related to neuronal development and synaptic signaling (Pinto et al., 2014). At the phenotype level, research networks dedicated to genotype-phenotype characterization have been established in the last 5 years (Zwaigenbaum et al., 2011) and genotype-phenotype associations are also coming up with interesting findings. For example, subsets of rare genetic hits for ASD are associated with paternal age, while other sets are associated with communication and verbal IQ, and yet others with adaptive functioning (Merikangas et al., 2014). Thus, the identification of a *single* biological or behavioural component/sub-phenotype substrate in this complex disorder is unlikely to occur. More and more (Bishop and Scerif, 2011), ASD is shown to be a collection of multiple rare diseases that lead to the same epiphenomenon.

Armed with the insights from genetic screens, the field is looking for convergence of pathways that result in the impairments in core domains though reverse translation efforts going back to cellular and animal models. However, at the same time, divergent phenotypes have resulted from the similar genetic hits (Ching et al., 2010, Bishop and Scerif, 2011), possibly accounting for the influence of common variants (Anney et al., 2010, Devlin and Scherer, 2012) and until recently, the overlooked influence of shared environmental factors (Herbert et al., 2006, Hallmayer et al., 2011). As the identification of these causal factors occurs and as population studies soon give way to an exponential increase in genetic findings (Buxbaum 2014), the

significance and implications of these hits are found through assays examining the neurobiology of these genes through assays from cell culture to mutant mouse models. The characterization of these findings will further our understanding of the mechanisms that underlie these disorders. However, the prioritization of both genes and assays is still debated (Hoischen et al., 2014).

Over the last 5 years, research networks dedicated to a cross disorder, reverse translation approaches to understand the biological basis for symptoms have been formed (http://www.eu-aims.eu/; (Ashwood et al., 2014))(http://www.pond-network.ca/). Previously, borrowing therapeutics and treating comorbid symptoms was the standard of management of these disorders; finding treatments of largest effect size to a heterogeneous population was not very effective, since it rarely addressed the biological cause. Because of the identification of convergent pathways, the therapeutic strategy is moving from solely treating comorbid symptoms to attempting to address the core domains. For example, the POND network locally in Ontario has been a model organization to facilitate the research translation in parallel with clinical trials, the first in Canada that targets neurodevelopmental disorders. Stratifying children via genetic differences, endophenotypes and treatment response across diagnostic categories, paired with our understanding of mechanisms in model systems, we will be able to develop medications and interventions that are more effective. With more clarity into the subgroups using a diagnostic agnostic approach, stratified treatments for various samples will give way to a higher rate of success. This approach, if successful, will advance the treatment and management of neurodevelopmental disorders by understanding the contextual factors, leading more precision in medical practice.

Reverse Translation

There are the typical challenges in model systems because of the inherent complexity of physiological interactions and the limited understanding of biology (Zoghbi, 2013). Over and above those challenges, a challenge specific to modelling ASD stems from the genetic heterogeneity and phenotypic variability simultaneously seen in the clinic. This is reflected in the

evolving definition and understanding of the spectrum as discussed above. Currently, the diversity of phenotypes in mouse models of ASD mice is similar to the variable expressivity seen in ASD patients (Ellegood et al., 2014, Ey et al., 2011). Given the variability at both the genotype and phenotype levels, approaches characterizing models at multiple levels, endophenotypes, are needed to find common targets – convergence in one or more of these levels. The endophenotypes that have an underlying biological process that is understood will have the most utility in this translation process. For example, having face validity for ASD in behaviours such as sociability deficits does not mean there is the existence of an autistic mouse, nor does it necessary lead to the conclusion, by itself, that the model is useful. In addition, a specific challenge with the classic sociability outcomes measures is that quantitative information may not translate directly to quality of interaction (Carter et al., 2011). A difference in the *quality* of interaction is a requirement for ASD but the *lack* of sociability is not. The former is difficult to measure in a mouse, and hence, up and coming metrics, such as the significance of USV call types (Zeskind et al., 2011) may be helpful in this regard (Wohr et al., 2013). Overall, however, above and beyond the face validity, recognizing the biological embeddedness of mental states and measurable biological processes that correlate to aspects of symptomology – endophenotypes – is the way forward. Utilitarian value can be conferred in reverse translation only when that is established and when clarity regarding construct validity from both the clinical and animal model is found. Thus, the goal of modelling disorders is ultimately to understand the biological processes and leverage that for treatment and management rather than merely reconstituting the disorder.

The POND approach of using diagnosis agnostic modelling, that is, modeling across diagnostic boundaries, has the potential to assist in finding relationships between behavioural outcomes related to face validity and biological mechanisms related to construct validity through endophenotypes. The work of our lab in part with the POND project aims to address this question at the level of mouse behaviour and neuroanatomical phenotype. We are completing an early life

behavioural pipeline followed by brain imaging under the same conditions in various background strains of mice as well as genetic mutant lines, which, included in this thesis, the *FMR1-KO* mouse is one. This study will allow for the comparison of different assays, behaviour measures and neuroanatomical changes and associations between these outcomes.

The *FMR1-KO* Mouse as a Model for Discovering ASD Treatment Targets

As mentioned above, strength of the *FMR1-KO* mouse model is its construct validity. Typically, as discussed above, outcomes after a gene knockout reflect the function of what is remaining and the differences in function cannot be merely localized to the single lesion but the interaction between that lesion and the rest of the brain (Teitelbaum and Pellis, 1992). In the *FMR1-KO* mouse, the genetic lesion found in patients with FXS is replicated – namely, the lack of the gene product. Therefore, the utility of the *FMR1-KO* mouse model is clear. FXS is the leading heritable single gene cause of ASD to date and the processes that are governed by the FMR1 gene product are relatively well understood. Research with *FMR1-KO* mice elucidating the function of FMRP at the synapse and circuit level has been advancing well in the past two decades and that understanding has opened many potential therapeutic avenues (Fung et al., 2012, Berry-Kravis, 2014). Currently, therapeutic targets have identified and are being tested using the *FMR1-KO* mouse (Maurin et al., 2014). The studies in this dissertation clearly demonstrate that underlying trans-diagnostic mechanisms between FXS and ASD make the *FMR1-KO* mouse a useful model to find ASD targets and similar translational strategies might be used in other brain disorders (Fernandez et al., 2013, Hagerman et al., 2014, Yu and Berry-Kravis, 2014).

Conclusions

In this dissertation, I have extended the current body of knowledge regarding the *FMR1-KO* model. My results showing the transient changes in the expression of ASD risk genes furthers

the case that the *FMR1-KO* mouse may help in our understanding of gene interactions in the development of brain disorders. I have shown that the subtle transient changes in early life communication are not a robust phenotype and not a strong predictor of social behaviour in the 4th week of life. Furthermore, I demonstrate that sex is an important modifier, both of gene expression and behavioural outcomes throughout the prepubertal period in this mouse model. Altogether, these point to usefulness of the *FMR1-KO* mouse model to understanding the processes during neurodevelopment.

These studies show that the phenotype expression of the FMR1 gene is influenced by multiple factors that interact in a complex interplay. These factors include genetic modifiers in background strain, early life adverse exposures, and sex; each contributes to risk or resiliency to disease. These interactions will manifest in various ways in different contexts. Specific contributions of my work that are valuable to the gaps include demonstrations that (1) strain by sex interactions in brain and behaviour are present in mouse models, (2) shared neurobiological pathways between FXS and ASD may be through the NLGN/NRXN family, and (3) the *FMR1-KO* mouse model is useful in exploring endophenotypes and find treatment targets for neurodevelopmental disorders.

Future Directions

Understanding neurodevelopment through the approaches in the thesis and strategies discussed above will lead to an appreciation of the complexities of brain development and the factors that influence it. That knowledge can be leveraged to treatment and therapy for those with neurodevelopmental disorders. To understanding of neurodevelopmental disorders specifically and brain development as a whole, research must move away from a criteria that gives clinical utility focused on managing symptoms to a paradigm based on etiology to modify disease (Rapin, 2014). This move away from a general pharmacology that addresses broad symptoms to precise circuity-based therapy will be possible in the future. We will be able to, based on a set of endophenotypes, predict which patients most likely will benefit from a certain treatment. The

studies in this thesis show that the *FMR1-KO* mouse model, first among many, is useful in the endeavor of back translation from the clinical to preclinical mouse, and then, finding useful outcomes, back to the clinic in the form of novel treatments and therapies.

In the future, similar work prioritizing translational models based on highly penetrant genes, such as the FMR1 gene, or other severe environmental risk factors may be an efficient strategy. Obtaining clarity as to the biology in these cases will benefit the larger group, assuming a sharing of mechanism in subgroups or cluster of ASD cases. In addition, better understanding of behavioural tasks and agreement on useful endophenotypes will serve the field well in the near future. There needs to be ethologically valid tasks (Wohr and Scattoni, 2013) which the biology of the complex behaviour is understood. This will lead to potential targets in translation work (Crawley, 2012).

Furthermore, this dissertation demonstrates the importance of contextual factors in developmental neurobiology, be it sex, strain, or environment, on the developmental trajectory of various outcomes across time. The early life developmental trajectory is dynamic and an understanding of the mechanism and influences to shape the trajectory of an outcome needs to be examined (Veenstra-VanderWeele and Warren, 2015). Recent work in examining the transcriptome, including work with non-coding RNAs, demonstrates the lack of understanding of their role in development (Tebbenkamp et al., 2014). Specific to mouse models, environment interactions are poorly understood (Homberg, 2013, Burrows, 2011) and need to be studied in a similar way as “rare genetic variants with larger effect sizes” (Edwin Cook, 2013, personal communication). A recent twin study reported that the genetic contribution in ASD may have been over-reported in the past and there was an environmental liability of 55% in twins (Hallmayer et al., 2011). This lower genetic heritability suggests shared environmental factors by twins more than siblings (*in utero* or perinatal) are more influential, and those factors may interact and act on other genes. Some of these include parental age (Hultman 2011), birth complications (Gardener 2009), immigrant populations (Ericksson 2012, Guinchart 2012, Kolevzon 2007), in utero

exposures to teratogenic compounds (Christianson et al., 1994, Williams and Hersh, 1997, Williams et al., 2001, Rasalam et al., 2005, reviewed in Roullet et al., 2013) and maternal infection (Chess, 1970, 1977, Atladottir et al., 2010). Thus, more research needs to examine the role of the environment, particularly, the early life period on the trajectory of outcomes in neurodevelopmental disorders.

Studies in the rodent literature have shown large effects due to environmental enrichment (Restivo et al., 2005, Branchi and Alleva, 2006, Nag et al., 2009, Yang et al., 2011, Buxbaum et al., 2012, Oddi et al., 2014). In the case of the *FMR1-KO* mouse, simple home cage enrichment was reported to rescue some deficits (Restivo 2005), thus, the role of environmental interventions cannot be downplayed. In the toddler and preschool children with ASD, early intensive behavioural intervention improves cognitive and early functional outcomes (Warren et al., 2011) and neuroanatomical changes (Dawson et al., 2012). With these therapies, most children do not move off the spectrum (Dawson et al., 2010) but importantly, secondary impairments in intellectual disability and language are prevented. It is significant in the management of these disorders to bend the trajectory of certain outcomes that would lead to further cascades of mental disorders and loss of adaptive function.

Lastly, another consideration stemming from a developmental understanding in mouse models is that once we identify a window, we must take care in our translation from the mouse: do we match developmental window, duration, brain development, cognitive ability or developmental milestone? That, once again, highlights the limited understanding of differences/similarities between mouse and man and calls for more clarity into the complexities of neurobiology. These studies emphasize the precision required in housing these intricately designed systems which mental processes are embedded.

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