Expression of Interleukin-6 (IL-6) in The Cerebellum is not altered in the Absence of Fragile X Mental Retardation Protein (FMRP) or with Motor Skill Learning

by

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Abstract

The ability of the brain to change structurally and functionally with experience is called brain plasticity. High levels of pro-inflammatory cytokines impair normal memory formation and consolidation. To better understand the role of pro-inflammatory cytokines in learning, the contribution of the cytokine interleukin-6 (IL-6) to a motor skill learning task investigated. The *Fmr1* Knockout (KO) mouse, an animal model of Fragile X Syndrome, has demonstrated impaired neural plasticity and learning. *Fmr1* KO and control wild-type (WT) mice were trained on the dowel and flat beam runways to study motor skill learning and motor activity respectively. The cerebellum from the animals was examined for IL-6 protein using ELISA. No significant differences in the levels of IL-6 in the cerebellum of the *Fmr1* KO and WT normal mice were found. The expression of IL-6 was not altered by the behavioural training. These results suggest lack of association between IL-6, and FMRP and motor skill learning.

Keywords: Fmr1 KO mice, IL-6, Motor Skill Learning, The Cerebellum

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Dedicated to

My Parents and sisters who love me from the depth of their heart

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Chapter 1

Brain Plasticity

The brain is capable of reorganizing and adapting in response to environmental experiences, such as in learning. A growing body of knowledge has been obtained about brain plasticity. It was once believed plastic changes only occur in the developing brain. An emerging evidence, however, supports the occurrence of brain plasticity in the adult brain, although it differs from which takes place in early development. A key understanding regarding brain plasticity is that similar mechanisms within the brain, such as morphological changes in synapses, or alteration of proteins, mediate different types of plasticity, including plastic changes underlying learning, developmental plasticity, or disorder-related plasticity. That is, the brain has a redundant tendency, through which the similar mechanisms mediate functional and structural changes in response to different experiences.

Enriched Condition

Early studies of experience-dependent plasticity investigated the influences of rearing rats in rich and complex environments compared to keeping rats in standard laboratory cages. The complex environment paradigm was initially introduced by Donald Hebb (Hebb, 1947). He reared rats in his house as an enriched condition (EC) and found the rats explored his home for several weeks (as pets) and in doing so, he observed they demonstrated better problem-solving ability than rats who were kept in the standard laboratory cages. Keeping rats in an EC environment, which provided new and diverse learning opportunities, appeared to impact behaviour. Following this observation, researchers attempted to further investigate this

phenomenon and established complex enriched environments in laboratories. In the laboratory, an EC environment consists of introducing different toys and objects which provide opportunities for social interaction, activity, and exploration (Rosenzweig, Bennett, Hebert, & Morimoto, 1978). Exposing rats to enriched environments affects the structure of their brain. At the gross anatomical level, the brains of EC rats had thicker, and heavier cortices relative to the brain of rats kept in standard laboratory cages (Rosenzweig, Krech, Bennett, & Zolman, 1962). This significant experience-dependent alteration in the cortex was observed via cellular level analysis where increases in the size of the neuronal cell bodies (Diamond, Lindner, & Raymond, 1967). Enhanced dendritic branching complexity in the visual cortex of rats exposed to the enriched environment (Greenough & Volkmar, 1973) could explain the increased cortical weight and thickness. If there is an association between structural changes in the brain and enhanced learning ability, it may suggest establishing new or strengthening of existing neural network is a part of the structural changes underlying this phenomenon. Since the synapse is the key structure for neural communication, alterations in behaviour may correspond to changes in the synapses themselves.

Formation of new synapses between neurons, which is called synaptogenesis is one form of plastic changes occurring in response to experience. Globus, Rosenzweig, Bennett, and Diamond (1973) showed an increased number of spines per basilar dendrite of pyramidal neurons in the occipital cortex. In order to examine synapse morphology, electron microscopy techniques were used to detect ultrastructural changes occurring in response to the complex environments. Turner and Greenough (1985) reported an elevation in the average numbers of synapses per neuron in the occipital cortex. Since synapses are the key sites of neuronal communication, the addition of new experience-induced synapses may underlie the formation of

a new visual memory in particular. Exposure to enriched environments not only adds new synapses, but changes the shape of the new or existing synapses. For example, Greenough, West, and DeVoogd (1978) found enhanced numbers of synaptic spines with discontinuities, called perforated synapses, in the occipital cortex of the EC rats, suggesting complex environments influence the brain size and synaptic ultrastructure. Exposure to EC influences the structure of the brain.

It is important to understand the context of previous studies discussed, where the rats were approximately 25 days old (e.g. Greenough & Volkmar, 1973; Greenough et al., 1978). These studies raise the possibility that the observed structural changes were due to a combination of the complex environment and unique features of early brain development. An emerging question in brain plasticity research is whether environment influences the adult brain in a similar way as for young developing brains. Riege (1971) examined the effects of exposure to a complex environment in adult rats on enzymatic activity and total brain weight at 285 days of age. He found the rats demonstrated increased cholinesterase activation in the visual cortex and increased cortical weight enhancements relative to control rats. Greenough, Juraska, and Volkmar (1979) demonstrated exposure to an enriched environment enhanced dendritic arborization in the visual cortex of rats at 80 days of age that was qualitatively similar to that of rats exposed to the environment at 25 days of age. These findings illustrate the adult brain can change in response to experience, although these plastic changes were not as robust as those observed among the young rats.

Structural modifications are the biological basis of learning and memory. In accordance with this idea is the notion that neural reorganization does not occur ubiquitously across the brain in response to specific experience, but that structural changes related to specific behaviour(s)

should be observed in regions of the brain related to the behaviour(s). Faherty, Kerley, and Smeyne (2003) found elevated neural growth in dentate gyrus pyramidal cells in the hippocampus of adult rats in a complex environment. These changes, however, did not occur in pyramidal cells of the motor cortex or spiny neurons within the striatum. The observed plastic changes in the hippocampus occurred in response to the spatial learning required for the animal to navigate around a changing environment, suggesting morphological plasticity is restricted to the brain regions engaged in the neural connections associated with a particular experience.

Although much of the research on experience-dependent plastic changes in the brain has focused on structural changes of neurons, supportive tissue elements, in particular, astrocytes show various plastic changes in response to experience. The association between these structural and functional plastic changes in astrocytes following exposure to enriched environments and after learning a new motor skill has been examined by means of quantification of glial fibrillary acidic protein (GFAP) within astrocytes. GFAP is a major component of astrocytic filaments. The association between the neuronal changes and enhancement in the volume fraction of astrocytes (i.e. hypertrophy) (Sirevaag & Greenough, 1987) suggesting non-neural plastic changes in conjunction with neural changes in response to experience. Structural changes in astrocytes in EC rats co-occurred with neuronal plastic changes induced in the EC paradigm. Jones, Hawrylak, and Greenough (1996) reported four days of differential rearing induced increases in surface density of GFAP-immunoreactive astrocytic processes in the occipital cortex of rats by an amount similar to the previously observed increase in dendritic growth in the same layer (i.e. layer II/III of the visual cortex) (Wallace, Kilman, Withers, & Greenough, 1992). This evidence demonstrates the hypertrophy of astrocytes in the occipital cortex of the rats subjected to EC is coincident with synapse formation, which can be attributed to the EC environment.

Exposure to complex EC provides rats with opportunities for different sensory and motor experiences, such as physical activity, social and exploratory behaviours, relative to the control rats. This multi-layer paradigm of manipulation, however, makes it hard to attribute a plastic structural change to a particular component of the complex environment related to learning. In light of this challenge, training animals in different learning and memory tasks, in particular, motor skill learning, is a more precise approach which attempts: 1) to minimize the influence of different factors on neural plasticity, and 2) to focus on the process of motor skill learning. Skilled motor tasks hold potential to provide a more accurate evaluation of the types of influences that a particular motor behaviour has on plastic changes in the brain.

Learning and Memory

Memory can be defined as the ability to retain and remember previous experiences and respond to them appropriately. Learning processes, through which prior experiences lead to persistent structural and functional alterations in the central nervous system (CNS), form memories. In humans, memory formation occurs in three successive stages: encoding, storage, and retrieval (Atkinson & Shiffrin, 1968). An interruption in one of these steps disrupts memory processing. Encoding allows data from the environment to be perceived as physical or chemical stimuli. At this stage, information should be modified, such as being processed and combined before being put into the next stage. In the second step of memory processing, which is storage, data are kept temporarily. The last stage is the recovery of data, which has previously been stored. In humans, this information needs to have access to the consciousness in order to be retrieved.

Memory can be divided into three main categories. Sensory, short-term and long-term memory. Sensory memory maintains relevant information for about a second after an object is perceived and approximately 12 items can be stored in this form of memory. This memory is an instant response to environmental stimuli. In humans, short-term memory refers to the ability of a person to temporarily remember and process information at the same time. Short-term memory capacity is usually limited to approximately seven items in a readily available state, usually from seconds to a week. Conscious effort is necessary to retain this information to become a long-term memory, which can then be maintained even large amounts of information for an infinite duration. The transfer of information to long-term memory for more permanent storage can be improved via mental repetition of the information or by associating it with other previously obtained knowledge.

Short-term memory is mediated via a temporary activity of neuronal connections, whereas long-term memory is sustained by more permanent alterations in neural communications extended all over the brain. The hippocampus is necessary for the consolidation of data from short-term to long-term memory, however, this brain structure does not store information itself. As observed in a patient, HM, who had both of his hippocampi removed (Scoville & Milner, 1957), the absence of the hippocampus, impairs the storage of new memories into long-term memory. After removal of his hippocampus, HM could still remember his childhood memories, but not events occurred during the years before the surgery. This observation suggests long-term memory is not dependent on the hippocampus, whereas the more recently encoded memories appear to do so (Smith & Kosslyn, 2007). The hippocampus contributes to the consolidation of memories, such that interactions between the hippocampus and different cortical regions store memories outside the medial temporal

lobe, where the hippocampus located, via the formation of connections between the cortical representations of the experience.

In terms of the type of information to be processed, there are two types of memories, declarative and procedural. Declarative memory stores information concerning principles and facts and require some conscious procedures to recall information. Procedural memory, on the other hand, is an unconscious process involving motor skills and how to do things, specifically in the case of motor learning, movements of the body, or the use of objects, such as playing guitar. These memories are usually obtained through practice and repetition and are composed of automatic sensorimotor behaviours, which are so profoundly embedded that individuals are no longer aware of them. Once learned, these body memories allow a person to conduct routine motor actions more or less automatically. When a person or an animal improves in performing a particular task because of repetition, the subject has unconsciously accessed aspects of the already learned experiences. Procedural memory is related to motor learning and relies on the basal ganglia and the cerebellum. A feature of procedural memory is that learned skills are unconsciously transferred into behaviours, and the process may be hard to describe.

The most frequently used test in the literature investigating learning and memory functioning in rodents is the water maze task, a hippocampal-mediated task, which measures spatial learning. Morris (1984) originally developed the water maze test where animals are situated in a circling pool and are trained to locate a hidden platform, which is positioned in the pool. The animal should recall the spatial location of the hidden platform, immersed under the non-transparent water surface, using visual cues, and learn how to reach the hidden platform to terminate swimming. After the acquisition of the task usually a transfer test or probe test is performed. In this task, the platform is removed from the maze. During a brief trial, the animal is

located in the maze without the platform where the frequency, during which the animal traverses the previous spot of the platform, or the number of time the animal swims in the spot, where the platform was previously placed, demonstrate the acquisition of the spatial memory. The indicator of spatial learning is the latency to discover the platform, meaning the quicker the animal finds the platform, the better the spatial memory is. The animal, thus, should learn both the procedure, that is to climb on the platform, and the process of finding the spatial position of the hidden platform.

Motor skill learning improves cognitive, perceptual, or motor performance as a consequence of training. Complex motor learning involves the activity of different joints and limb coordination (Sanes, 2003), in addition to cognitive processes. There are many paradigms, which have been used to study motor skill learning. Two commonly used in related research are the acrobatic and the dowel tasks. The first paradigm involves teaching an animal to reach via a narrow slit to obtain a food pellet. This action requires a repetitive series of movements in the shoulder, elbow, wrist and digits of the rats (e.g. Whishaw & Pellis, 1990). Animals demonstrate improvements in their speed and accuracy of reaching, which is a hallmark of learning in this motor skill. In the second paradigm, acrobat training, which was initially introduced by Greenough and his colleagues (e.g. Black, Isaacs, Anderson, Alcantara, & Greenough, 1990), animals are trained to run along a complex runway including various obstacles, where the rats should climb over or balance on. In this particular paradigm, rats the in control group perform a motor activity that is similar in length to the acrobat course. The other motor skill learning paradigm, the dowel task, is adopted from the most complex components of the acrobat courses as a motor skill task for training rats. In the dowel task, rats/mice have to run across a complex motor runway containing a number of dowels protruding from the runway at different angles

(e.g. Derksen, Ward, Hartle, & Ivanco, 2007; Larson, Hartle, & Ivanco, 2007). These previous studies have shown at the beginning of the training, the dowel rats took more time to doing so than control rats required to run on a simple flat beam runway. After repeated testing, rats subjected to the dowel task demonstrated an increase in their speed, indicating the occurrence of motor skill learning.

Motor Skill Learning

Teaching animals on the dowel task is an accurate way of studying motor skill learning because of the specificity of the manipulation. Through teaching an animal a novel motor skill, it is possible to examine motor skill learning component, via controlling motor activity using the simple flat beam task. Several brain regions are involved in motor learning, such as the primary motor cortex, cerebellum, striatum, and brain stem which can be investigated for differences in gross anatomy and molecular characteristics.

The cerebellum is a brain structure that plays a major role in motor functions, such as motor control and motor skill learning (Linas & Welsh, 1993). The cerebellum is not involved in the initiation of a movement, but plays a role in accurate timing, coordination, and precision. This region of the brain receives sensory inputs from the spinal cord and other areas of the brain and incorporates these sensory inputs to fine-tune motor activity. At the level of gross anatomy, the cerebellum is composed of a folded layer of cortex. In terms of the surface appearance, the cerebellum is divided into three lobes: from top to bottom, the anterior lobe, which is situated above the primary fissure and mediates motor functions and unconscious proprioception, the posterior lobe, located beneath the primary fissure and mediates fine motor coordination, and the flocculonodular lobe located under the posterolateral fissure. The medial part of the anterior and

posterior lobes is composed of the spinocerebellum, which mainly mediate limb and fine-tune body movements (Rapoport, van Reekum, & Mayberg, 2000). This area receives proprioceptive input from the spinal cord, auditory, and visual systems, and in turn projects to both the brain stem and cerebral cortex modulating descending motor systems. The spinocerebellum can expand proprioceptive information in order to predict the future position of the body throughout the course of a movement.

Purkinje cells (PCs) constitute the only inhibitory output from the cerebellar cortex in the form of a bundle of projections to the cerebellar nuclei and also vestibular nucleus, which in turn project axons to the rest of the brain. The outermost layer, is a key layer of the cerebellar cortex. This area comprises the parallel fibers (PFs), which are derived from the granule cells. Two main excitatory afferents are the mossy fibers deriving from the brain stem and spinal cord, and the parallel fibers (PFs) originating from granule cells and single climbing fiber (CF) arising from the inferior olive nuclei in the medulla. The input from CF to Purkinje neurons alters the response to mossy-fiber inputs in a prolonged period of time (Ito, Sakurai, & Tongroach, 1982). CFs regulate the input of PFs to PCs through inducing *long-term depression* in the synapses between PFs and Purkinje neurons that are simultaneously activated via the CFs. Parallel activation of CFs and PFs reduces the responses of PCs to further stimulation of the same PFs. Learning occurs in the in the deep cerebellar nuclei and the cerebellar cortex (Ito et al., 1982; Marr, 1991). Inputs from CFs produce instructive signals leading to alterations in the strength of synapses in the cerebellar cortex. Long-term depression of the synapses between PFs and PCs, in which CFs cause plasticity, is associated with learning (Ito, 1989). Other sites of synaptic plasticity throughout the microcircuit, such as the deep cerebellar nuclei are also linked with

learning. Learning a motor skill, thus, occurs via complementary synaptic alterations in the deep nuclei and the cerebellar cortex.

Motor skill learning leads to increased number of synapses of PFs density to PCs in the outermost molecular layer (Anderson, Alcantara, & Greenough, 1996). Also, the length of spines along the distal dendrites of PCs are significantly increased in the cerebellum of motor skill-trained rats (Kim et al., 2002; Lee, Jung, Arii, & Imoto, 2007). Learning a new motor skill also induces synaptogenesis in the cerebellar cortex by increasing the proportion of synapses per PCs in the paramedian lobule, which is also responsible for coordinated limb movement (Nishiyama, 2014). Learning a motor skill reorganizes the synaptic or dendritic morphology in the cerebellum causing constant alterations in neuronal activity. Examining changes in these features produced by motor learning may provide information about the key contribution of the cerebellum to learning.

Similar to exposure to an enriched environment, motor skill learning induces selective structural changes in the brain. Greenough, Larson, and Withers (1985) found reach training-induced increased dendritic branching in layer IV and layer V motor cortex pyramidal neurons. In layer V, the authors found the apical dendrites reorganized in the hemisphere contralateral to the reaching arm, however, the reorganization in layer IV just occurred in the basilar dendrites of pyramidal neurons in bilateral hemispheres. Motor skill learning also affects gross anatomy of the brain. Anderson, Eckburg, and Relucio (2002) found enhanced cortical thickness in the medial region of the two most anterior coronal planes of the motor cortex of the rats trained on an acrobat course relative to motor control animals, who had free access to a running wheel. Research indicates motor skill learning induces neuronal reorganization and consequently

anatomical changes in the cerebellum.

Learning a new motor skill can induce synaptogenesis and altered synapse morphology in specific regions mediating motor skill learning, such as the cerebellum and the motor cortex. Black et al. (1990) found a considerable elevation in synapses per PCs in the cerebellum of rats exposed to acrobatic training relative to control group after one month of motor skill training. The authors reported an increase in capillary density in rats with high activity compared to that of the rats in acrobat task, suggesting synaptic alterations were induced by learning. The same training method induced more multiple synapses (e.g. a presynaptic neuron in contact with more than one postsynaptic neuron) in PFs of the cerebellar cortex (Federmeier, Kleim, & Greenough, 2002). Derksen et al. (2007) found an increase in synaptophysin protein, which is an indicator of synaptogenesis, after the first five days of training on a complex motor learning task in the motor cortex of rats. Motor skill learning induced synaptogenesis such that increased synapse number and/or increased synaptic activity and consequently, indicate synaptogenesis, which was specific to motor learning. These structural and functional changes are due to the acquisition of new motor skills and not simple motor activity.

Long-lasting structural changes associated with motor learning have also been examined and demonstrate that training rats for ten days in acrobatic conditions results in increased synapse per PCs in the cerebellum, which is observable for 28 days (Kleim, Vij, Ballard, & Greenough, 1997). Precise assessment of volume density using an unbiased stereological technique, which provides three-dimensional and quantitative data from tissues, demonstrated a decline in PCs density after ten days of training. This evidence suggests the cerebellum sustains its new synaptic connections when a skill is learned, whereas the cerebellum does not maintain the altered neuronal density over 28 days of training. If decreases in neuronal density are due to

dendritic growth pushing cell bodies further away from each other, this suggests that the function of cerebellar plasticity is to maintain specific connections. This was supported by a persistent reduction in neural density in the motor cortex after ten days of reach training, suggesting the cortex might be changing, and requiring constant activity to keep its synaptic connections (Morales, Pinto-Hamuy, Fernandez, & Diaz, 1999). Briones, Klintsova, and Greenough (2004) also found rats exposed to an enriched environment for one month sustained an increased synapse per neuron in the visual cortex when removed from the complex environment for one month. It is likely the persistence of experience-dependent neural modifications varies between brain regions and is based on the type of experience inducing the change.

Other non-neural components of CNS, such as astrocytes, also undergo plastic changes during learning. In addition to various roles the astrocytes play in neuronal plasticity, the learning-induced structural plasticity of astrocytes accompanied with the number of synapses. For instance, Anderson et al. (1994) found in motor skill learning tasks, which resulted in the addition of synapses in the cerebellum, the volume of astrocytic processes for each neuron was enhanced with, and was associated with the number of synapses per neuron. The authors indicate motor activity itself does not cause astrocytic hypertrophy or synaptogenesis, but indicate increased astrocytic volume was due to learning-specific synaptogenesis, and not was induced as a result of a general increase in activity of neurons. Motor skill learning-related morphological alterations in synapses are associated with structural alterations in these glial processes.

Motor skill learning occurs in at least two different stages. An *acquisition phase*, which is a rapid improvement in their motor performance (i.e. accuracy and speed) occurring just after a few training sessions. This improvement plateaus after persistent training, which is referred to As a *maintenance phase* (Nudo, Milliken, Jenkins, & Merzenich, 1996; Kleim, Lussnig, Schwarz,

Comery, & Greenough, 1996). Different functional plastic changes associated with motor learning occur within these two distinct phases of motor skill learning. As an illustration, the expression of synaptogenesis in the motor cortex of acrobat rats (Kleim et al., 1996) and alteration of the motor map in the brain (Kleim et al., 2003) take place within the second phase of learning, but not in the acquisition phase. This evidence suggests these temporally characterized phases of motor skill learning reflect a difference in the molecular requirements for neuronal structural changes during early and late phase learning.

Much research on plasticity focuses on neuronal morphological changes as an ultimate mechanism, however, protein synthesis is necessary to mediate these structural changes. The first evidence demonstrating the necessity of protein synthesis for memory retention came from Flexner, Flexner, Stellar, and Haba (1962), who showed administration of a protein synthesis inhibitor, puromycin, inhibited learning a task that was easily learned by rats did not receiving the protein inhibitor. Since puromycin is toxic and might confound the finding, Flood, Rosenzweig, Bennett, and Orme (1973) replicated these results with less toxic inhibitor (i.e. anisomycin). Observed learning impairment was associated with the lack of new protein, rather than as impact caused by the toxicity of the treatment. These results indicate the importance of protein synthesis for learning.

One approach to investigating cellular mechanisms underlying plasticity of the brain in response to experience is using an electrophysiology model of learning and memory, long-term potentiation (LTP). This model was first introduced by Bliss and Gardner-Medwin (1973), who demonstrated high-frequency, short bursts of stimulation of hippocampal neurons led to a constant rise in the strength of synapses in the dentate gyrus. LTP is an N-methyl-D-aspartate (NMDA) receptor-dependent strengthening of a synaptic network, induced by electrical

stimulation requiring protein synthesis for the late stages to happen (Otani, Marshall, Tate, Goddard, & Abraham, 1989). Stanton and Sarvey (1984) tried to generate LTP in the hippocampal slices incubated in protein synthesis inhibitors. The authors found incubating the preparation for less than an hour impaired the induction of LTP, which was seen in these slices in a dose-dependent manner. Their results indicate hippocampal LTP requires protein synthesis and indicates the necessity of synthesis of protein for long-term memory and synaptic transmission.

LTP studies indicate neuronal activity increases mRNA within dendrites selectively at sites of synaptic activation (Cole, Saffen, Baraban, & Worley, 1989). LTP is a suitable model to investigate the process of activity-dependent synthesis of protein because LTP studies use neural circuitry with identified termination areas associated with a population of neurons. The mRNA should be in dendritic spines, or should be moved from the cell body through activity-induced signalling in order to allow protein synthesis. Stimulation of afferents to the dentate gyrus lead to an enhancement in the expression of immediate-early gene (IEG), and Arc, in activated dendritic segments (Steward, Wallace, Lyford, & Worley, 1998). Augmentation of the proteins and mRNAs in dendritic segments appears to be associated with neural activation, and the mRNAs locally translate proteins needed for supporting activity-dependent learning and memory, suggesting similar alterations in mRNA and proteins could be learning-induced.

Expression of proteins occurs during and after motor skill learning. Irwin et al. (1998) reported the Fragile X Mental Retardation Protein (FMRP) could be induced during motor skill learning on the acrobatic task. Recently, Wang, Lin, Chen, and Lin (2014) examined the synthesis of immediate-early genes activity-regulated cytoskeleton-associated protein (Arc), the maker of recent learning- dependent neuronal activity, following a cerebellar-dependent motor skill learning paradigm. The animals were trained to run on a runway, which was covered with

pegs, for five consecutive days. The authors found the expression of Arc was dramatically enhanced in the cerebellum of the rats after motor skill learning, whereas the levels of Arc protein did not change with motor activity, suggesting the expression of Arc protein in the cerebellum is associated with acquiring complex motor skills. These results provide support for the contribution of expression of various proteins as the neuronal substrate mediating acquisition and consolidation of complex motor skills.

Local Protein Synthesis Mediates Plastic Changes

The location where proteins synthesized is a subject of several studies. The traditional theory of activity-associated protein synthesis is the cell body produces proteins, which are then transferred out into the dendrites for regulating structural changes. Early studies found polyribosomes, which include necessary machinery for protein synthesis, in dendrite spines of hippocampal neurons in the rat (Steward & Levy, 1982) and during increased synaptogenesis or synaptic plasticity (Steward & Falk, 1986). Aakalu, Smith, Nguyen, Jiang, and Schuman (2001) used green fluorescent protein (GFP)-based synthesis reporters to identify brain-derived neurotropic factor (BDNF)-induced protein synthesis in isolated dendrites. Normal dendrites exposed to a GFP synthesis-based reporter demonstrated an enhancement in fluorescence after BDNF treatment. This increased fluorescence, which was due to GFP, indicate the BDNF triggered the synthesis of the reporter was not observed in dendrites that did not receive this treatment. These results suggest protein synthesis underlies synaptogenesis and dendritic growth.

There are still various unanswered questions on how an interruption in the synthesis of proteins, or their mRNA contributes to learning and memory deficits. Animal models of impaired brain plasticity and learning deficits provide a great opportunity for researchers to

examine the brain changes associated with experience, in particular, learning and memory. Known impaired neural plasticity in different neurodevelopmental disabilities, such as Fragile X Syndrome (FXS) and other related disorders, including autism spectrum disorders (ASD), has given researchers a good tool to investigate how deficits in cellular mechanisms affect learning and memory.

Fragile X Syndrome

FXS is a neurodevelopmental disorder and is the most prevalent genetic form of intellectual impairments. This disorder is caused by an expansion of trinucleotide CGG repeat in the fragile mental retardation1 (*FMR1*) gene to over 200 repeats, resulting in limited or no production of FMRP, in particular, in the brain (Verkerk et al., 1991). In the general population, the *Fmr1* gene contains 5 to 50 repetitions of the CGG nucleotide sequence. In FXS with the full mutation, hundreds to thousands of CGG repetitions suppress the expression of the *FMR1* gene, which encodes for FMRP (Warren, 1997). The physical characteristics of FXS are macroorchidism, large ears, an elongated face, and protruding jaw (Hagerman, 2002). The behavioural features of people with FXS include mild to severe intellectual disabilities, learning impairments, impaired sensory reactivity, anxiety, hyperactivity, and poor motor coordination (Hagerman, 2002). Recently a meta-analysis study reported the prevalence rate of FXS is approximately 1 in 11,000 females and 1 in 7000 males (Hunter et al., 2014). Since FXS is an X-linked disorder, females demonstrate milder symptoms than males because of compensation from the non-affected X chromosome in females.

Fmr1 KO Mouse Model

There are two inbred strains based on which the *Fmr1* Knockout mice, the well-known animal model of FXS are generated, FVB and B6 genetic backgrounds. The *Fmr1KO* mouse was initially introduced by the Dutch-Belgium Fragile X Consortium (Consortium et al., 1994). The *Fmr1* KO mice on FVB background are obtained from B. Oostra from 129/OLA embryonic stem cells. Then, a targeting vector composing of an interrupted *Fmr1* DNA sequence was incorporated into embryonic stem cells and carried into female mice. FVB (129p-+<Pdeb-rd1> Fmr1<tm1Cgr>) heterozygote *Fmr1* females and transgenic males were backcrossed for a number of generations (Consortium et al., 1994), to produce *Fmr1* experimental animals. Finally, these mice were created with other background strains, including the FVB inbred mouse strain. In humans, the trinucleotide expansion, which silences the expression of *Fmr1* gene, causes lack of synthesis of the FMRP (Ashley et al., 1993). Although human with FXS is different from the mouse model, in which the *Fmr1* gene is knocked out, and there is no FMRP, this mouse model has shown similar synaptic impairments to those seen in people with FXS.

Synaptic Characteristics of FXS and the *Fmr1* KO mice

Spines serve as an anatomical substrate for synaptic transmission and memory storage. Morphological studies indicate abnormal spine morphology throughout the lifespan, such as thin, long spines in FXS and *Fmr1* KO mice due to loss of FMRP (reviewed in Irwin, Galvez, & Greenough, 2000). A greater density of dendritic spines, along with morphological alterations, have been reported in the brain of patients with FXS compared to normal populations (Irwin et al., 2000), likely due to unstable synapses caused by an imbalance in excitatory-inhibitory synapses, or loss of synaptic pruning (Portera-Cailliau, 2012). Synaptic impairment is common

in several other neurodevelopmental disorders, such as ASD (Spooren, Lindemann, Ghosh, & Santarelli, 2012), suggesting the synaptic impairments are the key features of FXS and other related disorders impacting learning.

Similarly, the *Fmr1* KO mice present similar spine abnormalities seen in people with FXS (Nimchinsky, Oberlander, & Svoboda, 2001). Nimchinsky et al. (2001) indicated the *Fmr1* KO mice demonstrated elongated and enhanced spine density in their barrel cortex at the age of one week old, which was not detectable at one month of age. The adult *Fmr1* KO mice, however, demonstrated immature elongated spines relative to the WT mice (Comery et al., 1997). The expression of FMRP is necessary for normal development of dendritic spine structure, and the absence of FMRP impairs the normal development and morphology of the synapse, which may explain learning and memory deficits in this mouse model.

Motor Skill Learning Problems and Motor Deficits in FXS and the *Fmr1* KO mice

There is a large body of evidence on the behavioural and cognitive characteristics of people with FXS. A few study has focused on the motor features of FXS. Baranek et al. (2005) investigated the motor characteristics of individuals with FXS at 9-12 months old and reported abnormal motor patterns including repetitive leg movement and posturing problems. Rogers, Wehner, and Hagerman (2001) investigated the motor development and cognitive abilities in children FXS and autism, and with FXS without autism found lower scores on motor scales in the FXS group. Zingerevich et al. (2009) found lower fine motor scores in children with both FXS and autism than those without, suggesting motor impairments in individuals with FXS. Normal development of motor skills allows children to participate in social and physical interactions. Although children with FXS show low tone and endurance, influencing the normal motor development and prepossess them to encounter problems in developing cognitive and social interaction, and self-care activities, no study has

examined the molecular basis of these motor skill problems due to the loss of FMRP in this population.

Recent studies have shown motor skill learning deficits in the *Fmr1*KO mice. Padmashri and colleagues (2013) reported impairments in motor skill learning in the *Fmr1* KO mice using the forelimb reaching task. The researchers trained the mice to obtain a food pellet via a tiny slit using their preferred hand. The authors found the *Fmr1* KO mice had a lower number of retrieves from all reaches compared to the WT mice, suggesting the motor skill learning impairment. Padmashri et al. (2013) suggested the motor skill learning impairment was due to the impaired functional and structural synaptic plasticities, such as impaired learning-induced formation of dendritic spines, and high rates of dendritic spine turnover in the *Fmr1* KO mice relative to the normal WT mice. Using a simple reaching task, Reiner and Dunaevsky (2015b) reported*Fmr1* KO mice had motor skill learning deficits in the forelimb reaching task due to impairments in motor learning- dependent clustering of new dendritic spines. Anatomically, *Fmr1* KO mice have shown cerebellar pathology and aberrant cerebellar function. Ellegood, Pacey, Hampson, Lerch, and Henkelman (2010) reported *Fmr1* KO mice demonstrated anatomical changes only in the cerebellum, such as neuronal loss and decreased volume in the deep cerebellar nuclei. The *Fmr1* KO mice also show abnormal PC morphology with longer dendritic spines in their cerebellum (Koekkoek et al., 2005). Behaviourally, *Fmr1* KO mice exhibited impaired eye-blink conditioning, a cerebellum-associated type of associative learning, similar to FXS patients (Koekkoek et al., 2005). *Fmr1* KO mice appear to have a deficit in cerebellar-dependent motor skill learning.

Conclusion

The brain is a plastic organ, and its structure and function is responsive and sensitive to experience. A key characteristic of the brain is to constantly reorganize itself in order to adapt to environmental demands. Plastic changes are not restricted to development, but can be extended into adulthood. Both neural and non-neural cells of the brain undertake plastic changes in response to experience. Synthesis of proteins are necessary to mediate these changes in the brain in response to experience. The *Fmr1* KO mouse, which has no FMRP, provide a good tool to investigate the effect of absence of this protein on their impairments in learning and memory, in particular, motor skill learning.

Chapter 2

The immune system and immune proteins play various roles in the brain. Studies with animal models of inflammation support the causal relationship between inflammatory signalling and memory deficits. The majority of animal research on the effect of the immune system on learning and memory processing has emphasized on the role of IL-6, IL-1β and tumour necrosis factor alpha (TNF-α). In normal circumstances, immune mechanisms positively mediate the plasticity of neural circuits, enhancing neurogenesis and memory consolidation. These advantageous influences of the immune system are regulated via communications among immune cells of the brain and neurons with peripheral immune cells. In circumstances, under which the immune system is intensely stimulated due to injury, infection, or diseases, immune cells of the brain alter their functioning and morphology and consequently produce a large number of pro-inflammatory cytokines leading to impairments in memory and neuroplasticity.

Pro-inflammatory Cytokines

Cytokines are small cell-signaling molecules made of proteins with attached sugar molecules. These immune signaling molecules, which are mainly secreted by the immune cells, are involved in cell-to-cell interaction within the immune system. Cytokines mediate different facets of the immune response by binding to their particular cell surface receptors. Upon binding to receptors, the cytokines initiate a downstream cascade of messengers leading to alteration of the receptor's function. These proteins can modulate the production or suppression of other cytokines via up-regulation or down-regulation of various genes and their products. Depending on the functional properties of cytokines, these molecules are categorized as a) pro-inflammatory, such as IL-6, IL-1β, and TNF-α, all of which promote inflammation, and b) anti-

inflammatory, acting against inflammatory processes. The pro-inflammatory cytokines regulate numerous normal behavioural responses, such as learning and memory. Generally, receptors of pro-inflammatory cytokines present in the main regions of the brain, which are involved in learning and memory, such as the cerebellum and the hippocampus (Gadient & Otten, 1994; Kinouchi, Brown, Pasternak, & Donner, 1991). These findings suggest the contribution of these cytokines to learning and memory.

Pro-inflammatory Cytokines Signaling in the Brain

In the brain, the origin of cytokines can be via local synthesis at neurons or glia, or transmission of peripherally created cytokines via the blood-brain barrier (BBB) (Banks, 2005). Since pro-inflammatory cytokines are large proteins, they may passively reach the brain through circumventricular organs (i.e. CVOs- due to their location adjacent to the ventricles of the brain), and the choroid plexus, where there is a lack a BBB. The capillary bed, in these two areas of the brain, does not form a BBB, but, instead, the vessels leak. The capillary bed, in these two areas of the brain, does not form a BBB, however, the vessels leak. Circulating substances can enter these areas of the brain, and communicate with other regions. Inducing lesion to a CVO close to the hypothalamus could inhibit cytokine- related fever, demonstrating the passage of cytokines via leaky sites of the BBB (Blatteis et al., 1983). Cytokines can also pass the BBB through a saturable transport system (Banks, 2005). Necessary substances, such as amino acids and glucose, are transferred across the BBB through saturable transporter system. Since these substances have a higher concentration in the peripheral blood relative to the brain interstitial fluid, they pass in the blood-to-brain direction. A facilitated diffusion is a two-way or bidirectional procedure, in which, net movement is directed from a place with the more to the

less concentration. Cytokines have a saturable uni-directional transport system. As an illustration, the saturable transport for IL-6 (Banks, Kastin, & Gutierrez, 1994), and IL-1β (Banks, Ortiz, Plotkin, & Kastin, 1991) are in the blood-to-brain direction. The transfer systems for cytokines are specific for closely associated cytokines, such that IL-1β, IL-6, and TNF-α have their specific transporters. The transporters can demonstrate how molecules as big as the size of cytokines could traverse the BBB. Molecular charge, weight, as well as the degree of binding to circulating proteins may also play a role in the determination of the rate of entry. The transporters are not ubiquitously distributed all over the CNS nor are all pro-inflammatory cytokines transported in a similar manner to a particular region of the brain. Another possible way of the passage of the peripheral immune message to the brain is by vagal afferent pathways. Laye et al. (1995) reported peripheral injection of lipopolysaccharide (LPS) led to the production of IL-1β in the hippocampus and central expression of IL-6, TNF-α, and IL-1β, mRNA, which were blocked due to vagotomy, the surgical removal of the vagus nerve. Cytokine activation of peripheral sensory neural afferents results in central cytokine production, which suggests the vital contribution of the vagus nerve to cytokine signal transmission. Understanding how the immune and central nervous systems interact are important to understanding different communication pathways between brain, behaviour, and the immune system. The question is whether these pathways are redundant or complementary. These interaction pathways may work together and regulate various cytokine-related behaviours. It is not clear how these different mechanisms of interactions communicate, as well as under what situations each mechanism may dominate or recede relative to the others.

Pro-inflammatory Cytokines and Learning and Memory

During illness, IL-6, IL-1β, and TNF-α regulate a group of behavioural symptoms composing sickness behaviour syndrome (Dantzer, 2004). The sickness behaviours are adaptive, helping the animal/person to recover from the illness. The sickness behaviour symptoms are composed of changes in sleep patterns, loss of body weight, psychomotor retardation, decreased exploratory behaviour, and impaired pain perception, to name a few. The contribution of the pro-inflammatory cytokines to mediating these symptoms has been supported by evidence demonstrating associations between the increase in the levels of cytokines in the different clinical situation and the occurrence rate of the sickness behaviour symptoms (Dantzer, 2004). In addition to the different behavioural symptoms, cytokine-related sickness behaviours also coincide with cognitive deficits, specifically, memory and learning , suggesting the key contribution of cytokines to learning and memory.

IL-6

IL-6 is a key signalling protein in the immune system. This cytokine is involved in wide range of biological activities in the CNS. In normal conditions, IL-6 plays a key role in brain plasticity and brain development, such as stimulation of cerebellar and hippocampal differentiation (Oh et al., 2010), and enhancement of neural growth in the cerebellum through its protective effects against excitotoxicity (Peng, Qiu, Lu, & Wang, 2005). Research into the origins of the immune proteins resulted in the recognition that some non-neural cells of the brain, particularly glial cells, secrete immune proteins. Immune proteins, which are produced within the CNS, are called neuro-immune proteins to differentiate them from those of immune proteins created via peripheral immune cells because neuro-immune proteins traverse through the CNS.

Different cells in the brain produce IL-6. Astrocytes are the primary cellular sources of IL-6 in the CNS in both humans (Choi, Lee, Lim, Satoh, & Kim, 2014) and mice (Nakamachi et al., 2012). Neurons are also another source of IL-6, which secretes IL-6 under different circumstances, especially, during strong neuronal activity. In the brain, IL-6 is mainly expressed in PCs in the cerebellum, as well as in some cells of the hippocampus and cerebral cortex (Gadient & Otten, 1994). The secretion of IL-6 in glial cells occurs through a classical pathway, where IL-6 sequestrates into membrane-bound organelles, which are then transferred to the membrane where exocytosis occurs (Andersson & Matsuda, 1989). Studies on cortical neurons, however, demonstrated another pathway. Tsakiri, Kimber, Rothwell, and Pinteaux (2008) found strong neural activation could trigger IL-6 to be transferred to synaptic bottoms and secreted at or near the synapse. Activity-induced expression of IL-6 by glia or neurons leads to elevation of IL-6 protein in the CNS.

Motor learning-induced plastic changes in astrocytes, in particular, alterations in the morphology and function of astrocytes, may suggest the involvement of IL-6 in motor skill learning. This increased expression of IL-6 in response to an intense neural activity could be specific to learning, not the activity itself. As Chennaoui, Drogou, and Gomez-Merino (2008) demonstrated intense running on treadmill reduced the expression of IL-6 in the cerebellum of rats. IL-6 differentially response to motor activity and motor learning.

A growing body of literature on both humans and animals shows the link between IL-6, learning, and memory. IL-6 plays a dual role in memory (reviewed by Donzis & Tronson, 2014). Under some conditions, this cytokine exerts a supportive role in memory functioning, whereas in other conditions IL-6 plays a detrimental effect on learning and memory. The beneficial effect of IL-6 has been evidenced in a clinical study, in which the effect of exogenous IL-6 on cognition

and memory performance was assessed through administration of IL-6 to patients with chronic fatigue syndrome and a control population (Arnold et al., 2002). The authors found no memory disturbance after 6.5 h of IL-6 administration, and they found improvement in memory performance in both patients and control groups. The positive effect of IL-6 on cognition was supported by two other human studies, evaluating the effect of IL-6 in systemic lupus erythematous (SLE), which is an autoimmune disorder (Kozora, Laudenslager, Lemieux, & West, 2001), and in surgical patients (Shapira-Lichter et al., 2008). Kozora and his colleagues found a positive relationship between IL-6 in the plasma and cognitive function in SLE patients. Higher plasma levels of IL-6 in this patients were linked to better cognitive functioning, such as attention and concentration, and higher learning scores. The influence of surgical stress on cognitive performance indicated elevated levels of IL-6 one day following surgery, were correlated with improved surgical-induced declarative memory impairments (Shapira-Lichter et al., 2008). The positive effect of IL-6 on learning and memory has also been supported by animal studies. The injection of IL-6, two hours before ischemia, leads to enhanced passive avoidance memory, a hippocampal-dependent memory (Matsuda et al., 1996). Bianchi, Sacerdote, and Panerai (1998) showed an injection of IL-6 could inhibit the impact of the amnesic medication in the passive avoidance task, all of which indicate the beneficial effect of exogenous IL-6 on cognitive functions in some learning paradigms.

Aging is linked to enhancement in the expression of IL-6 protein (Ye & Johnson, 1999). Since aging is accompanied by cognitive deficits, it can be concluded enhanced IL-6 levels disrupt memory processes. IL-6 affects learning in an age-related manner, as evidenced in a study showed the role of IL-6 in memory functioning in mice with overexpression of IL-6 protein in the hippocampus (Heyser, Masliah, Samimi, Campbell, & Gold, 1997). Those mice

were trained in the active avoidance test at different time points (e.g. 3, 6, and 12 months of age). The homozygous mice showed disrupted learning at three months of age, but heterozygous mice had no problem in learning the task compared to the control mice. The homozygous IL-6TG mice demonstrated more learning problems at six months of age relative to their performance three months earlier. Both heterozygous and homozygous IL- 6TG mice showed further learning impairments by 12 months of age, which was not distinguishable, suggesting the detrimental effect of IL-6 on hippocampal-dependent learning in an age- dependent manner.

The positive effect of disrupted signalling of IL-6 on memory and learning was investigated in IL-6KO mice, in memory tasks. Although Braida et al. (2004) reported no deficits in the memory functioning of IL-6KO at 4-month-old and normal mice in the passive avoidance task, the IL-6KO mice demonstrated less susceptibility scopolamine-induced amnesia. The authors also reported better memory functioning of IL-6KO mice relative to the age-matched control mice in the radial arm maze, which is a more complicated spatial test. Balschun et al. (2004) showed acute inhibition of IL-6 signaling could improve the formation of spatial learning. In particular, the authors found the injection of anti-IL-6 antibodies, one hour following the acquisition of a hippocampal-dependent spatial memory, forced alternation task, led to an improved retention of this memory 24 h later. The evidence of improved memory functioning after either acute or chronic blockade of IL-6 signalling indicate IL-6 may play a modulatory role in the inhibition of memory formation.

In conclusion, on the one side, IL-6 is related to negative influences on memory, evidenced by the relationship between increased levels of IL-6 by age and memory loss, and studies demonstrated disrupted IL-6 signaling is related to improvement in memory. On the other side, in some situations, enhanced levels of IL-6 produced protective effects on memory. This

evidence suggests IL-6 could be considered as either an anti-inflammatory, or pro-inflammatory cytokine, and that the effect of IL-6 in memory relies on the particular condition, during which it is increased, and on the duration and intensity of the elevation (e.g. Acute vs. chronic).

IL-1β

Some studies show that IL-1β is necessary for learning and memory, specifically for memory consolidation that relies on the proper function of the hippocampus (Yirmiya & Goshen, 2011) suggesting the involvement of IL-1β to the learning process. Goshen et al. (2007) assessed the induction of IL-1β mRNA at different time periods after contextual fear conditioning, which is a form of associative learning measuring freezing response associated with an aversive stimulus, such as an electrical shock. They found gene expression of IL-1β in the hippocampus increased 24 h following contextual learning. Another approach to study the effect of IL-1β is giving an exogenous injection to animals. In those beneficial conditions, the doses of IL-1β injected are low and specific conditions used in the memory tasks. Low dose administration of IL-1β could improve contextual fear memories in the contextual fear conditioning test (Goshen et al., 2007). The contextual fear memories rely on the normal functioning of the hippocampus. The induction of IL-1β during learning and memory consolidations as well as the regulatory role of low dose administration of IL-1β on hippocampal-related memory suggest IL-1β has a positive effect on memory and cognition under normal condition.

Exogenous injection of IL-1β leads to learning and cognition deficiency. Oitzl et al. (1993) reported intra-cerebroventricular injection of a high dose of IL-1β, 60 minutes before starting the spatial memory task in water maze training led to short-term memory disruption during the first day of the training. In the spatial active avoidance paradigm, which is a

hippocampal-dependent learning, the mice administered with IL-1β demonstrated impaired avoidance learning, as they needed more practice to perform the avoidance response relative to control animals (Banks, Farr, La Scola, & Morley, 2001). Administration of IL- 1β can impair non-hippocampal- dependent learning and memory, such as motor skill learning. Larson et al. (2007) showed an injection of IL-1β to motor cortex immediately after training on the dowel task for two days disrupted the acquisition of the motor skill learning in rats. High levels of IL-1β exert negative effects on different types of learning and memory regulated by the hippocampus and the motor cortex.

TNF-α

TNF-α has positive effects on learning and memory under certain conditions. Brennan and colleagues (2004) showed intraperitoneal administration of TNF-α led to an enhanced number of escape and avoidance responses in the passive avoidance tasks. Gerber et al. (2004) demonstrated an inability to memorize a hidden platform (i.e. spatial learning) in TNF-α knockout (TNF-α KO) mice, who survived from an infectious bacterium after treatment with an antibacterial drug, compared to surviving wild-type controls. The results demonstrated the beneficial effects of TNF-α in wild-type controls on the recovery of memory performance after the infection. It can be concluded when there is a homeostasis in the brain TNF-α plays a negative role in learning, whereas when homeostasis does not exist, TNF-α plays a supportive role in learning and cognitive functioning.

Several studies have shown inhibitory effects of TNF-α on learning and cognitive functioning. There are inconsistent findings of the role of TNF-α in memory. Matsumoto, Watanabe, Suh, and Yamamoto (2002) found intrahippocampal administration of TNF-α for a

week disrupted working memory. High concentration of TNF-α in the brain could not exert any effect on learning and memory in young mice and was associated with age-induced memory loss (Aloe et al., 1999). The TNF-α KO mice, which have TNF-α deficiency, also demonstrated opposing effects on learning and memory. Golan, Levav, Mendelsohn, and Huleihel (2004) reported enhanced performance in spatial memory using the water maze test in the TNF-α KO mice relative to WT controls. The adverse effects of TNF-α on memory and learning are age dependent.

Mechanism Mediating the Roles of Pro-inflammatory Cytokines on Learning and Memory

The body may influence the brain in several ways. A number of pathways, under which increased levels of cytokines negatively affect proper functioning of the brain, which may lead to impaired learning and memory, have been identified. The primary source of immune signaling is peripheral. The immune activation signals have to be transferred to the brain by immune-to-brain circuits, through which lead to the release pro-inflammatory cytokines in the CNS (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008; Maier & Watkins, 1998). The elevated levels of cytokines presented in the brain activate various cellular mechanisms, and influence brain and behaviour (i.e. learning and memory) via their effects on neurogenesis, neuronal hyperexcitability, as well as glutamatergic neurotransmission function.

Inflammatory cytokines play a dual role in the induction and maintenance of neural plasticity and neurogenesis. In normal circumstances, pro-inflammatory cytokines from the immune system positively regulate neurogenesis. Under inflammatory conditions, pro-inflammatory cytokines exert adverse impacts on neural plasticity. Monje and colleagues (2003) showed exposure of hippocampal precursor cells, which can be differentiated to different cells,

to IL-6 reduced neurogenesis by 50%, suggesting IL-6 is an inhibitor of neurogenesis. The authors reported IL-6 mediated the anti-neurogenic impact of stimulated microglia on neurogenesis, when neural precursor cells were exposed to cell cultures obtained from activated microglia. Monje et al. (2003) suggest IL-6 decreased cell survival and neuronal differentiation. The authors also found treatment with anti-IL-6 antibodies could block the inhibitory impact of stimulated microglia on neurogenesis. Inflammatory signaling also lead to impaired neurogenesis. There is evidence showing the neurogenesis impairments following inflammatory event have long-term outcomes. As an illustration, Valero, Mastrella, Neiva, Sanchez, and Malva (2014) found approximately 65% reduction of synaptic networks in the new-born neurons in the hippocampus and impaired spatial learning in rats six weeks after injection of LPS. Cytokines can exert their detrimental effects on the processes of learning and cognition through influencing neural plasticity and neurogenesis.

Neural plasticity, learning, and memory rely on controlled form of neuronal activity. A well-timed and controlled stimulation of immune cells and production of pro-inflammatory cytokines exert positive regulatory effects on neural plasticity, neurogenesis, learning, and memory. As an illustration, local interactions among neurons, microglia, and astrocytes in the hippocampus mediate consolidation of memory. The excessive immune activation in the brain and secretion of high levels of cytokines can lead to hyperexcitability of neuronal circuits, and consequently lead to delirium, neurodegeneration, and excitotoxicity (reviewed by Yirmiya & Goshen, 2011). The strong neuronal activation taking place in epileptic seizures causes additional stimulation of immune cells, and consequently, high secretion of pro-inflammatory cytokines (Vezzani, Balosso, & Ravizza, 2008). Abnormal hyper-excitability is related to impairments in neural plasticity, neurogenesis learning, and memory. In susceptible people

whose immune functioning is already vulnerable due to neurodegenerative condition, or typical aging, the neuro-inflammation-mediated hyper-excitability causes further impairment in cognitive performance (Murray et al., 2012). Excess levels of cytokines impair the development of learning and memory, and neural plasticity.

Neuro-inflammation in FXS and the *Fmr1* KO mice

Little is known about whether cytokine levels in the brain of individuals with FXS and the *Fmr1* KO mice are altered. There is one study reporting elevated levels of IL-6 and IL-1β in the plasma and the peripheral blood mononuclear cells of male children diagnosed with both FXS and autism relative to patients with FXS alone (Ashwood, Nguyen, Hessl, Hagerman, & Tassone, 2010; Careaga et al., 2014). Recently, an animal study reported an increased activity of the astrocytes, which was demonstrated by the overexpression of GFAP in the cerebellum of the *Fmr1* KO mice relative to the WT normal mice (Pacey, Guan, Tharmalingam, Thomsen, & Hampson, 2015). Generally, the activation of astrocytes can lead to the secretion of pro-inflammatory cytokines, in particular, IL-6 (Klein et al., 1997) suggesting IL-6 protein can also be overexpressed in the cerebellum of the *Fmr1* KO mice as a result of the reactivity of astrocytes in the cerebellum of this animal model.

FXS and ASD

ASD is a complex neurodevelopmental disorder, which shares some features of FXS, including social deficits, repetitive behaviours, and motor impairment (Kaufmann et al., 2004). Approximately 21% of children with FXS are diagnosed with ASD (Hatton et al., 2006). Both patients and animal studies have shown excessive elevations of IL-6 relative to normal control

population/animals, particularly, in their cerebellum (as reviewed in Wei, & Alberts, 2013). Wei et al. (2012) demosntrated mice with over-production of IL-6 in their cerebellum through delivery of an adenoviral gene demonstrated impaired cognitive, learning deficits, as well as autistic-like behaviours. These results indicate increase in IL-6 in the cerebellum could mediate behavioural and learning deficits. Since FXS is a key risk factor for ASD and shares high genetic comorbidity between these two disorders, similar increase in the expression of IL-6 in the cerebellum of the *Fmr1* KO mice, which could be associated with motor skill learning deficits, is possible.

Conclusion

The immune system in collaboration with the CNS mediate neural plasticity, learning and memory in both normal and abnormal conditions. Under quiescent, normal conditions, the immune system can positively moderate learning, and memory via regulating normal neural functioning . Under abnormal conditions, however, when the immune cells of the brain is highly stimulated by either exogenous challenges, such as severe psychological stressors or pathogens or endogenous stimuli, such as stroke, or autoimmune processes, these cells produce excessive levels of pro-inflammatory cytokiens. This abnormal elevation of cytokines disrupts the precise balance between the neuro-glial connections, leading to disrupted neurogenesis, neural plasticity, as well as learning and memory problems. Dysregulated neuro-inflammatory signaling and impaired immune functioning seen in FXS and the *Fmr1* KO mice suggest the link between pro-inflammatory cytokines and learning deficits.

Chapter 3

The immune system can influence neural plasticity and behaviour, including learning and memory. Pro-inflammatory cytokines mediate cognitive and behavioural influences of immune activation. Over almost last three decades, several studies focused on the association between cytokines, learning, and memory. The majority of studies have investigated the effect of cytokines on hippocampus-associated learning. There are, however, a few studies on the role of cytokines on motor skill learning, which is mediated by the cerebellum and motor cortex.

The capacity for dynamic and efficient neuronal plasticity is required for the normal functioning and development of neuronal connections. Neuronal plasticity is the capability of a neurons to change structurally in response to experience, such as learning a new skill. Problems arise when a genetic mutation causes abnormal development of neuronal connections. In particular, in FXS, the impaired neuronal plasticity, such as long and immature dendritic spines and impaired functional plasticity result in impaired learning and memory (reviewed in Penagarikano, Mulle, & Warren, 2007). This impaired neural plasticity is due to the lack of FMRP, a protein mediating brain plasticity via regulating the synthesis of other proteins in the brain, which are necessary for normal brain function (reviewed by Sidorov, Auerbach, & Bear, 2013). Using the mouse model of FXS, which demonstrates a lack of FMRP, impaired synaptic plasticity and abnormal behaviours similar to what seen in people with FXS, allows us to study the function of FMRP in the brain.

The *Fmr1* KO mouse is a well- known genetic model of FXS. The *Fmr1* KO mice have shown learning and memory deficits, such as impaired motor skill learning (Padmashri et al., 2013), eye blink conditioning, which is dependent on the function of the cerebellum (Koekkoek

et al., 2005). The *Fmr1* KO mice demonstrated impaired neural plasticity and motor skill learning impairments, as well as enhanced activity of astrocytes, which is one of the main cellular source of IL-6, in their cerebellum (Pacey et al., 2015), all of which make this animal model a good tool to study the role of IL-6 in motor skill learning.

The majority of studies used animal models of inflammation have shown a deleterious effect of increased level of IL-6 on learning and memory (reviewed by Yirmiya & Goshen, 2011), specifically, hippocampal-dependent learning tasks. There is a lack of research, however, on the effect of IL-6 on cerebellar-mediated learning and memory (i.e. motor skill learning). Among different learning tasks, hippocampal-dependent learning tasks have been mostly used to investigate the effect of cytokines on learning likely due to the key involvement of the hippocampus in memory formation and learning. The presence of the receptors of pro-inflammatory cytokines in the hippocampus (Gadient & Otten, 1994), provide a mechanism to impair hippocampal- dependent learning and memory. There is evidence showing the presence of IL-6 receptors in another brain region, the cerebellum, which mediates motor skill learning. Larson et al. (2007) have determined the administration of IL-1β impairs the ability of animals to learn the complex dowel task. Since IL-6 and IL-1β are closely functionally associated, and IL-1β can stimulate an increased expression of IL-6 (Reyes & Coe, 1998), it can be suggested IL-6 may also play a putative role in motor skill learning.

Motor skill learning involves diverse cortical and subcortical brain regions, including the cerebellum, motor cortex, and basal ganglia. Synaptic plasticity in the cerebellum has been shown to be particularly important. Animal studies reported different neural plasticity underlie motor learning-induced behavioural improvements. Training rats on the acrobatic training, which engages whole body coordination to traverse obstacles, induces synaptogenesis at the level of

PCs, whereas motor activity could induce angiogenesis in the cerebellum (Black et al., 1990). Kleim et al. (1998) reported the increase in molecular layer volume of the cerebellum after acrobatic training as a result of increased number of PF synapses onto PCs. Lee et al. (2007) also reported the presence of multisynaptic boutons of PFs on PCs spines due to motor learning. This synaptic plasticity reflects the structural changes reported in the cerebellum during a motor skill learning task.

Hypotheses

The overall aim is to extend our understanding of the role of neuro-inflammatory cytokines in learning and memory. In particular, the contribution of IL-6 to motor skill learning in the *Fmr1* KO and the WT mice as normal control mice. I hypothesized the *Fmr1 KO* mice have higher levels of motor skill learning relative to the WT control mice. My second hypothesis was behavioural training on the complex dowel or simple flat beam task alter the expression of IL-6 in the cerebellum of mice relative to inactive control group.

Methods

Animals in this study were 20 adult male *Fmr1* KO and normal WT mice (three WT mice were excluded from the study due to intensive fighting), which were bred from ko-ko and wt-wt pairs at the University of Manitoba in Dr. Ivanco's breeding colony, and were housed 2-3 per cage. At the time of starting the experiment, the animals were two months of age, which are categorized as young adults. Young adults were chosen as per study of Derksen et al. (2007), in which they used young adult rats for training on motor skill learning tasks. At this age, mice are physically mature enough to be trained on the dowel task, which is a complex motor skill

learning the task. Mice were kept in cages in an animal room on a 12-hour light-dark cycle (7 am/7 pm). All procedures conducted during the light part of the cycle. Ethical approval was granted by the Fort Garry Animal Care Committee. Food and water were available ad lib. Weights of all mice were measured daily starting the day handling began and continued for the duration of the experiment.

Behavioural Training

Apparatus.

The motor tasks consisted of two different runways to evaluate motor performance. Both were 184 cm length and 3 cm width and were raised about 125 cm from the floor. The simple flat beam used in both the pre-training and control condition. The dowel task consisted of a group of dowels (0.3 cm diameter), were spaced 2 cm apart. The dowels were placed at different angles such that the width of the runway, which was perpendicular to the pathway the mouse traversed, was equal to the 3 cm width of the simple flat beam task. Three stands elevated the runways. Two of them had a tiny platform (7 cm-10 cm) indicated the starting and ending locations for the mice (see Fig.1A, B).

Procedure.

One week before starting the experiment, animal handling procedures for all the mice were performed as follows. Every morning at 8:00 am, all the mice picked up from their home cage and were located on a wire cage for five minutes, transferred to an empty cage, and returned to their home cage. After handling, all the mice were distributed as evenly as possible across three groups: motor activity (n=6), motor learning (n=6), or inactive control (n=5) group.

Pre-training.

Mice were approximately eight weeks old at the beginning of pre-training (as per Derksen et al., 2007; Larson et al., 2007) All animals, but the inactive control group were pre-trained on the flat beam task for three days. Exposure to this task provided the opportunity for the mice to get familiar with the nature of the task and new environment. On each day, mice were placed at the ending platform for 2 minutes so that they get familiar with the goal of the task. The mice then were positioned at the starting platform of the runway and were trained to traverse the flat beam to reach the goal platform. If the mice refused to run, the experimenter tapped on the runway in front of the mice or gently held their tail to make them run down the beam. The behaviour of the mice on this beam was monitored to ensure they did not go back to the start platform. For each pre-training session, the mice should run down the beam ten times per day (as per Larson et al., 2007). After each training session, the mice were transferred to their home cage. The mice in the inactive group were only handled.

Motor Skill Training.

Following pre-training (on the 4th day of the experiment), mice were randomly assigned to either motor activity (flat beam), or motor learning (the dowel). The mice in the motor activity group had to traverse a simple flat beam as they did in the pre-training (please refer to Fig.1 A). The motor skill learning training took place for five consecutive days to ensure the acquisition and maintenance of motor skill learning, (as per Derksen et al. 2007; Larson et al. 2007). Mice in the motor learning condition had to transverse the runway with the up-ended dowels, ten trials per day (please refer to Fig.1 B). To facilitate the acquisition of this task, the experimenter tapped the dowels. If necessary, the experimenter placed the front paws of the rats

onto the first dowel. If the mouse was hesitant to run on the runway to reach the finishing platform, the animal was pushed on the tail and hindquarters to encourage a correct movement. The experimenter expected the mice to be familiar with the goal of the task due to pre-training. Timing began when the back paws touched the beam and timing ended when the front paws reached the end platform. Similar runways to the dowel task used in other studies (e.g. Seeds, Williams, & Bickford, 1995; Wang et al., 2014) reported the involvement of the cerebellum in such runway tasks. These runways are the mouse version of what have been used previously by Larson et al. (2007) to examine the effect of pro-inflammatory cytokine on motor function, and by the researchers Derksen et al., (2007) to evaluate the expression of proteins following and after motor skill learning.

The inactive control group served as a control group and provided with limited opportunities for motor-skill learning or motor activity. The mice in the inactive group were only handled for eight consecutive days, which was equal to the number of the days the behavioural groups were trained. The inactive group was handled in the same way as the mice in the behavioural group to avoid confounding variables, such as stress resulting from moving the animals, and then they were kept in standard laboratory cages in the same room as the other two groups.

Tissue Collection

Approximately 24 hours after the last day of behavioural training, all the mice were anesthetized with 4% isoflurane for approximately five minutes. When animals were not responsive to tail pinch, they were removed from their cage and prepared for tissue collection. Incisions were made beneath the diaphragm, and saline solution was injected into the heart of the

mice to release blood from their heart and to ensure IL-6 is not in the circulating system, but in the brain tissue. The mice were then decapitated, and the brains of the mice were removed from the skull. Tissues from both the right and left anterior and posterior lobes of the cerebellum and the parietal lobe as the control tissue were dissected. The motor cortex, another possible region involving in the motor skill learning, was also dissected. Tissue samples were immediately placed on the ice and were frozen at -80°C until all the tissues were collected from all 17 mice.

Tissue Analysis

Before conducting ELISA, the Bradford protein assay was used to measure the total amount of protein in 17 samples obtained from each of the anterior and posterior lobes of the cerebellum. Tissue samples were thawed. A lysis buffer was used to break down the sample , then grounded and centrifuged to obtain a saturated protein liquid. The liquid was then divided as either sample to be used for the Bradford protein assay, or sample to be used for the ELISA. Samples for the Bradford were then loaded into a 96 well microplate filled with a Bradford reagent stock solution. A gradient of known concentrations of Bovine Serum Albumin (BSA) was used as a standard to compare sample concentrations against. Samples were then transferred into a microplate. The microplate was then placed in the iMarkTM Microplate Absorbance Reader (Bio-Rad). The plate was read at 595 nm. The absorbencies were obtained and recorded. A standard curve was calculated using the absorbencies of the known values of BSA. Total protein concentration was calculated using the standard curve. Each condition was coded such that the experimenter was blind to conditions.

ELISA.

One week after performing the Bradford assay, the samples were prepared for analysis of IL-6 concentration using Enzyme-linked immunosorbent assay (ELISA). This assay used an antibody specific for mouse IL-6 coated on a plate with 96-wells. Samples and standards were loaded into the wells and IL-6 exist in a sample was attached to the wells via the immobilized antibody. The wells were rinsed four times, and biotinylated anti-mouse IL-6 antibody was added to each well and incubated for 2.5 hours. Following rinsing unbound biotinylated antibody, HRP-conjugated streptavidin was loaded to each well, and incubated for another 45 minutes. The wells were again washed four times, a TMB substrate solution was added to the wells and incubated in dark for 30 minutes. Finally, Chromogen Substrate was added for the sake of bringing out the colour. Colour developed relative to the concentration of IL-6 bound. The colour was altered from blue to yellow by adding the stop solution. The optical density of colour was read on the microplate reader at 450 nm, and concentrations of IL-6 were calculated from a standard curve. Assays were done in duplicate to allow the experimenter detect any variation within the dilutions of the test samples, and between the assays.

Statistical Analysis

Behavioural data was analyzed using a 2 Task (motor activity/ motor learning) × 2 Genotype (WT/KO) × 5 (Day) × 10 (Trials) repeated measure analysis of variance (ANOVA). Group and Genotype were the between subject variables and Day and Trial, were the repeated variables, and the running time as the dependent variable. For the analysis of ELISA, we used 3 Group (Inactive /motor activity/motor learning) × 2 Genotype (WT/KO) × 2 Area (Anterior/ Posterior lobe) ×2 (Duplicate wells) repeated measure ANOVA. Group, Genotype, Area, and

Duplicate wells were the between subject variables and level of IL-6 was the dependent variable. Comparisons were considered significant if $p < 0.05$. The raw data was shown as mean ± SEM (Standard error of the mean). All the statistical analysis conducted by Statistica software.

Using the dowel task, the contribution of IL-6 to a complex motor skill investigated. It was predicted the *Fmr1* KO mice would have higher levels of IL-6 in their cerebellum compared to the WT mice. It was expected IL-6 would impair motor learning, such that the *Fmr1* KO mice would be slower to finish the dowel task than the normal WT controls would, and the mice in the simple flat beam (i.e. control for motor skill learning) task would complete the task similarly regardless of their genotype. It was also expected motor skill learning enhances the expression of IL-6, such that dowel mice would have higher levels of IL-6 in their cerebellum relative to the inactive control and the flat beam group. Conversely, we expected motor activity reduces the expression of IL-6 in the cerebellum of the mice, such that the flat beam mice would have less IL-6 relative to the inactive control mice.

Results

Alteration of IL-6

No main effect for task, $F(1) = .278$, $p = 0.762$ and no main effect for genotype, $F(1) = 0.335$, $p = 0.574$ was found. A marginal trend toward significance was found for the main effect of areas, $F(1) = 4.54$, $p = 0.056$, with higher levels of IL-6 in the posterior than the anterior lobe. A non-significant interaction between genotype and task was found, $F(1, 2) = 1.12$, $p = 0.359$, demonstrating no difference in IL-6 levels between genotypes and tasks.

Behaviour

A main effect of task was also found to be significant, F (1) =54.65, $p < 0.001$ demonstrating a significant difference in running times dependent on the task. No main effect of genotype was found, F (1) = 2.08, $p = 0.187$, demonstrating no significant change in running times depending on the genotype of the mice. A main effect of days was found to be significant, F (4) = 10.89, $p < 0.001$ demonstrating a change in running times across days. A main effect of trials was found, F (9) = 5.86, $p < 0.001$ demonstrating a change in running times across trials. A significant two-way interaction was found for task and trials, F (9, 36) = 6.42, $p < 0.01$, demonstrating across running times there was a significant difference between trials and runway. Further, a significant two-way interaction was found, F (4, 16) = 10.45, $p < 0.01$ for task and days demonstrating running times were significantly different across days depending on the task. No significant interaction was observed, F (4, 16) = 1.09, $p = 0.379$ for genotype and days, demonstrating no significance difference in running times across days depending on the genotype of the animals. A two-way interaction for genotype and trials was not found, F (9, 36) = 0.6, $p = 0.79$, demonstrating no significant difference in running times across trials and genotype. A significant interaction was found, F (36, 72) = 4.19, $p < 0.01$ for days and trials, demonstrating a change in running times across days and between trials.

The results showed no three-way significant interaction between task, genotype and day, F (4, 32) = 1.51, $p = 0.224$ demonstrating no difference in running time depending on the genotype of the mice or the tasks they were assigned. A three-way interaction for the task, genotype and trials were found, F (9, 72) = .57, $p = 0.819$, demonstrating no significant change in running times across trials, depending on genotype and task. A significant three-way interaction was found, F (36, 144) = 3.70, $p < 0.001$, for task, days and trials demonstrating a

significant change in running times across trials, days and between tasks. No significant three-way interaction was found, F (36, 144) = 0.87, p = 0.684 for genotype, days and trials demonstrating no significant change in running times across trials, days and genotype. No significant interaction was found, F (36, 288) = 0.89, p = 0.653 for the task, genotype, days and trials demonstrating no significant change in running times across trials, days, tasks assigned to and genotype of the animals.

Discussion

The goal of this research was to understand the effect of neuro-inflammatory mediators on motor skill learning. Specifically, we aimed to determine whether IL-6 is elevated in the *Fmr1* KO mouse model relative to the WT normal mouse. There was no significant difference in IL-6 levels in the cerebellum of the *Fmr1* KO and control WT mice. We also found no motor skill learning deficits in the *Fmr1* KO mice. As expected, all mice subjected to the simple flat beam task performed similarly regardless of their genotype. The interaction between genotype and learning condition was not significant indicating the effect of genotype and the learning conditions are independent in this experiment. The findings of this study indicate IL-6 levels does not change in response to motor training, and the expression of IL-6 protein is independent of FMRP.

Data from ELISA indicated there was no significant difference in the expression of IL-6 in the cerebellum of the *Fmr1* KO and WT. Pacey et al. (2015) reported the high production of GFAP, in the cerebellum of female *Fmr1* mice from the postnatal day 14 to adulthood (2-4 months). This increased reactivity of astrocytes in the cerebellum of the *Fmr1* KO mice could suggest potential elevation of IL-6 in the cerebellum of the *Fmr1* KO mice. A possible

explanation of the lack of inconsistency between results could be reactivity of astrocytes may not necessarily lead to increased expression of IL-6 since it is not the only source of IL-6 in the brain. Accumulating evidence in both animal models of ASD and patients with autism show elevated levels of IL-6 in the cerebellum of autistic brains, and since the *Fmr1* KO mouse is a related model, it was expected to observe higher levels of IL-6 in the cerebellum of the *Fmr1* KO mice compared with the WT mice. This discrepancy between the findings may suggest additional works on the molecular and behavioural comorbidities between these two neurodevelopmental disorders.

Behavioural trainings did not alter the expression of the IL-6 protein in the cerebellum of mice, as there was no significant difference in the levels of IL-6 after either motor skill learning or motor activity in the cerebellum of the *Fmr1* KO mice and the WT control mice. Based on various research studies indicated expression of different proteins, associated with neural plasticity, as the direct result of the elevated neural activation, which is specific to motor learning, not motor activity (Chennaoui et al., 2008; Derksen et al., 2007; Kleim et al., 1996; Padmashri et al., 2013; Seeds et al., 1995; Wang et al., 2014), it was expected motor skill learning enhances the expression of IL-6 in the cerebellum of the dowel mice. This hypothesis was not supported as there was no significant differences between the level of IL-6 in the mice subjected to the dowel task and the inactive control group.

Assuming learning-induced neural activity takes place due to behaviour, changes in IL-6 expression was expected in the dowel animals. Jankowsky, Derrick, and Patterson (2000) found 20-fold induction of expression of IL-6 gene four hours after in vivo induction of LTP, which is a model of learning and memory. Balschun et al. (2004) also showed dramatic enhancement of expression of the IL-6 gene, one-three hours after electrical stimulation of hippocampal slices,

and eight hours after high-frequency stimulation in freely moving rats. In our study, however, the tissues were collected for the analysis of IL-6 protein, 24 hours after the last day of behavioural training. At this time point, we may have missed earlier expression of the IL-6 protein, which could have been occurred within few hours after neural activity as per studies of Jankowsky et al. and Balschun, suggesting learning-dependent effect could be detectable at an earlier stage of learning. Another explanation is expression of an mRNA is not always correlated with expression of its product. The increased expression immediately after the neural stimulation may not lead to elevated production of the IL-6 protien.

The pattern of expression of IL-6 during and after motor skill learning has not been demonstrated in the literature yet. Based on research by Balschun et al., (2004), which documented immediate and dramatic elevation of IL-6 mRNA after induction of LTP, which can be equivalent to the acquisition phase of motor skill learning, it could be possible once the animals learn the task, IL-6 expression is highly expressed, but when they get accustomed with the motor skill learning task, and the initial phase of learning has terminated, activity of neurons may return to basal levels, consequently, reducing the level of IL-6 to the basal levels during the maintenance phase. Thus, a detectable change in IL-6 expression may occur within a closer time frame to neural activity, which could be immediately upon the acquisition of motor learning. Investigation of the expression of IL-6 within different time points, from the acquisition to the maintenance phase of motor skill learning may better illustrate the pattern of learning- induced expression of IL-6. Our hypothesized pattern of expression of IL-6 protein during learning may not follow what we suggested because neuronal activation in the cerebellum induced by behaviour is more complicated than what stimulated by electrophysiological stimulation. Whereas electrophysiology studies assess specific and small region of the brain with known

neural pathway, behaviourally-derived neural activation is diffuse and not limited to a particular region of the brain.

In our study, a slight significant difference in the expression of IL-6 between the anterior and posterior lobes of the cerebellum was found, with higher levels of IL-6 in the posterior lobe than the anterior lobe of the cerebellum. Clinical studies demonstrated the critical role of the posterior lobe in non-motor functions, such as cognitive and affective processes (Tavano et al., 2007). Higher levels of IL-6 in the posterior lobe relative to the anterior lobe suggest the association between IL-6 and cognitive rather than motor functioning. It has been shown by others that the *Fmr1* KO mice have severe impairment in cerebellum, such as disruption in eye-blink conditioning (Koekkoek et al., 2005), which involves paravermis in posterior lobe, is caudal to the paramedian sulci, as well as impaired rhythmic oromotor movements (Roy et al. 2011), which mostly involve lobule VIII, i.e. posterior lobe. The middle part of the audiovisual system in the cerebellum is localized in lobule VI/VII, which is a part of the posterior lobe. Although there was no difference between the *Fmr1* KO and WT mice, higher levels of IL-6 in the posterior lobe relative to the anterior lobe might partially explain the cerebellar- dependent learning impairments associated with the function of the posterior cerebellum. Since the posterior lobe is mainly involved in non-motor functions and higher level functioning, higher levels of IL-6 in this area relative to the anterior lobe may suggest the association of the posterior lobe of the cerebellum with motor skill learning. Also, since the anterior lobe of the cerebellum mediate motor functions, this area might be related to motor activity. Using different sensitive quantitative method, such as the immune-histochemical analysis, perhaps would help us to look closer at neural levels to specifically demonstrate the presence and location of IL-6 in tissue

sections, and probably reveal differences in expression within different regions of the cerebellum, which are mostly involved in motor learning.

The absence of differences in the levels of IL-6 between different groups in our study, could also be explained by the fact that behavioural data from task similar to the dowel task (e.g. Metz & Whishaw, 2002) and alterations in the expression of proteins reported previously in our lab (Derksen et al., 2007) indicate the complex dowel task is also mediated by other regions of the brain, such as the motor cortex, and sensory cortex involvement. Investigating those regions along with the cerebellum would help us better determine the pattern of the expression of IL-6 in different regions of the brain, which largely mediate motor skill learning.

Chennaoui and his colleagues (2008) demonstrated physical activity reduces the expression of IL-6 in the cerebellum of rats. It was expected motor activity in the flat beam task reduces the levels of IL-6 relative to the inactive condition. This hypothesis was not supported in our study, as the levels of the IL-6 in the cerebellum of the mice in the flat beam was not different from the inactive control mice. Differences in the intensity, duration and the type of the tasks between the two studies may explain the observed discrepancy. More specifically, Chennaoui and his colleagues trained rats on a motorized treadmill for five days, per week for seven weeks. The animals were progressively habituated to the exercise for one week, for five days, followed by the training program, in which the animals had to exercise one hour per day, for two consecutive weeks. At the end of the training program, the rats were subjected to an intense exercise. Alternatively, the mice in our study were exposed to less intense motor activity. The animals were firstly pre-trained for three consecutive days on simple flat beams, followed by running on the flat beam for five days. The intensity, duration, and type of exercise may differentially affect the expression of IL-6 in the cerebellum.

Based on research showing the expression of proteins following motor learning, but not motor activity (Derksen et al., 2007), it was expected the mice (e.g. both the *Fmr1* KO mice and the WT mice) in the dowel task will have higher levels of IL-6 in their cerebellum relative to their motor control mice in the flat beam task. In contrast, our results did not show a difference in the levels of IL-6 protein in the cerebellum of the mice assigned to the dowel or flat beam tasks. In spite of similar motor tasks, age of animals, and procedures Derksen and his colleagues applied to investigate the expression of synaptophysin protein in the motor cortex during motor skill learning on the dowel task, the levels of IL-6 protein did not change in response to motor skill learning. Different molecular techniques, such as Western blotting and immunohistochemistry versus ELISA, different region of the brain (i.e. motor cortex versus the cerebellum), and the insufficient statistical power may explain the inconsistent results.

All animals subjected to the dowel task took significantly longer to finish the dowel task relative to the flat beam task, suggesting the dowel animals experienced a greater challenge in navigating their way running across the dowel laden runway than navigating across the simple flat beam. The observation that the dowel animals showed more improvement over the five training days indicates they experienced a significant amount of motor skill learning. There were no significant differences in the performance of the mice subjected to the dowel task, demonstrating no motor skill learning impairment in the *Fmr1* KO mice relative to the WT normal mice.

The *Fmr1* KO mice demonstrate motor skill learning deficits due to potentially high levels of IL-6 in their cerebellum. Since we did not find the elevation of IL-6 in the cerebellum of the *Fmr1* KO mice relative to the WT mice, we could not argue that high levels of IL-6 might influence the motor skill learning. According to the previous literature, there is one study

implying detrimental effects of IL-6 on motor learning. Richwine et al. (2005) reported a reduction of IL-6 production in the brain of mice that were on a diet containing anti-inflammatory properties, resulted in improved motor learning relative to control mice. It was expected IL-6 exerts a detrimental effect on motor skill learning, such that the *Fmr1* KO mice would be slower at traversing the dowel task than the control WT mice. This hypothesis was not supported as there was no main effect of genotype, indicating no differences in the expression of IL-6 in the cerebellum of the *Fmr1* KO and WT control mice.

The lack of motor skill learning deficit in the *Fmr1* KO mice found in our study is also in contrast with literature demonstrating motor skill learning deficits in the *Fmr1* KO mice (Padmashri et al., 2013; Reiner & Dunaevsky, 2015a). These contradictory results could be due to the fact that in both studies a different paradigm (i.e. forelimb reaching task) used. These two tasks differ regarding the experimental design and procedure. In the forelimb reaching task, animals were constantly trained to extend their forelimbs via a narrow, small slit to grip and hold food rewards located at a particular location, and their motor performance improved over time and plateaued as a result of repeated training. The forelimb reaching task measure specific components of operant conditioning, whereas, the dowel task, learning of inter-limb balance and coordination in mice were examined. The differences in the nature of motor skills may account for the discrepancy between results. The forelimb reaching task mainly mediated by the hippocampus (Hong et al., 2007), primary motor cortex (Kleim et al., 2004), and other regions, but not the cerebellum. Similar tasks to the dowel task suggested the involvement of the cerebellum (Seeds et al., 1995; Wang et al., 2014). The inconsistent results may also be due to different regions of the brain mediate the dowel task.

We found no significant differences in the motor activity of the *Fmr1* KO mice and the control WT mice. Roy et al. (2011) reported no significant impairment in motor abilities, such as motor coordination and balance in adult male *Fmr1* KO mice on the FVB strain. The *Fmr1* KO mice, however, have demonstrated severe impairments in cerebellar-dependent motor tasks, such as impaired eye-blink conditioning (Koekkoek et al., 2005) and rhythmic oromotor movements (Roy et al. 2011), suggesting a cerebellar pathology in these mice. No significant impairment in the motor skill learning, however, was observed in our study.

When studying mouse models, it is necessary to consider the severity of motor deficits may significantly differ with genetic background. Dobkin et al. (2000) showed that behavioural and learning impairments associated with the absence of *Fmr1* expression in mice relies on genetic background. The contradictory results observed about the motor skill learning problems, could also be due to the different strains of the mice used in previous studies. Both studies by Reiner and Dunaevsky (2015) and Padmashri et al. (2013), reported motor skill learning deficits in the *Fmr1* KO mice on B6 genetic background, whereas in our study the mice were bred on the FVB genetic background.

The findings of this study indicated no association between lack of FMRP and expression of IL-6 levels in the cerebellum of mice. Our results suggest motor behaviours do not induce the expression of IL-6, indicting a lack of association between motor functioning and alteration in the expression of IL-6. The higher levels of IL-6 in the posterior lobe of the cerebellum than the anterior lobe indicate IL-6 might be more associated with non-motor, such as cognition and learning, than motor functions. From these results it can be concluded the expression of IL-6 in the cerebellum of mice is not altered in the absence of FMRP and with motor skill learning.

Chapter 4

In this study the association between IL-6 and motor skill learning in the cerebellum of the *Fmr1* KO and WT mice was examined using the dowel task. The anterior and posterior lobes of the cerebellum were examined using ELISA, because the anterior lobe mainly mediates motor functions, and the posterior lobe regulates fine motor skills as well as non-motor functions. The results of this study demonstrated levels of IL-6 were not increased in the cerebellum of the *Fmr1* KO mice relative to the WT control mice and that IL-6 was also not altered in response to either the complex motor learning or motor activity. We also did not find significant differences in the levels of IL-6 between the anterior and posterior lobes in both genotypes. Our findings, although preliminary, suggest no association between the expression of IL-6, and FMRP and motor skill learning. Investigating the contibution of other cytokines to motor skill learning in the *Fmr1* KO mice, may yield significant relationship between the levels of pro-inflammatory cytokines, and FMRP and motor skill learning.

Limitations

The limited time frame to complete the study did not allow the experimenter to have large sample size. Animal models are useful tools to study brain-behaviour association in controlled conditions. In particular, the *Fmr1* KO mouse model has helped researchers understand how an impairment in brain structure and function, including the abnormal structure of dendritic spines, as well as impaired protein synthesis, affect learning and memory. There is, however, limitation in generalizing findings to humans with FXS.

Future Directions

A key complexity in interpreting the effect of inflammatory signaling on the regulation of memory would be that cytokines do not act alone. Instead, alteration of any individual cytokine leads to changes in inflammatory signaling at network-level via influencing the production of other cytokines. Future research is required to investigate patterns of cytokine expression and activity in response to a particular experience to fully understand the complication of activity and function of cytokines at network-level in response to learning tasks. Further studies with larger sample sizes are also required to ensure a representative distribution of the groups and generalizability of the results to people with FXS. Since the intensity, duration, and type of motor task may differentially affect the expression of IL-6 in the cerebellum, future studies may also use motor tasks with different levels of difficulties in order to precisely examine the motor behaviour-induced expression of IL-6. Investigating the expression of IL-6 at different stages of motor skill learning can help researchers fully determine the alteration in the IL-6 protein across different stages of motor skill learning. Investigating the expression of IL-6 levels during development to adulthood can better illustrate the assciation between expression of IL-6 and motor skill learning problems. Future research could also consider different regions of the brain, in which pro-inflammatory cytokines are expressed in those regions in order to illustrate the association between levels of IL-6 and motor skill learning across different relevant regions of the brain assocaited with motor skill learning. Since *Fmr1* KO mouse has particular strain-specific learning problems, future studies could also study learning deficits in different strains (i.e. FVB and B6) of the *Fmr1* KO mice to better understand the behavioural and molecular profile of the *Fmr1* KO mice on different genetic background.

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Appendix

List of Abbreviations

Arc activity-regulated cytoskeleton-associated protein

ASD autism spectrum disorder

BBB blood brain barrier

BDNF brain-derived neurotrophic factor

CF climbing fiber

CVO circumventricular organs

EC enriched condition

ELISA enzyme-linked immunosorbent assay

FMR1 Fragile-X Mental Retardation1

FMRP Fragile-X Mental Retardation Protein

FXS Fragile X Syndrome

GFAP glial fibrillary acidic protein

GFP green fluorescent protein

IEG immediate-early gene

IL-1β interleukin-1 beta

IL-6 interleukin-6

KO knockout

LPS lipopolysaccharide

LTP long-term potentiation

Pc Purkinje cell

PF parallel fiber

MAP2 microtubule-associated protein

NMDA N-Methyl-D-aspartate

TNF-α tumor necrosis factor alpha

WT wild-type

Fig. 1. Flat beam runway used for the pre-training and control (A). The Dowel runway with a series of up-ended dowels used for the motor skill learning task (B).

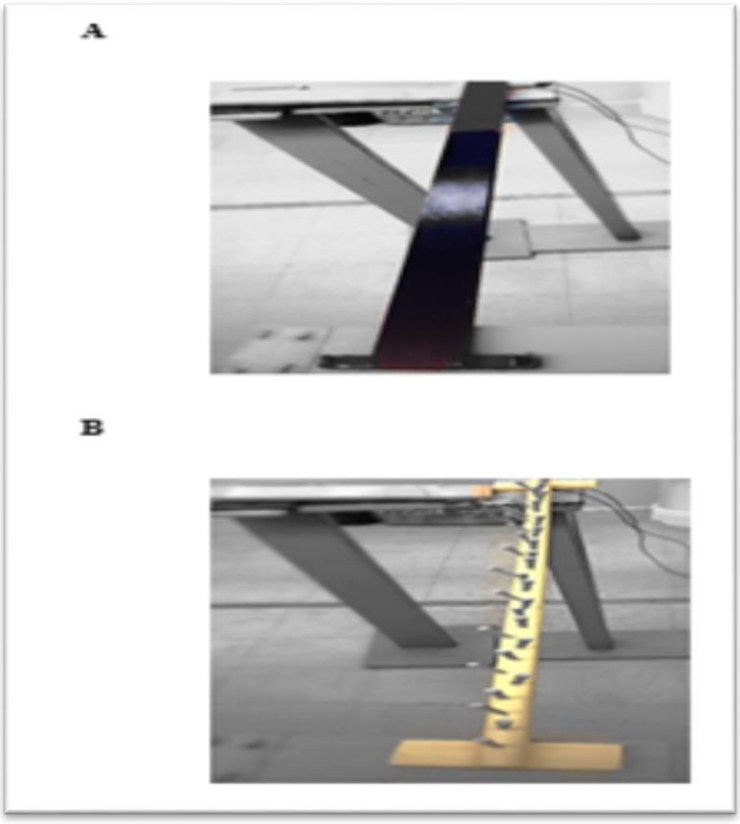

Fig. 2. Line graph of behavioural data from the Fmr1 KO and WT mice with average time (M ± SEM) to complete the dowel and flat beam tasks over 5 days.

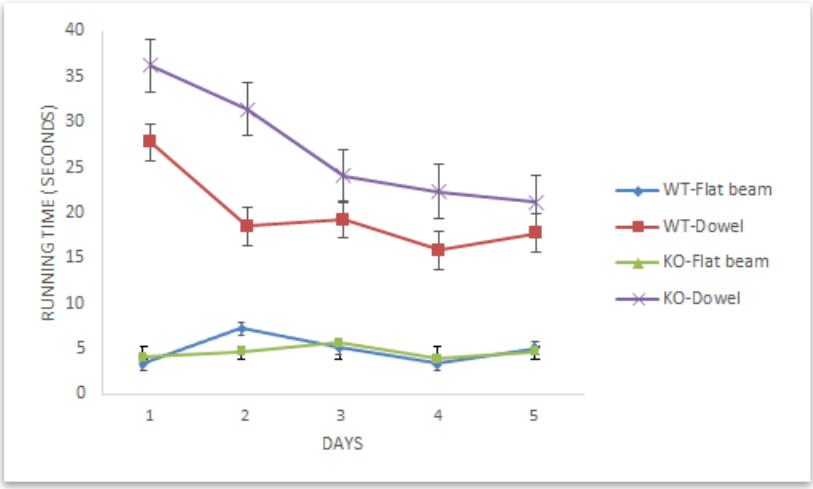

Fig.3. Bar graph of average amount of IL-6 in the right posterior lobe of the cerebellum of the *Fmr1* KO and WT mice, across different groups (i.e. Inactive/ Dowel/ Flat beam) from ELISA analysis (M ± SEM)

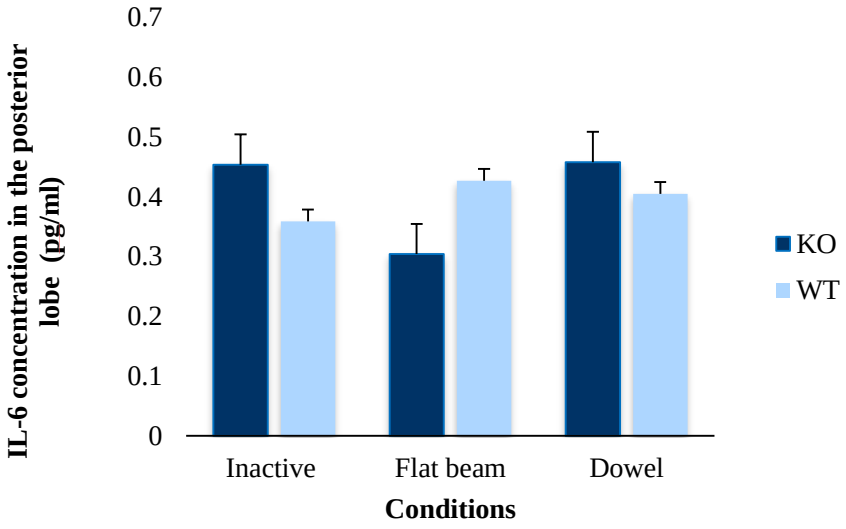

Fig.4. Bar graph of average amount of IL-6 in the right anterior lobe of the cerebellum of the *Fmr1* KO and WT mice, across different groups (i.e. Inactive/ Dowel/ Flat beam) from ELISA analysis (M ± SEM)