Role of hippocampal myocyte enhancer factor 2 (MEF2) in the neuroplastic and cognitive effects
of environmental stressors

by

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A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial fulfilment of the
requirements for the degree:

Master of Science

in

Neuroscience

Carleton University
Ottawa, Ontario

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Abstract

Recently, the role of transcription factor myocyte enhancer factor 2 (MEF2) has prevailed as a pro-survival agent against neuronal apoptosis, promoter of neuronal development and key player in memory consolidation. The current study examines whether three different types of stressors, namely psychologically relevant, immune and chemical stress would differentially influence MEF2 expression within the hippocampus. The study examines MEF2 associations to alterations in neurogenesis and performance in cognitive task (Y-maze). Our findings show non-significant differences of MEF2 expression within animals under psychologically relevant stress and those given immune insults. However, animals treated with environmental toxin paraquat showed declines in MEF2 levels within subgroups that scored low on the cognitive task. Moreover, we report novel findings of lowered neurogenesis within the hippocampus as a direct result of administration of environmental toxin and immune stressor. Our results add to the growing MEF2 body of work and open the door to future exploration.

Acknowledgements

First off, I would like to thank all members of the Hayley lab, past and present, with whom I've had the pleasure of meeting and working. You have made my life much more interesting and I have learned so much from you. In particular I want to thank Darcy Litteljohn and Chris Rudyck for their guidance and aide. And how can I forget Teresa Fortin, the driving force behind the smooth operation of our lab. I am proud to say that when I was in the Hayley lab, I was not amongst colleagues, I was amongst friends. Truly, I wish you all the best in your future endeavours and may your achievements shine brightly.

I would like to thank my friends and family for their endless support and encouragement. Particularly, I want to acknowledge my parents, Anwar Syed and Mahrukh Hasan, who have stood beside me from day one, throughout the trials and tribulations. I would also like to thank my brother Zuhair, especially for those late night drives back from university. I want to give a shout out to all my neuropeeps for making these two years fun and interesting. From those countless coffee runs, impromptu lunches, philosophical discussions to those late nights at school, you made my time at Carleton unforgettable. I also want to thank all my fantastic friends for their counsels and for listening to me ramble on and on about my research, especially Nick and Arsalan. I have grown over these past two years and I am thankful to each and every one of you for being part of my experience.

I'd like to also thank my committee members Hymie Anisman and Matt Holahan for their guidance. Finally, and most importantly, I would like to thank my supervisor Shawn Hayley. Thank you for accepting me as a student in your lab and for teaching me. Thank you for supporting me and for pushing me to try my best. I am grateful that I had the opportunity to be part of your lab and learn under your tutelage.

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List of Abbreviations

6-OHDA=6-hydroxydopamine

CDK = cyclin-dependent kinase

CNS = central nervous system

DA = dopamine

DCX = doublecortin

DG = dentate gyrus

DNA = deoxyribose nucleic acid

ERK = extracellular signal regulated kinase

HDAC = histone deacetylase

LPS = lipopolysaccharide

LTP = long-term potentiation

LTM = long-term memory

MEF2 = myocyte enhancer factor 2

MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MWM = Morris water maze

PD = Parkinson's Disease

PKA = protein kinase A

PQ = paraquat

SN = substantia nigra

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Introduction

A large number of individuals who are admitted into intensive care units for critical illnesses sustain moderate to severe physical and cognitive impairments following recovery from the primary pathology (Hopkins, 2013). These cognitive impairments are characterized by problems with memory and attention, as well as disturbed executive functioning (Hopkins & Jackson, 2009). Patients diagnosed with post-traumatic stress syndrome, schizophrenia, dementia, and major depressive disorder have all been shown to display impairments in learning and declarative memory (Al-Uzri et al., 2006; Bae & Hyun, & Lee, 2014; DeCarolis & Eisch, 2010; Kontaxaki, Kattoulas, Smyrnis, & Stefanis, 2014; Eagle, Fitzpatrick & Perrine, 2013). Similarly, systemic inflammatory responses to infection (sepsis) often leads to cognitive complications, including encephalopathy; damage in such cases is often focused in the hippocampus, and this is accompanied by memory deficits (Bozza et al., 2010; Widmann & Heneka, 2014). The unpredictable onset of impairments in memory and critical thinking skills, which are hallmarks of dementia, remain mostly mechanistically and etiologically uncharacterized. However, recent studies suggest environment stressors as having a significant influence in mediating mild cognitive impairment (Gates, Valenzuela, Sachdev, & Singh, 2014). Besides being one of the most malleable or plastic brain regions, the hippocampus is also especially vulnerable to a variety of stressors, with such insults provoking rapid structural, molecular, cellular and functional changes in hippocampal plasticity (DeCarolis & Eisch, 2010). Currently, we propose the transcription factor, myocyte enhancer factor 2 (MEF2), as a novel candidate regulating hippocampal plasticity (neurogenesis) and spatial memory (Y-maze performance) responses to psychological, immune and chemical stressors.

Myocyte enhancer factor 2

MEF2 proteins are a group of endogenous transcription factors that regulate a variety of physiological processes, most notably those involving muscle differentiation and development, immunological processes and development of plasticity within the central nervous system (CNS) (Mount et al., 2013; Potthoff & Olson, 2007; She & Mao, 2011; Yang & Mao, 2010; Yin et al., 2012). MEF2 proteins have been positively identified within the nucleus of neurons within the cortex, striatum, hippocampus, lateral septum, and bed nucleus of the stria terminalis (Neely et al., 2009; Rashid, Cole, & Josselyn, 2013; She & Mao, 2011). Initially MEF2 was shown to participate in the regulation of muscle-specific genes, particularly due to affinitive binding to AT-rich sequence in the muscle creatine kinase (Dodou, Sparrow, Mohun & Treisman, 1995; Gossett, Kelvin, Sternberg & Olson, 1989; Shore & Sharrocks, 1995; Yu et al., 1992). To date, vertebrates are known to exhibit four isoforms of this protein (MEF2A-D), each encompassing a highly conserved base structure, especially at the N-terminus which contains the DNA-binding domain (Wu et al., 2010). The alternative splicing that distinguishes the different isoforms from one another occurs in the 57-86 amino acid domain, otherwise known as the MEF2 domain (She & Mao, 2011; Yang & Mao, 2010). The C-terminal of the same protein is often cast within the role of a transcription activation domain, its topography varying between the MEF2 isoforms (Martin et al., 1994; Potthoff & Olson, 2007). MEF2 relies on the recruitment and cooperation of numerous other transcription factors (e.g. p35, p38, CDK5) to exert its full influence on gene expression (Potthoff & Olson, 2007).

These MEF2 isoforms have collectively been shown to be especially prevalent and active during muscle development, regulation of circadian cycle, immune cell fate response and neuronal development (She & Mao, 2011; Sivachenko, Li, Abruzzi & Rosbash, 2013). Due to its involvement in gene transcription, it is no surprise that MEF2 is highly regulated by post-transcriptional modifications and phosphorylation to ensure homeostatic levels of expression, (She & Mao, 2011; Yang & Mao, 2010; Yin et al., 2012). The MEF2 proteins appear capable of multi-faceted signaling effects within a plethora of cells, including cardiac, skeletal, endothelial and neuronal subtypes; where they play a particularly important role in the processes of differentiation, proliferation and morphogenesis (Pothoff & Olson, 2007). Accumulating research supports various roles of MEF2, including a pro-survival function of MEF2 as a calcium-dependent factor that protects post-mitotic neurons from apoptosis (McKinsey, Zhang, & Olson, 2002). As well, hyperthermia-exposed rat brains show increases in MEF2-DNA binding, supporting MEF2's role in stress responses (Maroni et al., 2003). And finally, MEF2's bidirectional regulation of genes through recruitment of co-repressors and co-activators speaks to a potential role of the transcription factor in memory storage (Schoch & Abel, 2014).

Several proteins have been found to associate with MEF2 and regulate its functions, including the mitogen-activated protein kinases, p38 and extracellular signal regulated kinase 5 (ERK5), cyclin-dependent kinase (CDK), Nur77, calpain, and various caspases (Han, Jiang, Kravchenko & Ulevitch, 1997; She & Mao, 2011; Yin et al., 2012). Indeed, the MEF2 protein has many sites or domains that allow for binding of a variety of kinases and phosphatases (She & Mao, 2011). Interestingly, p38 has been found to phosphorylate MEF2C when encouraged by calcium influx in response to extracellular signals (She & Mao, 2011; Yin et al., 2012). Phosphorylation of MEF2 through p38 results in the promotion of cell differentiation and growth

(Yin et al., 2012). Furthermore, MEF2 can induce neuroprotective effects by recruiting neurotrophins, such as brain-derived neurotrophic factor (BDNF) (Yin et al., 2012). As well, in the presence of an oxidative stressor, research shows an increase in MEF2-mediated activation of the ERK pathway, which had neuroprotective consequences (Mount et al., 2013; She & Mao, 2011).

One of the more prevalent negative regulators of MEF2 is the post-mitotic influencer, CDK5, which, unlike other cyclin-dependent kinase family members, is only inactive during cell division (She & Mao, 2011; Yin et al., 2012). Phosphorylation of the CDK5 protein activates its pathway, which results in expression of targets that are important for neural development, as well as synaptic plasticity (Mount et al., 2013; Yin et al., 2012). MEF2A and MEF2D are two known substrates of CDK5, which are controlled through phosphorylation-regulated inhibition at the SER444 location (She & Mao, 2011; Yang & Mao, 2010). Oxidative or extracellular stressors promote high levels of CDK5, which in turn increases its inhibitory effects on MEF2; in this way, MEF2's protective effects are diminished (Mount et al., 2013; She & Mao, 2011; Yang & Mao, 2010; Yin et al., 2012). The MEF2 and CDK5 connection is further complicated through the link to apoptotic caspase proteins. Caspases are responsible for effectively inactivating MEF2A by degrading the protein (Mount et al., 2013; She & Mao, 2011; Yang & Mao, 2010; Yin et al., 2012). However, the functional significance of MEF2-induced neuronal survival with regards to memory remains unclear.

Insert Figure 1 about here

MEF2 and neuroplasticity

Memory formation and long-term consolidation requires the acetylation and deacetylation of histone residues to promote expression of plasticity-associated genes. Nucleocytoplasmic histone deacetylases (HDACs) can act as negative regulators for formation of long-term memory and may even promote memory impairment (Sando 3rd, Gounko, Pieraut, Liao, Yates 3rd & Maximov, 2012). Recently Class II HDACs have been suggested to mediate their repressive effects within the nucleus, through formation of complexes with MEF2 thus suppressing MEF2 target genes (Di Giorgi et al., 2013). Indeed, the association of an HDAC with MEF2 within an un-stimulated cell, which may result in deacetylation of the nucleosomal histone area surrounding the MEF2 DNA-binding site, suppresses transcription of MEF2 target genes immediately downstream (Cohen & Greenburg, 2008; McKinsey et al., 2002).

Calcium signaling, namely through Ca^{2+} /calmodulin-dependent protein kinase (CaMK), underlies the bidirectional activity of the transcription factor MEF2. Upon synaptic firing, there is an influx of the secondary messenger Ca^{2+} into the neuron cytoplasm; intracellular CaMK facilitates the phosphorylation of two sites on the HDAC. The phosphorylated sites of the HDAC create a docking point for the intracellular chaperone protein 14-3-3; subsequently the HDAC disassociate from the MEF2 upon formation of the HDAC-14-3-3 complex and exits the nucleus (Cohen & Greenburg, 2008; McKinsey et al., 2002). It has been repeatedly hypothesized that during memory formation, the exit of HDACs from the nucleus relieves the MEF2 repression, thereby encouraging the recruitment of co-activators (eg. p300, NFAT) that allow expression of downstream plasticity related genes (Belfield et al., 2006; Fitzsimons et al., 2013; Youn, Sun, Prywes, & Liu, 1999). Indeed, Fitzsimons et al. (2013) showed that overexpression of HDAC4 impaired long-term courtship memory in *Drosophila* and that MEF2 was co-localized with

HDAC4 within the cell nuclei. Together these studies implicate MEF2 as a possible “pro-memory” transcription factor, and suggest that its repression might contribute to memory impairment.

A recent study revealed that overexpression of MEF2 severely dysregulated dorsal projections of ventral-lateral hippocampal axons, whereas MEF2 knockdown increased the branching of axons (Sivachenko et al., 2013). Similarly, MEF2 repression has been posited to modulate cocaine-induced remodeling of dendrites and spines within the NAc and striatum, which may contribute to addictive memories (Pulipparacharuvil et al., 2008). Recent research by Cole et al. (2012) also showed MEF2-transcription to facilitate the down-regulation of spine growth in the hippocampus. However, robust memory formation (via Morris water maze) was associated with higher levels of phosphorylated (pMEF2) within the dentate gyrus (Cole et al., 2012). Constitutively, MEF2 is native in an active unphosphorylated form. Interestingly, increasing MEF2 expression just prior to memory training, through microinjections into the upper blade of the mouse dentate gyrus (DG), blocked the formation of spatial memory (as demonstrated by a lack of preference for the target zone during a probe test). Similarly, post-training overexpression of MEF2 prevented the strengthening of acquired memory associated with the lack of spine density growth, as normally seen during robust memory formation (Cole et al., 2012). Thus, the role of MEF2 in memory formation and consolidation is clearly extremely complex, with evidence for both positive and negative mnemonic effects. Accordingly, a major aim of my thesis work is to further elucidate the relationship between hippocampus-dependent memory function and MEF2.

Stressors and MEF2

Physiological stress is an unavoidable aspect of daily life, and long-term exposure to stress has been linked to mental health problems and altered brain function (Krugers & Joels, 2014; Lucassen et al., 2014). Stress effects on cognition are complex and depend on the onset and nature of the stress stimuli. Chronic stress can lead to emotional disturbances, memory dysfunction, onset of anxiety disorders and changes in behaviour (Chen, Shen, Liu & Li, 2014; Pohl, Olmstead, Wynne-Edwards, Harkness & Menard, 2007). Among the cognitive impairments, memory disturbance is a dominant point for research, with special focus on the hippocampus. As alluded to above, the hippocampus belongs to the limbic system and is deemed essential in the consolidation and retention of memories, as well as spatial navigation. Chronic stress has been shown to negatively impact synapse morphology (e.g., diminished dendritic arborization) and hippocampus-dependent memory, and multimodal stress (multiple stressors) is more detrimental in this regard than unimodal stress (Grizzell, Iarkov, Holmes, Moim, & Echeverria, 2014; Maras et al., 2014). For example, Maras et al. (2014), observed that delivery of several simultaneous stressors resulted in significantly poorer performance in novel object recognition than restraint-alone stress or no stress. Another line of research reported decreased hippocampal volume (assumed cell shrinkage) and impairment in long-term potentiation (LTP) in patients exposed to chronic stress (Lucassen et al., 2014). Such stressor history has also been associated with a loss of hippocampal excitatory synapses from the CA3 to the CA1 region (Maras et al., 2014).

Other researchers have shown that stressor exposure, including prenatal stress, results in cognitive deficits proportional to the decreased number of neurons and synapses in the hippocampus (Hayashi et al., 1998; Ratajczak et al., 2013). In fact, chronic psychosocial

stressors, such as bullying, have been reported to interfere with hippocampal-based spatial working memory, as well as increase the risk of developing psychiatric disorders later in life (Novick, Miiller, Forster, & Watt, 2013). Acute stress has also been implicated in cognitive deficits and hippocampal pathology. For instance, Wagner et al. (2013) showed that mice subjected to a single social defeat had impairment in spatial object recognition even after an 8 hour recovery period. Similarly, human participants, when given acute psychosocial stress, displayed impairments in spatial working memory and attention immediately following the stressor (Olver, Pinney, Maruff, & Norman, 2014).

Essentially, organisms cope with social and environmental stressors by altering their behaviour and physiology, and such adaptations are likely supported by changes in neural plasticity. This neural plasticity is mediated by a number of intersecting brain systems, including monoamine neurotransmitter (i.e., dopamine (DA), serotonin and norepinephrine; Sørensen, Johanssen & Øverli, 2013) and neurotrophin signalling circuits (e.g., BDNF, NGF). Equally important (or perhaps most important, seeing as how transcription factors critically regulate the availability of signalling factors) is the activation and deactivation of transcription factors, which modulate gene transcription. In this regard, MEF2 may play a role in the pathways required for neuronal survival and neural plasticity (Dietrich, 2013; Rashid et al., 2014).

Immune processes and MEF2

The relationship between the inflammatory immune response and cognitive processes has received substantial attention in recent years, especially the link between inflammation and cognitive decline (Bettcher & Kramer, 2013). Indeed, administration of the endotoxins,

Legionella pneumophila and lipopolysaccharide (LPS), both induced decreases in exploratory behaviour (Kawano et al., 2013) and Morris water maze (MWM) performance after a single dose (Gibertini, Newton, Friedman & Klein, 1995; Shaw, Commins, & Mara, 2001; Valero, Mastrella, Neiva, Sanchez & Malva, 2014). Similar results were observed in rats that received 3 or 7 days of consecutive LPS administration, with animals displaying an increased latency to the MWM platform compared to vehicle-injected counterparts (Hou et al., 2014; Zhu et al., 2013). Further studies by Shaw et al. (2005) demonstrated that LPS-treated animals took a more indirect route to find the platform in the MWM, and Hou et al. (2014) reported decreased spontaneous alternation in the Y-maze spatial memory task. Similarly, peripherally administered LPS modified hippocampus-based context fear conditioning, such that conditioned mice froze less when administered the endotoxin (Weintraub et al., 2013). These data point strongly to the importance of brain-immune interactions in shaping fear memory processing.

The continuous addition of new neurons in the hippocampus (i.e. neurogenesis) is a normal function and is believed to contribute to neural adaptations to changing environmental conditions. The appearance of hippocampal neuronal precursors has been well documented within the DG, and is often assessed by quantifying the number of cells expressing the microtubule-associated protein, doublecortin (DCX) (i.e., a marker of immature (less than 3 weeks old) neurons) (Couillard-Depres et al., 2005). Interestingly, immune factors influence neurogenesis, as indicated by the decrease in DCX-positive DG cells that was observed up to 6 weeks after peripheral LPS administration (Hou et al., 2014; Valero et al., 2014). Importantly, during LTM and natural learning, the DG has repeatedly shown to display increases in DCX + neurons (Hargreaves, Cain & Vanderwolf, 1990). Hence, studies proposed in this thesis assessed

hippocampal DCX staining in the context of LPS treatment, as well as stressor and chemical toxin exposure, as will be discussed shortly.

Accumulating evidence suggests that, in addition to regulating neuroplasticity, MEF2 might be critical for immune signalling. For instance, increased MEF2A and MEF2D levels were observed in adult mice that were exposed to the viral mimic, poly(I:C), during their gestational period (Elmer, Estes, Barrow & McAllister, 2013). These changes were associated with decreased synaptic density; since the knockdown of MEF2A and MEF2D restored the synaptic density, these effects were proposed to stem from MEF2 functions (Elmer et al., 2013). As well, *Mef2* knockdown enhanced infection susceptibility and decreased recruitment of immune factors after septic injury in *Drosophila*, and these effects were reversed upon reintroduction of *Mef2* gene expression (Clark et al., 2013). Furthermore, the interaction of MEF2 with regulatory sites of antimicrobial peptides (*Drosicin* and *Metchnikowin*) was shown to be necessary for normal induction of peptides by the bacterial toxin LPS (Clark et al., 2013). Finally, Liopeta et al. (2009) suggested the role of MEF2 as a regulator of cytokine expression within T lymphocytes. This proposition was supported by in vitro studies using RAW264.7 (macrophage line) cells. Indeed, protein levels of MEF2C were significantly elevated in these cells following administration of the pro-inflammatory cytokine, interferon gamma (IFN- γ). Further, it was posited that MEF2C could be a mediator of the apoptosis in immune cells often evident after exposure to high doses of LPS or cytokines (Fu, Wei & Gu, 2006).

Environmental toxins and MEF2

The prevalence of environmental toxins within our society has increased over the past century and poses a concern for the development of neurodegenerative conditions within the CNS. Importantly, besides their effects on motor functioning, administration of chemical toxicants, such as MPTP and 6-OHDA, has been shown to impair spatial navigation in rodents (Ferro et al., 2005). Meanwhile, the pesticide, rotenone, is able to reproduce cognitive deficits characteristic of those observed in Parkinson's Disease (PD) patients, and these effects have been related to mitochondrial dysfunction and impairment of hippocampal-dependent long-term potentiation (Kimura et al., 2012; Sala et al., 2013). Similarly, chronic administration of paraquat (a commonly used herbicide) induced impaired spatial navigation with increased escape latency related to the MWM (Sun, Li, Chen & Zhang, 2011).

Paraquat exposure has been repeatedly linked to an increased risk of PD (Blandini & Armentero, 2012; Jackson-Lewis, Blesa, Przedborski, 2012; Jiao et al., 2012; Qi, Miller, & Volt, 2013; Tieu, 2011). Although paraquat and MPTP are similar in chemical structure, they do not act on DA neurons via the same mechanisms (Blandini & Armentero 2012; Jackson-Lewis, Blesa, Przedborski, 2012; Qi, Miller, & Volt, 2013; Tieu, 2011). Like MPTP, paraquat can pass through the blood brain barrier, and although the quantity that is found in the brain varies with age and dose, the mechanism in which paraquat enters the DA neurons is not fully understood (Blandini & Armentero, 2012; Tieu, 2011). Though mechanisms have been shown that indicate dopamine transporter (DAT) to shuttle the cationic PQ^{2+} into DA neurons (Rappold et al., 2011), it is suggested that there may be another transporter which paraquat uses to enter the DA neurons (Blandini & Armentero, 2012; Jackson-Lewis, Blesa, Przedborski, 2012; Tieu, 2011). Once inside the neuron, paraquat affects the mitochondrial complex I (Jiao et al., 2012; Qi, Miller, &

Volt, 2013; Tieu, 2011; Yang & Mao, 2010). In particular, through its mitochondrial interactions and redox cycling, paraquat induces the production of superoxides and other free radical species (Jiao et al., 2012). Overwhelming oxidative stress has been linked to the cell death observed in PD (Jackson-Lewis, Blesa, Przedborski, 2012; Jiao et al., 2012; She & Mao, 2011; Tieu, 2011). As well, paraquat has been reported to induce autophagic responses, which result in protein degradation and organelle deficits (Qi, Miller, & Volt, 2013; Yang & Mao, 2010).

Recently, MEF2 has been implicated in the regulation of neuronal survival, especially the MEF2A and MEF2D isoforms. In particular, repression of MEF2D transcriptional activity negatively affects hippocampal neuron survival (Salma & McDermott, 2012). Studies have shown that cAMP-mediated activation of protein kinase A (PKA) inhibits MEF2D's pro-survival function (Belfield, Whittaker, Cader, & Chawla, 2006; Salma & McDermott, 2012). Furthermore, overexpression of MEF2D confers some protection to hippocampal cells against reactive oxidative species such as hydrogen peroxide (H₂O₂), emphasizing the role of MEF2 as a prosurvival factor against toxic insults (Salma & McDermott, 2012). Similarly, Petrik et al. (2012) found that Mef2a/c/d is necessary for adult hippocampal neurogenesis

MEF2 might play an important role in the development of neurodegenerative disorders through its influence on cell cycle proteins. Indeed, a down-regulation of MEF2 activity was reported with MPTP treatment, and this effect was mediated by the cell cycle regulator protein, CDK5. Moreover, calpain inhibitors (PD150606 & PD151746) reversed the CDK5-dependent MEF2 phosphorylation (Verdaguer et al, 2005). As well, PC12 cells treated with 6-OHDA, a generator of reactive oxygen species, increased the degradation of MEF2D after 3, 5 and 7 hours. Yet, as would be expected, pre-treatment of PC12 cells with a CDK5 inhibitor (Roscovitine) prevented cell death and blocked the down-regulation of MEF2D (Kim et al., 2011). Evidence

for a MEF2-CDK5 interaction is further strengthened by the finding that developmental exposure to the anesthetic, isoflurane, increased nuclear CDK5 along with inhibiting MEF expression, and that MEF2 inhibition prevented these effects (Wang et al., 2013). Collectively, the available data suggest that MEF2 is critical for cell survival by acting through the CDK5 pathway, and that inhibition of MEF2 by neurotoxins may contribute to the loss of neurons.

Aim of thesis

In the present thesis, we were primarily interested in whether three distinctly different types of stressors, namely chronic psychologically relevant, immune and chemical would differentially influence MEF2 expression within the hippocampus. Secondly, we were interested in determining whether any changes in MEF2 would be associated with alterations of hippocampal neurogenesis and performance on a cognitive task (Y-maze). We address these research questions in three separate experiments utilizing behavioural and immunohistochemical assessments. Our primary hypothesis is that all three stressors will up-regulate MEF2 levels in the hippocampus and this will be associated with reduced hippocampal neurogenesis and impaired cognitive performance on a Y-maze task.

Methodology

Animals

Mice selected for the three studies were either the CD1 or the C57/BL6J strain (both provided by Charles River). At the time of study, male mice were aged 10-12 weeks, already acclimated to the vivarium for a week, singly housed in standard (27cm X 21cm X 14cm) transparent polypropylene cages and given *ad libitum* access to mouse chow. Mice were maintained on a 12 hour dark/light cycle. All test protocols were approved by the Carleton University Committee for Animal Care and were in accordance with the guidelines as outlined by the Canadian Council for the Use and Care of Animals in Research.

Treatment administration

Study 1: Psychological stressor exposure and MEF2

CD1 male mice (n=6) received 2 randomly assigned stressors per day (including cage restraint, tail pinch, tail hang, predator odour, hair dryer stress, wet bedding, elevated cage, continuous 24 hour lights, and social stress) for a total duration of 2 weeks (*Table 1*). The time gap between the two daily stressors was at least 3 hours and no more than 5 hours. The control mice (n=6) received no handling and were housed in a separate room. The animals were perfused half hour after the final stressor, which for all animals was restraint stress.

Stressors

Cage Restraint – Mice were restrained in a narrow cylindrical apparatus on a flat table surface with a small gate barring their exit. The tail was taped onto the table outside the gate and the mouse left in restraint for 30 mins. This was conducted approximately twice a week.

Tail Pinch – Mouse tail is wrapped in small piece of gauze, near the connection to the body and a paper clip is placed upon the gauze-wrapped tail. The paper clip presses down on the tail and places pressure on the mouse. The tail pinch is carried out for two minutes.

Tail Hang – The mouse is manually suspended by his tail one foot above its cage by its tail for a total duration of two minutes (twice a week).

Predator Odor – Fresh rat feces is placed into the mouse home cage for a duration of twelve hours twice a week.

Social Stress – The experimental mouse is placed inside the cage of a larger retired breeder (bully mouse). Once the two mice start fighting, we separate the mice once the bully mouse has dominated the experimental mouse three times. Separation is defined as the insertion of a mesh partition in the middle of the cage that allows for free diffusion of odor, sound and visual cues but no physical contact. After separation, the mouse is left in the bully mouse cage for 30 minutes. This was performed once a week.

Hair Dryer – The mouse is removed from its home cage and placed into an identical cage sans bedding. The mouse is then buffeted with high pressure air from a Conair hair dryer alternating between cold and hot air at low and high speeds. The stressor lasts for a duration of 5 minutes and the mice received this stressors twice a week.

Wet Bedding – The bedding inside the homecage of the stress-group mice was drizzled with water so as to sustain a wet surface without the pooling of water. The cage was left wet for a duration of 12 hours (overnight), i.e. during the active phase of the mouse's diurnal cycle. This was carried out once per week.

Elevated Cage – The homecage of the mouse was elevated at an angle of 45 degrees from the surface and the cages were left as such for a duration of 12 hours. This was carried out once per week.

Continuous Lights – The vivarium lights within the housing room of the stressed group were left on during the dark phase (active phase) of the mouse's diurnal cycle. In total, mice were under lighting for 24 hours. This was done once per week.

Study 2: Paraquat and MEF2

Mice (N=40) were separated into two cohorts (A and B) to examine a time-response. Each cohort consisted of a saline control group and a PQ experimental group for a total of four groups (n=10/group). Each group was scheduled to receive a total of six injections of the appropriate treatment over the course of two weeks. Cohort A animals (saline and experimental) were perfused one hour after the last respective injection whereas Cohort B animals were perfused seven days after the last injection. All mice received an interperitoneal (i.p.) injection of PQ (standard dose of 10mg/kg) or an equivalent injection of saline. There was at least one day of rest between injections so as to deliver 3 injections per week.

Prior to the first injection, mice were placed into the micromax locomotor activity chamber for an hour and their baseline movement measured. Afterwards, mice were tested in the micromax after the third injection and then finally after the fifth injection (t=1 hour). The discrete Y-maze was administered during the time between the fifth injection and the sixth (Figure 2.)

Figure 2. *Schematic outline for Study 2.*

Schematic outline for the Paraquat & MEF2 study includes time points for injections, behavioural testing and tissue collection for both cohorts.

Study 3: LPS and MEF2

Mice were segregated into three groups of mice (n=6) who received i.p. injections of either saline, high-dose LPS (10ug/animal) or low-dose LPS (2.5ug/animal). These mice were given a rest period of one hour post-injection. The discrete two-trial Y-maze was administered once the rest period was complete. Immediately after the Y-maze, the mice were sent away for perfusion. This was a study carried out over the duration of one day (Figure 3.).

Figure 3. *Schematic outline for Study 3.*

Schematic outline showing the single day experimentation of the LPS & MEF2 study which includes notable time points for experiment.

For more details on each study, please refer to *Table 1* at the end of this section.

Behavioural tests

Two Trial Discrete Y-Maze. This test is designed to evaluate spatial recognition memory and as such it utilises a modified version of the Y-maze. Based on innate preference, the test doesn't require conditioning (Oonk et al., 2011). The Y-maze apparatus is a structure that consists of three enclosed arms, each arm measures 40cm x 3cm x 13cm (length x width x height). The three arms converge onto an equilateral triangle in the centre. The maze has proximal cue stickers on the inner end of each arm and the testing room contains distinct distal cues fixed upon the walls. The testing consists of two phases: Trial 1 and Trial 2. The first phase (Trial 1) involves one arm of the Y-maze to be blocked and thus inaccessible for exploration. The mouse is placed into a Home arm and given 5 minutes of exploration in the two accessible arms. Half an hour later, Trial 2 commences and once again the mouse gets placed into the Home arm, however, the block has been removed and the mouse can explore all three arms. Same arm returns (SAR), alternate arm returns (AAR) and novel arm visits (NAV) were recorded when each animal had placed all 4 paws in the arm runway.

Figure 4. *Illustration for Y-maze.*

Schematic drawing for the Two-Trial Discrete Y-maze indicates Home arm and the Blocked arm. The arm block is retained for Trial 1 (t=5mins), while just prior to Trial 2 (t=5mins), the block is removed.

Home-cage locomotor activity. Measurements of spontaneous locomotor activity were taken using a Micromax (MMX) infrared beam-break apparatus (Accuscan Instruments, Columbus, OH, USA). The animals, kept within their home cages, were placed inside the external infrared beam-break apparatus; spontaneous locomotor activity was determined as a measure of photobeam breaks. There was a 30 minute acclimation period in the behavioural testing room prior to the one hour measurement. The duration of measurement was 1 hour and the measurements were started one hour after paraquat or saline exposure as described in the timeline during the 3 week paradigm.

Brain perfusion and tissue collection

Perfusion. Mice were perfused half an hour after the last restraint stressor for the first stress study whereas mice were sacrificed one hour or 7 days after the last paraquat or saline injection to establish a time-course. Within the LPS context, the mice were taken for perfusion 1.5 hours after initial injection. All perfusion methodology was identical.

Mice were perfused after being injected with sodium pentobarbital (80mg/kg) and upon opening of the chest cavity, trunk blood was collected for CORT analyses. The mouse brain was fixed with 4% paraformaldehyde in 0.1M Potassium Buffer (PB) during the perfusion and post-fixed for 24 hours in identical 4% paraformaldehyde-PB solution. The brains were washed twice in 10% sucrose-PB for 4 hours per wash. Afterwards, the brains were stored overnight (24 hours) in 30% sucrose-PB and then flash frozen for long-term storage in -80°C until processing. The trunk blood was centrifuged at 3,700rpm for 8 minutes and like the brains, stored at -80°C until CORT determination.

Tissue Collection. Mice brains were removed from -80°C storage and sliced immediately at a thickness of 40µm using a Cryotome FSE (Thermo Scientific). Upon slicing, brain slices were placed in 0.1M potassium buffer Saline with 0.01% sodium azide and refrigerated at 4°C until immunochemistry analyses.

CORT analyses

Animal blood was collected using a pipette during the perfusion prior to the application of protein fixing formaldehyde. The blood was immediately centrifuged at 3700rpm for 8 minutes. The separated plasma was aliquot into volumes of 10ul with anticoagulant EDTA and flash frozen at -80°C until HPLC analysis.

Immunofluorescence

Coronal brain slices were selected so as to contain the hippocampus sections with the DG readily apparent (bregma -1.28mm to -2.12mm). Slices were washed in 10mM PBS solutions thrice for duration of 5 minutes per wash. Next, the slices were incubated overnight (24 hours) in the primary antibody (230µl/slice) at a refrigerator temperature of 4°C. After the application of the

primary antibody, the brain slices were washed thrice in 10mM PBS solution for duration of 5 minutes per wash. After washing, the slices are incubated in the fluorescent secondary antibody (230µl/slice) at room temperature sans light (since the secondary antibodies are light-sensitive) for a duration of 2 hours. Promptly after incubation in the secondary antibody, the slices are washed again (3 x 5mins in 10mM PBS) however this time in the dark so as to minimize light exposure. Following the final wash, the brain slices were mounted and cover-slipped onto glass slides and left to dry in darkness for an hour minimum before microscope visualization.

Antibodies

Primary antibodies utilised included antibodies to DCX (1:200, C-18, Santa Cruz) and MEF2A(1:1000, H-300, Santa Cruz).

Statistical Analysis

The stress study was analyzed via simple t-test (“stress vs. “non-stress”) while PQ study data was analyzed using a 2 (paraquat vs. saline treatment) by 2 (“acute” vs. “chronic” time course points) between-subjects Analysis of variance (ANOVA). Finally, the LPS data was analyzed using a simple oneway ANOVA (“saline” vs. “LPS-low” vs. “LPS-high”).The data was further analyzed for an interaction effect using two-way ANOVA and Bonferonni pairwise comparisons when appropriate. All data were analyzed using the statistical software IBM SPSS Statistics 20 or StatView (version 6.0) and differences was deemed statistically significant when $p < 0.05$.

Table 1. Summary of the three studies conducted for exploring changes in hippocampal-MEF2 as a consequence of environmental stressors. The groups for the Paraquat contain numbers which refer to the two cohorts for this particular study: “1” refers one hour post-final injection while the “7” is seven days after final injection

Study	Mouse Species	n	Time Frame	Treatment	Groups	Behaviours	Stains	Brain Region
Stress	CD1	6 x 2	3 weeks	Random Chronic Stress	Stress;Non-Stress	None	MEF2	DG; CA3; Piri
Paraquat	C57	10 x 4	2 weeks+1 week	Paraquat (10ug/kg)	Saline1;Paraquat1; Saline7;Paraquat7	MicroMax; Y-Maze	MEF2; DCX	DG; SNc
LPS	CD1	6 x 3	1 day	LPS	Saline; LPS-Low (2.5ug); LPS-High (10ug)	Y-Maze	MEF2; DCX	DG

Results

Study 1: Chronic unpredictable stress does not affect hippocampal MEF2

Accumulating insights into the various roles of MEF2 within neuronal survival, neurogenesis, neuroplasticity and memory consolidation support the influence of environmental factors on expression of this transcription factor within the hippocampus. A great deal of evidence indicates that environmental factors – both chronic and acute – can negatively affect cognitive function. Here we assessed the extent to which MEF2 expression levels are influenced when mice are exposed to three weeks of unpredictable mild physical stressors.

Although the chronic unpredictable stress did not affect changes within MEF2 expression levels within the hippocampus, the treatment group displayed behavioural signs of chronic stress (weight data not shown) compared to their control non-stressed counterparts. An ANOVA test failed to show significant differences in MEF2 levels between the stress and non-stressed groups ($F < 1$) (Fig. 5).

Study 2: Paraquat influences behavioural exploration

Animals were assessed for spatial navigation using the spontaneous Y-maze test. Herein, an ANOVA examining percent visit frequency into the novel arm during the second trial of the testing protocol revealed a significant reduction in visitations for mice treated with the environmental toxin paraquat ($F_{1,36} = 4.23$, $p < 0.05$) (Fig. 6a). The home arm and alternate arm show no differences between saline and PQ-treatment in either duration or frequency of visits into said arm ($F < 1$). Similarly, assessment of time spent within the novel arm (ANOVA)

suggested a trend towards reduced duration by PQ-treated mice within the novel arm (albeit not significant) ($F_{1,36} = 3.56, p > 0.05$) (Fig. 6b).

The impact of paraquat upon neurogenesis within the hippocampus

To determine the influence of paraquat on neurogenesis, assessments of the immature neuronal marker, doublecortin (DCX), were made within the dentate gyrus portion of the hippocampus. Through the use of immunofluorescence, DCX-positive counts were assessed for separate groups of mice 1 hour or 7 days following the last paraquat injection. This separation was done to examine the influence that passage of time may have upon DCX expression. A 2-way ANOVA revealed no interaction between PQ-treatment and passage of time ($F < 1$), nor was there a main effect of time ($F < 1$). However, a main effect of paraquat treatment ($F_{1,33} = 89.75, p < 0.001$) was observed, with a significant reduction of DCX levels within the PQ-treated groups (Fig. 7).

The effects of paraquat and time on corticosterone levels within blood plasma

Trunk plasma blood retrieved just prior to perfusion analyzed through a 2-way ANOVA failed to show any changes between the groups. There was no interaction between treatment and time, nor were there any main effects conferred by either treatment or time ($F < 1$) (Fig. 8).

Impact of paraquat and time upon MEF2 expression within the DG

Accumulating evidence supports the notion that MEF2 expression within the hippocampus is influenced through signaling pathways associated with oxidative stress. Herein, brain slices were assessed through indirect immunofluorescence to attain MEF2-positive cell counts from the granular layer of the dentate gyrus. The effects of both treatment and time were examined. A 2-way ANOVA revealed no significant interaction between the two factors ($F < 1$) nor was a main effect of time observed ($F_{1,35} = 1.97, p > 0.05$). Additionally, the analysis revealed a non-significant trend ($F_{1,35} = 3.17, p = 0.084$), whereby MEF2 levels tended to be lower in PQ-treated animals with respect to their saline counterparts (Fig. 9).

We also examined the MEF2 data in relation to the Y-maze behavioural data. To this end, the y-maze visit frequency data was split along its median ($Mdn = 38\%$) into two separate groups: “high” exploratory mice and “low” exploratory mice. The high exploratory mice frequented the novel arm more than 38% for their total visits to all arms whereas the low exploratory group scored 38% or lower on their novel arm visitations. Separate ANOVAs, examining maze performance against treatment, conducted upon each exploratory group revealed non-significant role of treatment in the high group ($F < 1$). However, there was a significant effect of treatment within the low group ($F_{1,18} = 5.29, p < 0.05$), whereby pesticide treated animals exhibited lower levels of MEF2-positive cells (Fig. 10b). The immunofluorescent images in Fig. 10a are representative sections of the dentate gyrus that show PQ-influenced increases in MEF2 expression within the DG of animals who performed poorly in the y-maze.

Study 3: Behavioural outcomes in spatial exploration following LPS administration

Animals were assessed for Y-maze spatial performance upon administration of the endotoxin lipopolysaccharide (LPS). Contrary to expectations, there was no significant effect of LPS treatments (low and high) upon the percent duration in novel arm ($F < 1$), nor was there a significant difference between visitations into the novel arm ($F < 1$) (Fig. 11).

LPS influences neurogenesis within the hippocampus

Here we assessed the contribution of LPS to neurogenesis within the hippocampus, specifically within the inner blade of the DG. Accordingly, the 1-way ANOVA revealed a significant reduction in DCX expression within the LPS-treated animals ($F_{2,14} = 3.97$, $p < 0.05$). Further pairwise comparisons failed to show significant differences between the low and high LPS treatment groups ($F < 1$) (Fig. 12).

The effect of LPS on corticosterone levels within blood plasma

As previously described, corticosterone levels were quantified within circulating blood plasma. Comparisons through ANOVA revealed corticosterone levels to be substantially higher in the LPS-treated animals ($F_{2,15} = 13.46$, $p < 0.001$). Also, though not statistically significant, corticosterone levels appeared to be higher in the low LPS-treated mice relative to the high LPS-treated animals ($F_{1,10} = 3.12$, $p > 0.05$) (Fig. 13).

Influence of endotoxin LPS on MEF2 levels within the hippocampus

The scant evidence within the field supports the notion of differences in MEF2 expression as a function of immune insult. Herein, we estimate the impact of endotoxin LPS, administered as two separate doses, upon the expression of the MEF2 expression within the dentate gyrus. MEF2 staining revealed no differences in expression between the treatment groups (Fig. 14). Indeed, both the low LPS and the high LPS doses failed to induce significant changes ($F < 1$). Thus it appears that treatment with LPS neither diminishes nor amplifies MEF2 expression within the hippocampus.

Discussion

Traumatic life events, stressors and various illnesses can promote deficits in memory acquisition and consolidation (Bae et al., 2014; Hopkins, 2013; Kontaxaki et al., 2014) with associated neuronal damage (Bozza et al., 2010). Although the underlying etiology behind such effects is still unclear, accumulating evidences suggests MEF2 as a potential regulator of stressor induced cognitive deficits. Indeed, existing evidence suggests MEF2 as a pro-survival transcription factor that confers post-mitotic protection against apoptosis to the neuron and aides in mediation of cell fate decisions (McKinsey *et al.*, 2002; Youn *et al.*, 1999). Most importantly, MEF2 has been linked to neuroplasticity and memory consolidation (Belfield *et al.*, 2006; Cole *et al.*, 2012). However, limited research has focused on the link between stress-induced cognitive disturbances and the role of MEF2. As deficits in memory are symptomatic of many neurodegenerative diseases and neural disorders, it is important to understand their underlying mechanisms. In the present investigation, we set out to explore the cognitive effects of three various environmental stressors on MEF2 expression within the hippocampus.

MEF2 and spatial memory under environmental stressors

Contrary to our hypothesis, which predicted a MEF2-upregulation with administration of chronic physical stressors, there were no notable changes in MEF2 expression (within the dentate gyrus) following restraint stressor exposure. Contrastingly, hyperthermia-exposed rat brains show increased MEF2 expression within the cerebellum and hippocampus (Maroni *et al.*, 2003) raising the possibility of an underlying stress pathway that was not activated by the current stress paradigm. The noticeable lack of difference may also be attributed to a transient change in MEF2

within the hour following administration of stressor, which then returns to baseline rapidly thereafter.

In addition to traditional stressors, immune system activators have also been shown to have potent effects on cognitive processes. Interestingly, patients with multiple sclerosis, an immune-mediated disease that affects the CNS, show higher failure rates in information processing tasks (Messina & Patti, 2014). LPS, the principal inflammatory agent of the bacterial cell wall endotoxin, induces cognitive decline in well-documented studies of systemic inflammation (Hritcu *et al.*, 2011; Noh, Jeon & Seo, 2014; Qin *et al.*, 2007; Valero *et al.*, 2014) where spatial performance is consistently impaired. Though long-term effects of a single dose of LPS are reported up to seven weeks later (Valero *et al.*, 2014), including inhibition of neuronal differentiation in hippocampus (Smith *et al.*, 2014), the endotoxin may not confer its full influence when first administered. Indeed, researchers have reported the lack of significant changes in spatial memory within the Morris water maze despite significant neuroinflammatory responses within LPS-treated mice 1 hour after LPS administration (Noh *et al.*, 2014). Furthermore, we assessed MEF2 in response to LPS administration. However, paralleling the previously noted lack of effect of restraint stress on MEF2 expression, we found that LPS failed to induce any changes to hippocampal MEF2 expression.

Previous research reported the necessity of MEF2 for regulation of cytokine expression within T lymphocytes via calcium/calmodulin signaling (Boubali *et al.*, 2012; Liopeta *et al.*, 2009; Zhuang *et al.*, 2013). Similarly, others show dominant negative form (deleterious mutation) of MEF2 to prevent macrophage death in the face of LPS treatment (Fu *et al.*, 2006). Interestingly, downstream pro-survival targets of MEF2 (eg. *Nur77*) are also up regulated during T cell apoptosis and during the accelerated macrophage differentiation, resultant of LPS-induced

inflammation (Baek *et al.*, 2009). Hence, our future analyses will focus on the measurement of MEF2 downstream targets in order to attain a better view of MEF2 signaling in the presence of a neuroinflammatory response.

Hippocampal damage, thought as a primary cause of memory impairment, may be induced through the oxidative properties of neurotoxins (Chen *et al.*, 2010; Jiao *et al.*, 2010; Qi *et al.*, 2013). However, damage to the hippocampus is also likely modulated by impaired neural plasticity, of which, MEF2 is an important regulator (Rashid, Cole & Josselyn, 2014). While a decline in MEF2 levels has been demonstrated with neuronal apoptosis (McKinsey *et al.*, 2002), MEF2 has also been identified as a master regulator of neural responses in frog neurons (*xenopus laevis*) wherein it's expression drops drastically when faced with enhanced neural activity (Chen *et al.*, 2012). Indeed, lower MEF2 levels are associated with increases in caspase cleavage and shifts in plasticity thresholds in a calcium-independent manner without the induction of cell death (Chen *et al.*, 2012). To extrapolate, in the event of an environmental stressor (such as LPS) that up-regulates neuronal activity, it is possible that a shift in plasticity threshold would allow for more rapid plastic effects to occur. As shown previously, the MEF2 protein exists in a constitutively unphosphorylated form that changes function once phosphorylated. Operating under the assumption that transactivation of MEF2 increases through a LPS induced p38-activation (Han *et al.*, 1997), we expected higher levels of the phosphorylated MEF2 (pMEF2). Unfortunately, changes in pMEF2 do not necessarily translate into discernible changes within the overall unphosphorylated MEF2 and as such, we may have missed subtle changes in the transcription factor.

One proposed mechanism (Cole *et al.*, 2012; Flavell *et al.*, 2006) of memory impairment has been proposed to stem from the activation of the pro-plasticity protein, Arc, which is a

known substrate for MEF2. Previous research showed that nuclear over-expression of MEF2 resulted in elevated Arc down-stream expression. It has been suggested that Arc induces AMPAR endocytosis on the neuron cell surface, which can disrupt synaptic transmission and suppress excitatory synapse numbers (Cole *et al.*, 2012; Flavell *et al.*, 2006). Consequently, the impairments in memory are apparent when this occurs within the hippocampus. Similarly, lack of higher pMEF2 expression contributes to robust memory formation as suggested in previous studies (Sivachenko *et al.*, 2013).

It is possible that our failure to find a significant MEF2 change with LPS treatment might be related to timing. Indeed, an hour and half duration between peripheral LPS administration and mouse introduction to the first trial of the Y-maze may have been too short of a time for the LPS to markedly affect spatial memory and the related MEF2 levels. It has also previously been shown that acute administration of LPS, though it may induce sickness (i.e. hypothermia) and some immune response, does not impair performance ability on cognition-based tasks (Banks & Robinson, 2010; Noh *et al.*, 2014; Toyama *et al.*, 2014).

Paralleling the impact of the aforementioned treatments, the environmental toxicant, PQ, did not influence MEF2 expression. The differences in hippocampal MEF2-expression approached significance based on PQ-treatment, albeit there was no influence of time whatsoever. This is to say that MEF2 expression levels remained unchanged one week after the last PQ injection, indicating no time-based effects of PQ on levels of MEF2. However, Y-maze performance was correlated with MEF2 expression in the dentate gyrus. In this regard, PQ induced a significant decline in visit frequency into novel arm. Moreover, when mice were subdivided according to their Y-maze performance, then mice scoring in the lower half on this test had significantly reduced hippocampal MEF2 levels. This difference is likely based on

impaired memory function rather than immobility brought about by general malaise, as there were no obvious signs of sickness at the time of testing induced by the endotoxin. Due to the lack of obvious sickness (data not shown) prior to and during testing, we conclude the decline in novel exploration as a direct cause of PQ's effects on cognition. As previously mentioned, our findings are in line with those, which report chronic administration of PQ to impair acquisition and consolidation of spatial memory within the Y-maze task (Bortolotto *et al.*, 2014; Sun *et al.*, 2011).

To reiterate the results, animals categorized into the high exploration group on the Y-maze displayed no significant change in MEF2 as a result of PQ influence. Contrastingly, PQ-treated animals in the low exploratory group on the Y-maze exhibited a decline in MEF2 expression, whereby low spatial performance was associated with decreased MEF2 expression. Akin to its expression pattern in striated muscles, MEF2 genes are expressed in neurons which have already exited the cell cycle and are about to enter differentiation (Lyons *et al.*, 1995). As such, lower MEF2 expression may be indicative of neurons stuck within the self-renewal phase of neuronal progenitor cells, prior to differentiation into immature neurons (Meza-Sosa, Pedraraz-Alva, & Pérez-Martínez, 2014).

Previous research lends credence to the possibility that HDACs might be linked to MEF2 functions in the face of environmental stressor challenge (Fig. 15). Upon administration of PQ, the drug is expected to cross the BBB and into the brain via the neutral amino acid transport system expressed in brain capillaries (Shimizu *et al.*, 2001) and enter neuronal soma within hippocampal neurons. This transport relies on the carrier ability of amino acids to transport the PQ into the brain as opposed to damage to BBB permeability induced by the herbicide (Shimizu *et al.*, 2001). Once inside the neuron, PQ mediates the import of HDAC from the cell body, via

altered calcium signaling (Li *et al.*, 2007; Zaidi *et al.*, 2009), and into the nucleus wherein the HDAC binds onto the MEF2 rendering the complex inactive and incapable of transcription. Due to the induced conformational changes to MEF2, coactivators are unable to bind while concurrently corepressor complexes are recruited to the HDAC-MEF2 complex (Zhuang *et al.*, 2013). Given the co-localization of MEF2 and HDAC previously shown (Fitzsimons *et al.*, 2013), MEF2-induced transcription becomes inactive and downstream plasticity-inducing genes are not transcribed. Since memory enhancement cannot occur, by the lack of enhancement, memory consolidation suffers and the mouse is unable to remember the surroundings which it has previously explored. This proposed mechanism is supported with research that promotes the role of HDACs as negative regulators of memory through the formation of a complex at the MEF2 DNA-binding site (Cohen & Greenburg, 2008; McKinsey *et al.*, 2002; Sando *et al.*, 2012).

Related research confirms the degradation of class II HDACs as an augmenter of MEF2 transcriptional activity (Potthoff *et al.*, 2007). Similar research with MPTP indicates the positive influence on memory acquisition and consolidation upon the introduction of an HDAC inhibitor such as phenylbutyrate (Gardian *et al.*, 2004). Simultaneously, there may be a Cdk5-dependent pathway which renders MEF2 in a phosphorylated form thereby diminishing MEF2s protective effects (Mount *et al.*, 2013; She *et al.*, 2011). Indeed, PD research has shown the binding of MEF2 with its downstream transcriptional target *Nur77* to be involved in regulating neuronal cell loss following MPTP treatment (Mount *et al.*, 2013). *Nur77*-deficient animals were shown to be drastically sensitive to MPTP-induced neuronal degradation than controls (Mount *et al.*, 2013) hence implicating the downstream importance of MEF2 in neuroplasticity. This second proposed mechanism (Fig. 16) may come about as a result of PQ neurotoxicity of ROS within the hippocampus (Chen *et al.*, 2010). The oxidative stress would increase Cdk5 activity which

results in the subsequent phosphorylation of MEF2 resulting memory dysfunction. We suggest these pathways as a prelude to future work which seeks to tease out the nature of the MEF2-memory relationship.

Neurogenesis and memory in relation to MEF2

Our finding of lower DCX levels in LPS-treated animals was not correlated with any obvious behavioural changes. Neurogenesis being hallmarks of the hippocampus and olfactory system (Kemperman, Wiskott & Gage, 2004), the marked difference in DCX was expected to accompany differences in spatial navigation. However, since that was not the case, the aforementioned issue of a shorter time lapse between drug injection and Y-maze testing, could have influenced the outcomes. This decline in DCX within the LPS-mice may be explained as an inflammatory insult to the complex multi-step process from precursor cell to neuron (Kempermann *et al.*, 2004). Others have shown similar results in neonatal mice (Järlstedt *et al.*, 2013) and increased microglia activation (Ekdahl *et al.*, 2003). Brain inflammation is believed to be associated with cognitive impairments such as AD and dementia wherein spatial memory has been noted as impaired (Guariglia, 2007; Ekdahl *et al.*, 2003). Our data are consistent with model inflammation-mediated suppression of hippocampal neurogenesis.

Limited human studies and conflicting rodent results on neurogenesis in PD have largely focused on the effects of dopaminergic projections from the substantia nigra (SN) (Marxreiter, Regensburger, & Winkler, 2013). Research on neural precursor cells show stress onset to induce the production of new neurons in two specific regions within the brain, the subventricular zone (SVZ) and the subgranular zone of the DG with focus on the former region (which receives

dopaminergic projections from the SN) producing inconsistent results within PD-models (Lamm *et al.*, 2014). Indeed, an increase in neurogenesis has been noted after administration of dopamine-receptor agonist within the SN; progression of PD has been correlated with decrease in dopaminergic-induced modulation of neural stem cells in the forebrain (Lamm *et al.*, 2014, O'Sullivan *et al.*, 2011). Contrastingly, others showed anti-inflammatory agents (such as minocycline) to not induce neurogenesis within a 6-OHDA model (Worlitzer *et al.*, 2013) and no differences in neural stem cell expression between PD patients and controls were observed (Berg *et al.*, 2011). However, it has become more apparent that cognitive deficits in working and spatial memory are characteristic of early PD-onset, prior to the appearance of motor symptoms (Ferrer, 2009). While these early symptoms could be attributed directly to damage of the nigrostriatal system, it is plausible to consider these symptoms outcomes of damage or dysfunction of other systems (eg. limbic system) (Ferrer, 2009).

The underlying mode of action on the brain through which PQ induces PD-like symptoms is not entirely known but it is telling that its chemical structure resembles that of MPP⁺ (the active metabolite of the well known dopaminergic toxin, MPTP) (Wang & Slikker, Jr., 2010). Our examination of neurogenesis within the PQ-treated rodent brain is consistent with the literature, in that our results, for the first time, show a significant decline in DCX-positive neurons within the dentate gyrus of PQ-treated animals. Importantly, this effect was still maintained over a seven-day interval. Given our results, it is important to note that this reduction of neurogenesis was correlated with a decline in Y-maze performance. We consider the possibility that PQ may be affecting neurotrophic factors such as BDNF to provoke such changes. Indeed, our lab has previously demonstrated that administration of PQ causes time-dependent alteration of hippocampal BDNF (Litteljohn *et al.*, 2011), a trophic factor shown to play a role in memory-

related processes (Rudyk *et al.*, 2012). Further investigation into the relative amounts of BDNF and DCX in these PQ-treated animals may highlight a possible mechanism of spatial impairment. To date, our data is the first data to demonstrate the link between the herbicide PQ and reduced neurogenesis within the dentate gyrus.

Interestingly, prior studies have demonstrated the necessity of MEF2 in order to induce neurogenesis within the hippocampus. Mice injected with repeat doses of the small molecule, Isx-9, promote increases in adult hippocampal neurogenesis (Petrik *et al.*, 2012). These increases are related by common global mechanisms known to enhance neurogenesis. Upon selective MEF2-isoform knockout, administration of Isx-9 prevented neurogenesis, suggesting the intrinsic importance of MEF2 signalling in baseline neurogenesis (Petrik *et al.*, 2012). Similarly, research with human embryonic stem cells has shown dramatic increases in MEF2 levels as neural differentiation proceeded (Cho *et al.*, 2011). Furthermore, the knockdown of MEF2 results in the decline or delay in neurogenesis while increased activation of MEF2 produced a 3.2 fold increase in neurons compared to their control counterparts (Cho *et al.*, 2011). Through our own studies with the PQ neurotoxin, we show that relatively higher levels of DCX-positive cells corresponded to higher levels of MEF2. While our data are supportive, we cannot assume that the observed increased neurogenesis was primarily through the action of MEF2. To test this possibility we propose further *in vitro* research that modulates MEF2 expression within hippocampal cells, and even *in vivo* research examining dose-dependent changes with administration of activating kinases or transcription cofactors. The eventual clinical implication would be to measure the MEF2 response in clinical studies that aim to treat neurodegenerative diseases (such as PD) with cell-based therapies, such as neural grafts of fetal mesencephalic

tissue (Lindvall & Björklund, 2004), or through administration of embryonically-derived neuronal precursors into host brain (Cho *et al.*, 2011).

Corticosterone measurements under toxin conditions

Finally, we measured corticosterone differences between treatment in both LPS & PQ studies given that that increased corticosterone levels induced by such challenges are believed to strongly correlate with the state of distress that animals are experiencing (Kirsten *et al.*, 2013; Suzuki *et al.*, 1986). Indeed, we found marked corticoid elevations induced by LPS, however, PQ had no such effect. Importantly, previous studies on the neurodegenerative and motor symptoms of PD consistently show increases in corticosterone levels during the disease (Smith *et al.*, 2008). Simultaneously, PQ and MPTP-based research shows increased serum corticosterone in mice seven days after treatment with drug (Kaku *et al.*, 1999). Co-morbid effects in PD models, such as depressive-like symptoms, are associated with hypersecretion of glucocorticoids into the rodent hippocampus (Sapolsky, 2000). Interestingly, recent research has also shown the association between decreased blood plasma cortisol levels and increased HDAC activity (Jason *et al.*, 2011), which would complement the aforementioned mechanistic model.

Conclusion

In conclusion, the present findings suggest that MEF2-expression is not sensitive to chronic physical stress nor is it sensitive to LPS-induced activation of the immune system. Although initially, chronic exposure to environmental toxin PQ did not reveal differences in hippocampal

MEF2 expression, segregation into high and low performers on the Y-maze demonstrated that lower levels of MEF2 proteins were associated with low spatial performance. These findings refute the contention that environmental stress, regardless of method of induction, bring about differences in MEF2 transcription and consequent cognitive deficits. Consistent with previous studies, exposure to the environmental stressor PQ (herbicide) induced differences in spatial memory. In addition, our data shows the first evidence of neurogenesis reductions within the hippocampus as a consequence of environment toxin (PQ) (Järlstedt *et al.*, 2013). Taken together with emerging MEF2 data, such knowledge about very different environmental stressors, should help stimulate research on the pro-survival machinery and associated strategies to combat neurodegeneration.

Figure 1. *MEF2 integrated pathway drawing.*

An illustration of various roles that MEF2 employs under normative conditions and during outside influences.

Figure 5. *Chronic unpredictable stress does not affect hippocampal MEF2.*

(A) Three week paradigm of chronic unpredictable stress compared to their control counterparts; (B) immunofluorescence of MEF2 positive cell within the granular layer of the dentate gyrus representing the control non-stressed mouse brain and the chronically stressed mouse brain (magnification: 40x, slice thickness: 35 μ m).

Figure 6. *Paraquat influences behavioural exploration in Y-maze.*

Spontaneous Y-maze data, presented as a percentage, has been segregated according to treatment and further subdivided according to Y-maze arm (novel, home & alternate). The Y-maze performances of PQ-treated animals in each arm are being compared with their counterpart saline: (A) shows the frequency of visits for the mice respective to each arm, (B) shows duration of time that mice spent in each arm (** indicates significance of $p < 0.05$).

Figure 7. *The impact of paraquat upon neurogenesis within the hippocampus.*

(A) Immunofluorescent staining of DCX-positive cells within the inner dentate of mouse brain of saline-treated mouse brain and PQ-treated mouse brain (magnification: 20x, slice thickness: 35 μ m); (B) treatment of environmental toxin Paraquat show apparent decline in DCX-positive cells within the inner blade of the dentate gyrus examined at a thickness of 35 μ m whereas no effect of cohort was observed (***) indicates significance of $p < 0.001$).

Figure 8. *Corticosterone in PQ-treated animals over time.*

Comparison of corticosterone levels (ug/dL) measured across treatment and paraquat show no difference under the environmental toxin stress paradigm.

Figure 9. Overall MEF2 expression as influenced by PQ and time.

MEF2-positive staining data segregated according to treatment (Saline & Paraquat) and then subdivided accordingly into “1 hour” and “7 days” groups. Total MEF2 counts were compared to examine influence of treatment and cohort.

Figure 10. *MEF2-expression levels based on Y-maze performance in PQ-treated mice.*

(A) MEF2-positive stains as viewed by immunofluorescent staining represents the control animal brain viewed against toxin-treated group within the low exploratory group (magnification: 20x, slice thickness: 35 μ m); (B) MEF2-positive cell counts measured within the dentate gyrus of mice within the median-segregated exploratory groups. PQ treated mice performed significantly worse than the saline-treated animals (** indicates significance $p < 0.05$).

Figure 11. *Spatial navigation in Y-maze after LPS treatment.*

Shown is the spontaneous Y-maze data pertaining to (B) duration of mice within the novel arm during the second trial of the Y-maze protocol. The data is divided into saline, low LPS (2.5ug/animal) and high LPS (10ug/animal).

Figure 12. *LPS influences neurogenesis within hippocampus.*

(A) Expression levels of the neurogenesis marker, DCX, as compared between treatments within the inner blade of the hippocampus (magnification: 20x, slice thickness 35 μ m); (B) Expression levels of the neurogenesis marker, DCX, are compared between treatments. Saline treated mice show higher expression levels of DCX in comparison to those mice who received either dose of the LPS (2.5 μ g & 10 μ g) (** indicates significance $p < 0.05$).

Figure 13. *Corticosterone levels in blood plasma of LPS-treated animals.*
Corticosterone levels (measured using radioimmunoassay) show significant differences between the control (saline) groups and the treatment LPS-groups (** indicates significance $p < 0.05$).

Figure 14. *Influence of LPS on MEF2 levels in hippocampus.*

(A) Immunofluorescence of MEF2-positive cells and DCX-positive cells arranged so as to reflect the appropriate treatment (i.e. saline, low dose LPS, high dose LPS) within the perfused and sliced mouse brain (magnification: 20x, slice thickness: 35 μ m); (B) saline MEF2 expression levels within the dentate gyrus of mouse hippocampal slices show no significant differences when compared to the two endotoxin (LPS)-treated groups.

Figure 15. *Potential HDAC-MEF2 pathway in PQ-mediated changes in spatial memory.*

A potential pathway which suggests PQ action through the intracellular import of class II HDACs from the cytoplasm into the nucleus of hippocampal cells. The resulting HDAC-MEF2 complex prevents the transcription of downstream MEF2-regulated plasticity genes thus potentially impairing memory consolidation.

Figure 16. *Potential Cdk5-dependent pathway of neurotoxicity.*

A potential pathway that may involve the Cdk5-dependent phosphorylation of MEF2 into an inactive form (pMEF2). Oxidative stress may increase levels of pMEF2 leading to memory dysfunction

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