THE ROLE OF LIPID MEDIATOR PROSTAGLANDIN E2 IN EARLY NEURONAL DEVELOPMENT

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

AUGUST 2015

ABSTRACT

Lipid mediator prostaglandin E_2 (PGE_2) plays a pivotal role in early development of the nervous system. Abnormal PGE_2 signaling in neurodevelopment has also been implicated in autism. Increased levels of PGE_2 have been attributed to maternal infection and the inflammatory response. My *in vitro* work shows that elevated levels of PGE_2 cause an increase in cytosolic and growth cone calcium levels in differentiated neuroectodermal (NE-4C) cells and a dose- and time-dependent effect on neurite extension length. Furthermore, PGE_2 induced subcellular localization of the EP4 receptor to the plasma membrane in NE-4C stem cells and growth cones of differentiated NE-4C cells. My *in vivo* work shows that prenatal exposure to PGE_2 results in differential mRNA levels of two important developmental genes *Wnt3a* and *Fosl1* in the mouse brain at various embryonic stages. This furthers our understanding of the functional implications of abnormal PGE_2 signaling in the neurodevelopment of autism.

Acknowledgements

Contributing to research in the field of biology and neurosciences has been a lifelong dream of mine that has been made possible with the support of many people. I will always be grateful to my supervisor, Dr. Dorota Crawford, for her continual guidance and support, and for providing a solid foundation on which to build my future research career. Furthermore, I wish to thank Dr. Logan Donaldson for providing valuable feedback and guidance as my supervisory committee member, and to my examination committee Dr. Terrance Kubiseski and Dr. Lauren Sergio. My gratitude also extends to my fellow lab members, Christine Wong, Ravneet Rai-Bhogal, Eizaaz Ahmad, and Rona Bakri Nsouli for willingly sharing their knowledge and friendship, especially to Hongyan Li for all of her help! I would also like to thank my parents and family, Paul, Bonnie, Shane, and Shannon, for their continual support and belief in myself. Furthermore, many thanks to Antonio for all of the encouragement and advice along the way.

These studies were supported by the Natural Sciences and Engineering Research Council of Canada (NSERC).

TABLE OF CONTENTS

List of Tables

List of Figures

Abbreviations

$[Ca^{2+}]_E$, Extracellular calcium concentration
$[Ca^{2+}]_i$, Intracellular calcium concentration
AA, Arachadonic acid
AC, Adenylate cyclase
ACC, Animal Care Committee
AH23848, EP4 antagonist
ALA, Omega-3 alpha-lineolic acid
ANOVA, Analysis of Variance
ASD, Autism Spectrum Disorders
ATCC, American Tissue Culture Collection
Ca^{2+}, Calcium
cAMP, Cyclic adenosine monophosphate
CAY10580, EP4 agonist
COX-1, Cyclooxygenase-1 enzyme
COX-2, Cyclooxygenase-2 enzyme
CREB, cAMP response element binding the protein
C_T, Threshold cycle number
DAPI, 4′,6-diamidino-2-phenylindole
DHA, Docosahexaenoic acid
DIC, Differential Interference Contrast
$dmPGE_2$, 16,16-dimethyl PGE2
DMSO, Dimethyl sulfoxide
DNA, Deoxynucleic acid
dNTP, Deoxynucleotide triphosphates
DRG, Dorsal root ganglion
dT, Deoxy-thymine nucleotides
E, Embryonic day
EDTA, Ethylenediaminetetraacetic acid
EGTA, Ethylene glycol tetraacetic acid
EP, E-Prostanoid
EPA, Eicosapentaenoic acid
FITC, Fluorescein isothiocyanate
Fosl1, Fos-like antigen 1
Fura-2 AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy) ethane-N,N,N',N'-tetraacertic acid, acetoxylmethyl ester
Gapdh, Glyceraldehyde 3-phosphate dehydrogenase
GPCR, G-protein-coupled receptors
h, Hours
$HBSS_{red\text{-}free}$, Hanks balanced salt solution with no phenol red
hprt, Hypoxanthine phosphoribosyl transferase
IP_3, Inositol triphosphate

K_d, Dissociation constant for fura-2
LA, Omega-6 lineolic acid
LPS, lipopolysaccharide
Max, Maximum
MEM, Minimum Essential Medium
Min, Minimum
mM, Millimolar
mRNA, messenger ribonucleic acid
N, Total number
NBM, Neurobasal media
NE-4C, Neuroectodermal stem cells
Neuro-2a, Neuroblastoma cells
NGS, Normal Goat Serum
nm, Nanometer
nM, Nanomolar
NSERC, Natural Sciences and Engineering Research Council of Canada
P, Postnatal day
PBS, Phosphate buffered saline
PCR, Polymerase chain reaction
PFA, Paraformaldehyde
PG, Prostaglandin
PGE_2, Prostaglandin E2
Pgk1, Phosphoglycerate kinase
PI3K, Phosphoinositide 3-kinase
PKA, Protein kinase A
PKC, Protein kinase C
PLA_2, Phospholipase A2
PLC, phospholipase C
PMCA1, Plasma membrane Ca2+-ATPase
Pou5f, 1 Pou Class 5 homeobox 1
PUFAs, Polyunsaturated fatty acids
qRT-PCR, Quantitative real-time polymerase chain reaction
R, Ratio
R_{max}, Maximum 340/380 ratio
R_{min}, Minimum 340/380 ratio ()
RNA, Ribonucleic acid
ROI, Region of interest
RQ, Relative quantity
RT-PCR, Reverse transcription polymerase chain reaction
RT, Reverse transcription
SFM, Serum free media
TBS-T, Tris-Buffered Saline and Tween 20
Tubb3, Mouse BIII tubulin

TxA_2, Thromboxane A2
μM, Micromolar
VPA, Valproic acid
Wnt, Wingless-type MMTV integration site family
$\Delta 340/380_{max\text{-}min}$, Difference between $\Delta 340/380_{max}$ and $\Delta 340/380_{min}$
$\Delta 340/380_{max}$, 340/380 ratio maximum mean
$\Delta 340/380_{min}$, 340/380 ratio minimum mean

CHAPTER 1. INTRODUCTION

1.1. Environment and autism

Prenatal development of the brain is a critical period in which intricate processes may be disturbed by an imbalance of environmental stimuli. Autism is a heterogeneous neurodevelopmental disorder with a great range in presentation and severity of symptoms, thus may be referred to collectively as Autism Spectrum Disorders (ASD)[1]. The disorder affects as many as 1 in 68 children, and is almost five times more likely in boys than girls [2]. Although autism is believed to be primarily genetic in origin, an increasing amount of evidence suggests that autism is in fact a complex interaction between genes and environmental insult[3]. It is likely that many genes are involved in the etiology of autism along with a number of implicated environmental risk factors like infections such as the rubella virus [4], [5], mercury, oxidative stress [6]–[10], immunological factors [11], [12], dysfunctional calcium (Ca^{2+}) regulation [13], [14], and altered lipid metabolism [15], [16].

Drug use during pregnancy has also been linked to ASD including the anti-nausea medication used by pregnant women in 1957-1962, thalidomide [17], and an anticonvulsant and mood-stabilizing drug, valproic acid (VPA) [18]. Likewise, a drug administered to women to induce uterine contractions for early pregnancy termination [19] called misoprostol resulted in autism related characteristics termed Mobius syndrome [20]. Exposure to these drugs during the first trimester of pregnancy, more specifically around weeks five to eight of gestation, has been implicated in abnormalities of neurodevelopmental [21]. The brain undergoes its most rapid development from the prenatal period to the early postnatal period. Thus during this critical period, the brain is most vulnerable to environmental insults because it depends on

precisely regulated temporal and spatial developmental processes [22]. Neural development encompasses the process of neurogenesis, proliferation, migration, differentiation, synapse formation, and myelination [23]. Disruption at any point can have long lasting detrimental effects. Collectively, these findings suggest that the window of susceptibility for autism induction may be very early in gestation.

1.2. Lipid signaling in the nervous system

The human brain, by weight, is composed of approximately 60% lipids with over 20% polyunsaturated fatty acids (PUFAs) [24]–[26]. PUFAs are major components of the neural cell membrane phospholipids. Arachadonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the predominant PUFAs which are derived mainly from omega-6 lineolic acid (LA) and omega-3 alpha-lineolic acid (ALA), respectively [27], [28]. The conversion of these lipids is crucial for the integrity and proper functioning of the plasma membrane, such as modulation of ion channels, enzymes and receptor activity [29], [30]. It is also possible for AA to be synthesized from membrane phospholipids via phospholipase A2 (PLA2) where subsequent reactions can take place to make various prostanoids. Cyclooxygenase-1 enzyme (COX-1), constitutive form in the body, and cyclooxygenase-2 (COX-2), inducible form in body and constitutive form in the brain, metabolizes AA to the unstable PGG2 and PGH2 intermediates to five primary prostanoids via prostaglandin (PG) or thromboxane synthases: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2, and thromboxane A2 (TxA_2), whereby PGE_2 is the major signaling prostanoid. These lipid mediators interact with specific members of a family of distinct G-protein-coupled prostanoid receptors (GPCRs), designated EP, FP, DP, IP and TP receptors, respectively **(Figure 1)** [31], [32].

The released prostanoids play important roles in normal neural function including sleep induction, spatial learning, synaptic plasticity and long-term potentiation or inflammation [26], [33]. Of these, PGE_2 signaling has gained considerable attention for its involvement in activity-dependent synaptic plasticity [34], [35].

Clinical studies have reported significantly higher concentrations of PLA2 in the red blood cells of individuals with autism and Asperger's syndrome compared to healthy individuals [25]. Moreover, ASDs have been associated with the gene encoding the COX-2 protein [36]. Several studies have reported altered AA, DHA, and EPA levels in patients with autism. Children with autism have a higher AA: DHA ratio [24] and a higher AA: EPA ratio [37]. Significantly lower-than-normal levels of AA and DHA have also been found in blood plasma of individuals with autism [38], [39] **(Figure 1)**.

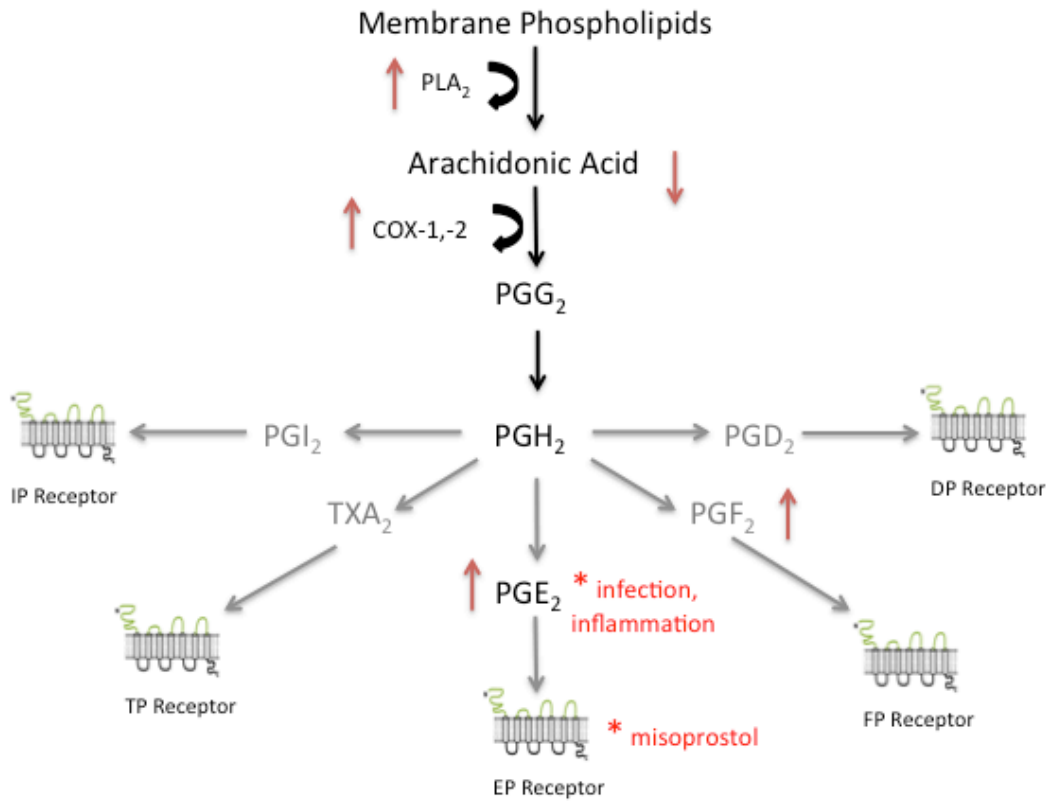

Figure 1. The lipid signaling pathway. Prostanoids (PGI_2, PGE_2, $PGF_2\alpha$, PGD_2) and thromboxane A_2 (TxA_2) are bioactive lipid metabolites that elicit cellular events through the action of their respective receptors (IP, EP, FP, DP, and TP). They are derived from a series of conversions from membrane phospholipids. PGE_2 is the major lipid signaling pathway. (*Red arrows* indicate an increase or decrease level in individuals with ASD; *asterisks* indicate a link to ASD). This figure was adapted from Wong and Crawford (2014) [16].

1.3. Autism and lipids in the brain

The cellular signaling and molecular mechanisms involved in the pathology of autism must be better understood to prevent or treat the disorder. Lipid signaling is involved in early brain development and maintaining its function. Abnormal lipid signaling due to genetic causes or environmental influences has been implicated in ASD [15], [17], [20] **(Figure 1)**. Prostaglandin E2 (PGE_2) is the natural, endogenous analogue of a drug implicated in ASD, misoprostol. Therefore the endogenous PGE_2 signaling pathway is a good candidate pathway to assess the neurodevelopment of autism. PGE_2 is the major lipid mediator in the brain and synthesized in the cell from phospholipids found in cell membranes. It typically acts within the local environmental to provide autocrine and paracrine stimulation to a number of signaling pathways in the nervous system.

1.4. Prostaglandin E_2 and development

During the embryonic stage of the development, there is an increase in the levels PGE_2 messenger ribonucleic acid (mRNA) [40]. Many physiologically important functions, such as synaptic plasticity [34], [35], dendritic spine formation, refining of mature neuronal connections [41]–[43], pain transmission [44], and cell survival [45] or death [46] are medicated by PGE_2 in the nervous system. Early research has reported abnormalities in mice offspring when exposed to PGE_2 during the embryonic stages [47]. Activation of the PGE_2 signaling pathway regulates the inflammatory response, fever, pain and sleep, including inflammation upon maternal infection [16], [48]. Elevation of endogenous PGE_2 levels has been found during events such as inflammatory response and oxidative stress [49]. It is possible that heightened inflammatory response during early development may lead to nervous system defects. The disruption of PGE_2 signaling could result in abnormal cellular functioning

throughout development. Research shows that immune activators such as prenatal exposure to rubella infections, anticonvulsants, perinatal hypoxia, and postnatal infections have all been identified as putative contributors to ASD [50]. Increased occurrence of maternal immune abnormalities during early pregnancy and greater incidence of familial autoimmunity have been suggested to alter fetal brain development and as ASD predisposing factors [51], [52], suggesting a role for dysregulation in PGE_2 signaling in the pathology of autism.

1.5. Prostaglandin E_2 signaling via EP receptors

PGE_2 acts on four cell surface GPCRs designated EP1-4[31, 32] depending on receptor binding affinity, receptor expression profile, differential coupling to signal transduction pathways, and cellular context [48]. PGE_2 binds to its four EP receptors at different affinities, predominantly on EP3 and EP4 (EP3>EP4>>EP2>EP1) [53], [54]. Ligand-dependent activation of each EP receptor promotes the activity of specific kinases and their constitutive metabolic pathways to result in either neuroprotection or neurotoxicity [55]. The EP2 and EP4 receptors can play a neuroprotective or neurotoxic role [56]–[58]. Disturbances in PGE_2 signaling as a result of altered level of endogenous PGE_2, due to inflammation or infection, or exposure to drugs such as misoprostol might have an adverse effect on various developmental stages due to differential expression and activation of the EP receptors [40].

1.6. EP receptor cellular localization

Neuroectodermal (NE-4C) stem cells endogenously express the four EP receptors. In NE-4C cells, EP2 has the highest mRNA expression followed by EP3γ and EP4 receptors. The endogenous EP1 and EP3β receptor expression is considerably low. These results were consistent for EP receptor protein expression as well [3]. In NE-4C cells, EP1 is localized in

the ER membrane; EP2 receptors are uniformly expressed around the nucleus and co-localized with the nuclear envelope; EP3 receptors were located at the plasma membrane; and EP4 receptors at the Golgi apparatus [3]. However, the localization of the EP receptors has not yet been studied in differentiated NE-4C neuronal cells.

In primary sensory neurons, the EP4 receptor is also localized in the Golgi apparatus [59]. Furthermore, it has been shown that PGE_2/EP4 signaling induces EP4 externalization, from the Golgi apparatus to the plasma membrane, during the inflammatory response in dorsal root ganglion (DRG) neurons [59]. It has been suggested that PGE_2-prolonged sensitization of DRG neurons may contribute to the transition from acute to chronic pain by facilitating EP4 receptor synthesis and anterograde axonal trafficking to the plasma membrane [60]. Thus the EP4 receptor trafficking in response to PGE_2 suggests EP4 likely plays a functional role.

1.7. EP receptors in the brain

The expression of EP receptors' mRNA in mouse embryonic development (E7, E11, E15, E17) is ubiquitously expressed within eight major regions of the brain (cortex, frontal cortex, thalamus, hypothalamus, hippocampus, brain stem, medulla and cerebellum). Most of the receptors including EP1, EP2, EP3α and EP3β were highest at E15, which is the peak of neurogenesis. Notably, the highest increase was the EP4 receptor at E7 followed by E15. This indicates that PGE_2 signaling may play an important role during early neuronal development via activation of these receptors [40].

Beginning early in the first month of gestation in humans, specific areas of the central nervous system begin to form with the neurogenesis and migration of cells in the forebrain, midbrain, and hindbrain [23]. Development of brain structures, such as medulla, pons, and cerebellum, which start at the early stages of the neurogenesis, followed by other areas, such as

the hippocampus, hypothalamus, thalamus, and entorhinal cortex [23], may potentially be affected by abnormal PGE_2 signaling via EP receptor activation. Zhu et al (2008) found that the four EP receptors are heterogeneously expressed in the hippocampus and their expression is differentially regulated by neuronal activities, suggesting that EPs may actively participate in hippocampus synaptic transmission and plasticity in rat pups [57].

Within the adult mouse brain, the EP1 and EP4 are more highly expressed in the frontal cortex compared to the entire cortex suggesting their potentially important role in this brain region [40]. Autism is a disorder that markedly affects executive function and high-order integration processes such as complex social interaction, associative thinking, and appropriate emotional reactions [61]. Given the higher expression of EP1 and EP4 receptors in the frontal cortex, it is possible these receptors play a role in higher functions, and may be dysregulated in autism contributing to the characteristic social deficits. Moreover, all of the EP receptors' mRNA was highly expressed in the medulla oblongata [40]. The medulla oblongata is a portion of the hindbrain that controls autonomic functions such as breathing, digestion and heart rate. This highlights the importance of EP receptors.

1.8. Prostaglandin E_2 and EP receptor signaling

The EP subtypes bind most potently to the prostaglandin PGE_2, with their signal transduction pathways being identified as G_i, G_s or G_q proteins **(Figure 2)**. EP1 receptor activation results in a release of intracellular calcium from inositol triphosphate (IP_3) via the G_q-phospholipase C (PLC) - IP_3 and activation of PKC. EP4 receptor has been associated with an increase in cAMP in cells, however with much less affinity than EP2 [62]. EP2 and EP4, are coupled to the stimulatory G_s protein and mainly mediate the increase in cAMP through activation of adenylate cyclase (AC) [63], [64], which in turn activates protein kinase A (PKA)

and mediates phosphorylation of cAMP response element binding the protein (CREB) transcription factor [54] (**Figure 2**). We recently also showed novel findings that it is also possible for EP4 signaling pathways to operate via G_i proteins as well [65]. Moreover, in response to PGE_2, EP2 and EP4 receptors demonstrate dissimilar patterns of desensitization and internalization [66]. The various EP3 receptor splice variants act to either inhibit or increase cAMP synthesis via G_i or G_s proteins, respectively. It has also been noted, however, that the EP receptors do not couple exclusively to the pathways described but often to more than one G protein and signal transduction pathway [48]. Ultimately PGE_2 activation of the EP receptors acts to regulate calcium levels, which will be discussed further below.

Short term PGE_2 exposure, due to environmental stimuli or drug exposure, may be detrimental to the developing embryo or nervous system since calcium homeostasis has been implicated in cellular functions such as differentiation and growth, membrane excitability, exocytosis, and synaptic activity [67]. Our lab has previously shown that misoprostol, a prostaglandin type E analogue, alters the calcium level in the cytosol via PKA-mediated pathway carried out by EP4 receptor signaling [65]. Tamiji and Crawford (2010) showed that blocking EP4 receptor or PI3K resulted in greater elevation of intracellular calcium in response to PGE_2 and misoprostol due to activation of the remaining EP receptors (EP1-3) [65]. Moreover, this suggests an unique involvement of the EP4 receptor in the inhibition of calcium intracellular levels in neuroblastoma (neuro-2a) cells.

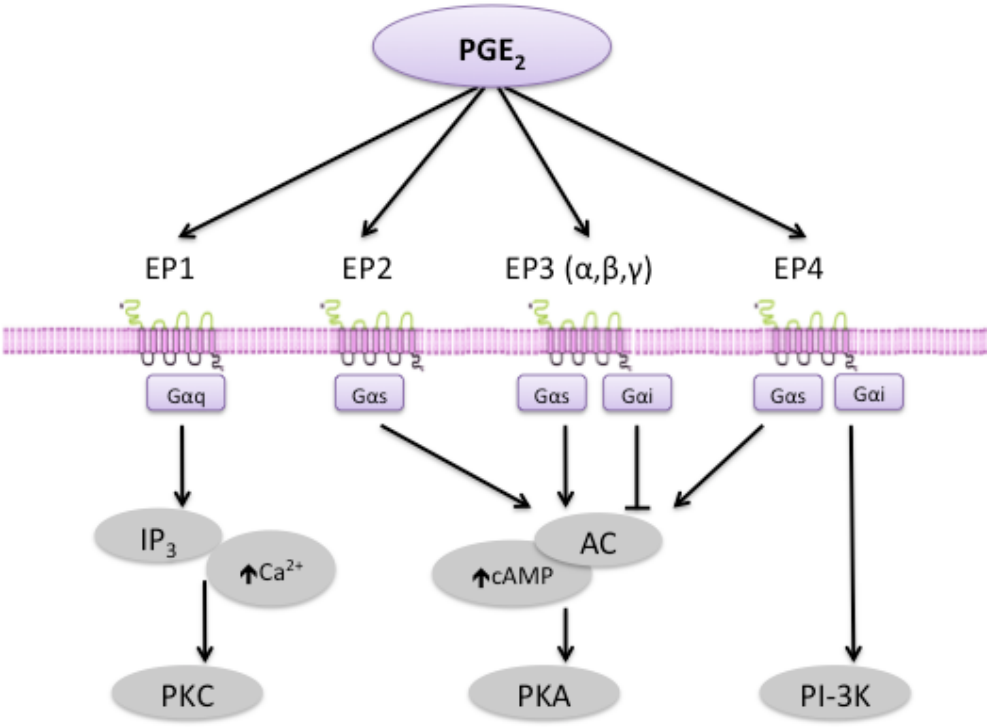

Figure 2. The PGE_2 signaling pathway. Stimulation of EP1-4 via PGE_2 leads to activation of PKC, PKA, or PI-3K via G_q, G_s, or G_i mechanisms. This figure has been adapted from Tamiji and Crawford (2010) [65] and Wong and Crawford (2014) [16].

1.9. Calcium signaling in the developing nervous system

Calcium plays a pivotal role in the early development of the brain [68]. Normally, calcium ions are key mediators to multiple cellular processes in early neuronal development including cell growth, differentiation, synaptic activity, gene expression, activity dependent signaling, and cell death [69]–[74]. In neurons, the level of calcium ions is very tightly regulated with a very low basal level (100 nanomolar (nM)) in the cytosol compared to up to 20 000-fold gradient in extracellular concentration (millimolar (mM)) [67], [75]–[77]. This allows minor perturbations or localized Ca^{2+} elevations to efficiently activate enzymes or neighboring ion channels that may result in profound pathological/physiological effects either short or long term [67], [78].

Environmental cues like growth factors and neurotransmitters modulate calcium homeostasis, which is a crucial component of neuronal development and signaling [69], [79]. Previous studies have revealed that the calcium oscillatory frequency (also referred to as transients), which is the influx and efflux of calcium, is regulated by neurotransmitters, growth factors and cytokines [80]–[82]. The calcium transients play equally important roles in the control of cell fate specification in the nervous system, cellular phenotypes and axon path finding [70], [83], [84]. During neural development *in vivo*, growth cone calcium transients are influenced and modulated by environmental factors like laminin, a suppressor molecule, expressed on axons, and stimulating molecules such as netrin which are expressed on the floor plate [28], [85], [86]. The growth cone is the motile tip of an axon that is responsible for responding to environmental guidance cues.

During neuronal differentiation in cultured mouse neural crest-derived cells, transient increases in intracellular calcium concentration ($[Ca^{2+}]_i$) occurs followed by a decline in spiking frequency as neurogenic activity declines [70]. This increase in calcium transient

activity and frequency was also observed in neocortical ventricular zone cells (neuronal precursors) [87]. Additionally, rapid calcium increases can induce differentiation of neuronal cells [88]. Calcium wave frequency, size and distance also increase in late neurogenesis [84], [89]. At later stages of development, regulation of expression of transcripts encoding cell adhesion molecules is partly achieved by calcium transients in cultured mouse DRG neurons [90]–[92]. Overall, calcium transients are fundamental in modulating neuronal differentiation, axonal extension, migration, gene transcription, and protein interactions during early neuronal development [69], [79].

1.10. Calcium localization in neurons

Calcium signaling in neurons is characterized by high spatial compartmentalization, which plays very different roles in information coding depending on the neuronal region affected, such as in the entire neuron versus localized signals in the growth cone [93]. It has been demonstrated both *in vitro* and *in vivo* that the global calcium increases usually control the production of neurotransmitters and maturation of potassium channels, while local calcium waves in the growth cone regulate neurite extension [70], [94], [95]. Decreased presynaptic calcium causes short-term depression, whereas elevated levels contribute to forms of synaptic enhancement like facilitation, augmentation, and post-tetanic potentiation [96], [97].

The calcium concentration in growth cones is regulated by both calcium influx through the plasma membrane and release from intracellular calcium stores [98], [99]. Neuronal growth cones maintain a baseline intracellular calcium concentration at the resting state, termed the resting $[Ca^{2+}]_i$ [100]. Neurons require high frequency, brief spiking along with intracellular calcium buffering in order to restrict calcium entry [101]. This calcium mediated signaling involves fluctuations of $[Ca^{2+}]_i$ above the resting level, which is observed as calcium

fluctuations and also referred to as transients. These $[Ca^{2+}]_i$ transients regulate motility *in vitro* and extracellular cues may modulate *in vivo* migration [94], [102]–[104].

1.11. Calcium's role in growth cones

Growth cone behaviours depend on various factors of calcium signaling including the global or local spatial distribution of calcium, the temporal dynamics of calcium changes, the resting $[Ca^{2+}]_i$, and effector molecules the growth cone may encounter, such as guidance cues. These oscillatory patterns and their spontaneous spiking frequency have been shown to regulate neurite outgrowth and migration [80], [103]. For example, it is the temporal $[Ca^{2+}]_i$ signaling pattern that regulates axon outgrowth and growth cone mobility [95], and localized $[Ca^{2+}]_i$ signaling serves as a directional cue for guidance of neurite extension [77], [105], [106] and branching [107]–[110].

It has previously been found when growth cones are exposed to stimuli that produce a large, sudden global $[Ca^{2+}]_i$ elevation, they usually slow down, stop, or retract in a calcium-dependent manner [100], [111], [112]. However, different neurons have optimal calcium ranges whereby lower and higher $[Ca^{2+}]_i$ concentrations slow outgrowth [104]. In *Xenopus*, localized calcium signals provide the intracellular directional cues for extension that is sufficient to initiate both attraction and repulsion of the growth cones [85].

By generating both global and local calcium signals, a growth cone could generate different turning responses under different environmental conditions during guidance [105]. In fact, the direction of growth cone steering is regulated by local $[Ca^{2+}]_i$ elevation in addition to the baseline $[Ca^{2+}]_i$ [85], [113], [114].The magnitude of each $[Ca^{2+}]_i$ elevation can cause both attraction or repulsion cues, depending the baseline $[Ca^{2+}]_i$ [85]. In contrast, other studies have also found that elevating $[Ca^{2+}]_i$ in growth cones can promote neurite outgrowth [115], [116].

This discrepancy in $[Ca^{2+}]_i$ regulation of extension length may be due to differences in the resting $[Ca^{2+}]_i$ level relative to its optimal resting level [100]. Growth cones have been shown to adapt to elevated baseline $[Ca^{2+}]_i$ conditions, which suggests that the Ca^{2+}-dependent targets that define the 'optimal range' can adjust their sensitivity or be down regulated [100], [104], [117].

1.12. Calcium signaling and disorders of the nervous system

Strict calcium regulatory mechanisms exist to ensure a low intracellular level that when altered may contribute to neuropathies. Disruption of calcium homeostasis influences both short and long term neuronal maturation and thus may promote pathological changes resulting in altered network organization [68].

Neurodegenerative disorders have been linked to calcium dis-homeostasis in different regions of the brain and cellular compartments including Huntington's disease, cerebellar ataxia, Alzheimer's disease, and Parkinson's disease[118]. For example, increases in $[Ca^{2+}]_i$ of substansia nigra pars compacta dopaminergic neurons in Parkinson's Disease have been attributed to slow, broad Ca^{2+} spiking and a lack of intrinsic buffering [119].

Calcium dysregulation is also present in autism. Mutations in genes encoding L- and T-type voltage-gated channels have been identified in individuals with autism [120]. A member of the novel P5 subfamily of transporters involved in calcium transport across biological membranes, ATP13A4, has also been implicated in autism [121]. Moreover, calcium dis-homeostasis and abnormal PGE_2 levels have been implicated in autism [16], [121]. Excessive calcium levels have been shown to be responsible for boosting mitochondrial aspartate/glutamate carrier activity, mitochondrial metabolism and oxidative stress in post-mortem temporocortical gray matter of autistic brains [122].

1.13. Objectives

The overall objective of my Master of Science research is to investigate the molecular basis of PGE_2 signaling in the early development of the nervous system using an *in vitro* experimental model system. More specifically, I will be looking at the effect of increased levels of PGE_2 on (1) mobilization of the intracellular calcium levels in differentiated neuronal cells (Chapter 2, Appendix A), (2) subcellular localization of the EP4 receptor in undifferentiated NE-4C stem cells and differentiated NE-4C neuronal cells (Chapter 3, Appendix B), and (3) expression of specific early developmental Wnt (wingless-type MMTV integration site family)-target genes in the mouse model after prenatal exposure to PGE_2 (Appendix C).

1.14. Hypotheses

The PGE_2 signaling pathway plays an important role in the early development of the nervous system. My current study stems from our previous findings in our lab that show that PGE_2 involvement in regulation of calcium homeostasis in mouse neuroblastoma (Neuro-2a) cells. We showed that increased level of PGE_2 resulted in an increase in intracellular calcium levels in differentiated neuronal Neuro-2a cells [65], and a decrease in neurite extension length [40]. Our lab also determined that the PGE_2 dependent intracellular calcium regulation occurs via an EP4-PI3K inhibitory mechanism [65]. In this study I use mouse neuroectodermal (NE-4C) stem cells as an experimental model of early development (see section 1.15). These cells are a good model for neuronal stem cells as they are also capable of differentiation to neurons and astrocytes.

Based on our previous findings I hypothesize that:

1. **Increased level of PGE_2 can alter calcium homeostasis in differentiating NE-4C cells. More specifically, I hypothesize that PGE_2 can**
    a. **alter basal cytosolic and growth cone calcium level,**
    b. **affect growth cone calcium fluctuation amplitude**
    c. **influence length of neurite extension**

(Study 1 – Submitted manuscript in Chapter 2 and additional data in Appendix A)

We have also previously shown that the EP4 receptor can regulate calcium homeostasis in neuronal Neuro-2a cells via PI3K inhibitory mechanism [65]. In addition, other studies show that the functional role of the PGE_2/EP4 signaling may depend on subcellular localization of the EP4 receptor [60]. Given the previous findings I hypothesize that

2. **Elevated levels of PGE_2 can induce subcellular trafficking of the EP4 receptor from it's normal location in the Golgi apparatus to**
    a. **the plasma membrane of undifferentiated NE-4C stem cells and NE-4C differentiating neuronal cells, and**
    b. **to the growth cones of differentiating NE4C neuronal cells**

(Study 2 – Submitted manuscript in **Chapter 3** and additional data in **Appendix B**)

Additionally, our lab has shown first evidence of crosstalk between the PGE_2 signaling and developmental Wnt pathways in the nervous system using NE-4C stem cells [3]. I contributed to a study conducted by a PhD student in our lab using genetically identical mouse off-springs (C57BL/6) prenatally affected with higher level of PGE_2 during the critical developmental period. The objective was to confirm that the interaction between PGE_2 and

Wnt also exists *in vivo*. Moreover, we intended to answer a fundamental question of whether external environmental risk factors (that are capable of increasing the level of PGE_2 during development) (i) can affect expression of early developmental genes, and (ii) if the expression varies between genetically identical pups. The general hypothesis for this study is that

3. Prenatal exposure to higher PGE_2 level can influence Wnt pathway *in vivo* via affecting expression of Wnt-target genes differently in each genetically identical off-spring (I characterized two genes *Wnt3a* and *Fosl1)*

(Study 3 – Manuscript in preparation as co-author in **Appendix C**)

1.15. Experimental model system

For *in vitro* studies, I will use NE-4C stem cells as my experimental model **(Chapter 2, 3, Appendices A, B)**. NE-4C cells were obtained from American Tissue Culture Collection (ATCC), which were cloned from primary brain cell cultures prepared from the for- and midbrain vesicles of 9-day old transgenic mouse embryos lacking functional p53 tumor suppressor protein. These NE-4C cells divide continuously and are able to differentiate into distinct neural cell types upon appropriate induction [123]. For *in vivo* studies, I will use mRNA collected from embryonic mouse brain as my experimental model **(Appendix C)**.

CHAPTER 2. LIPID MEDIATOR PROSTAGLANDIN E2 ALTERS INTRACELLULAR CALCIUM TRANSIENTS IN DIFFERENTIATED NEUROECTODERMAL STEM CELLS

Elsevier Editorial System(tm) for Molecular and Cellular Neuroscience Manuscript Draft
Manuscript Number: MCN-15-82
Title: Lipid mediator prostaglandin E2 alters intracellular calcium transients in differentiated neuroectodermal stem cells
Article Type: Regular Article
Keywords: Prostaglandin E2; Differentiated neuroectodermal stem cells; Neurons; Growth cones; Calcium fluctuations; Neurite extension; Autism Spectrum Disorders
Corresponding Author: Dr. Dorota Anna Crawford, PhD
Corresponding Author's Institution: York University
First Author: Jennilee M Davidson
Order of Authors: Jennilee M Davidson; Hongyan Li; Dorota Anna Crawford, PhD

Note: Jennilee Davidson performed all experiments, collected and analyzed all data, and wrote the manuscript.

2.1. Abstract

Lipid mediator prostaglandin E_2 (PGE_2) is an endogenous signaling molecule that plays an important role during early development of the nervous system. Abnormalities in the PGE_2 signaling pathway due to genetic or environmental factors have been associated with neurodevelopmental disorders. Previous studies have shown that stimuli that can affect PGE_2 levels such as infections, inflammations, toxic chemicals or exposure to some drugs all are associated with Autism Spectrum Disorders. Our previous study shows that higher PGE_2 levels can affect migration, proliferation and differentiation of neuroectodermal (NE-4C) stem cells through cross-talk with another pathway crucial in early development, called the canonical Wnt signaling pathway. In this study we use ratiometric fura-2AM calcium imaging to show that increased concentrations of PGE_2 elevates basal cytosolic and growth cone intracellular calcium levels in NE-4C stem cells differentiated to neurons. PGE_2 also increased the minimum and maximum level as well as amplitude of calcium fluctuation in the neuronal growth cones. Furthermore, we found that PGE_2 affected the neurite extension length in cells with the highest growth cone calcium amplitude. In summary, our results show that PGE_2 may adversely impact intracellular calcium dynamics in differentiated neuronal cells and affect early development of the nervous system.

2.2. Introduction

Proper development and function of the nervous system relies greatly on the release of bioactive lipid metabolites such as prostaglandin E_2 (PGE_2) from membrane phospholipids [31]. PGE_2 is the major lipid mediator critical in the developing nervous system. During embryonic brain development, PGE_2 is involved in synaptic plasticity, dendritic spine formation, refining of mature neuronal connections, and cell survival and death [124]–[126]. PGE_2 signaling also regulates important biological functions such as the inflammatory response, fever, pain and sleep, whereby elevation of endogenous PGE_2 levels has been found during inflammatory response and oxidative stress [16], [127], [128]. Abnormalities in the PGE_2 signaling pathway due to genetic or environmental causes have been implicated in Autism Spectrum Disorders (ASD) [15], [16].

PGE_2 level in the brain is regulated by the activity of phospholipase A_2 and cyclooxygenase -1 or -2, (PLA_2, COX-1, COX-2) enzymes that regulate its release from phospholipid membranes and synthesis, respectively [31]. PGE_2 exerts its diverse effects through four G protein-coupled E-prostanoid receptors designated EP1-4 [129]. EP1 receptor activation results in a release of intracellular calcium from inositol triphosphate (IP_3) via the Gq-PLC-IP_3 and activation of PKC. The remaining receptors EP2, EP3 and EP4 mainly mediate the increase of cAMP via Gs-cAMP/PKA pathway. EP4 receptor activation can also result in the decrease or increase of Ca^{2+} levels via G_i or G_s, respectively [65]. We have previously shown that changes in PGE_2 level can cause neurite retraction and affect calcium transients in differentiated neuroblastoma (Neuro-2a) cells [40], [65]. We have also determined that increased PGE_2 level can alter migration, proliferation and differentiation of neuroectodermal stem (NE-4C) cells through cross-talk with the Wnt pathway, which is crucial for early brain development [3].

This study investigates the effect of PGE_2 on Ca^{2+} dynamics in differentiated NE-4C cells used here as an experimental model system for early neurogenesis [130], [131]. Our results show that PGE_2 elevates the cytosolic calcium level and the calcium fluctuations in the growth cones. This study adds further insight into the contribution of PGE_2 in regulation of calcium dynamics in neuronal cells during early developmental events such as differentiation.

2.3. Materials and Methods

2.3.1. Cell Culture

Mouse NE-4C cells were obtained from American Tissue Culture Collection (ATCC) and grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1X penicillin-streptomycin mixture (Invitrogen). Cells were maintained in an incubator containing 5% CO2 at 95% humidity at 37°C. Cells were plated on 0.01% poly-L-lysine (Sigma) coated 100mm culture plate (BD Falcon) and were subcultured at a 1:10 ratio. Supplemented MEM was changed every 2 days.

2.3.2. Differentiation and PGE_2 treatment

Differentiation of NE-4C cells was induced on day 0 using Neurobasal media (NBM; supplemented with L-glutamate, 1× Pen Strep, and 1 × B-27; Invitrogen) in poly-L-lysine (Sigma, MW 70000–150000 kDa) pre-coated 100mm culture plates. By day 6, neurospheres (clusters of neural stem cells) (unpublished results) were mechanically dissociated, seeded onto glass chamber slides (BD Falcon) and differentiated until day 12. Supplemented differentiating media was replaced every 2 days. Pou Class 5 homeobox 1 (*Pou5f1*) and Tubulin beta-3 chain protein (*Tubb3*) were used on day 12 as stem cell and late neuronal cell markers to confirm

differentiation using PCR. PCR primers are as follows: *Pou5f1* forward 5'-ctggctaagcttccaagggc-3' and reverse 5'-ccagggtctccgatttgcat-3'; *Tubb3* 5'-agcagctacttcgtggagtg -3' and reverse 5'- gggcttccgattcctcgtca -3'. As compared to day 0, which showed presence of the *Pou5f1* marker, we confirmed that on day 12 only the *Tubb3* marker was detected (data not shown). On day 12 NE-4C cells were treated with 0.1 micromolar (μM), 1 μM, and 10 μM PGE_2 (Sigma) for 3 and 24 hours (3 h and 24 h). Control and treatment groups had media changed every 2 days.

2.3.3. Calcium Imaging

Real-time calcium imaging was performed using the ratiometric method of intracellular calcium ($[Ca^{2+}]_i$) measurement with fura-2-acetoxymethyl ester (fura-2AM, Invitrogen) calcium indicator with a Nikon Eclipse Ti-E microscope as we previously described [40], [121]. To ensure suitable physiological conditions while completing analysis, conditions were replicated from our previous experiments. The fluorescence ratio of 340/380 in fura-2AM loaded (Ca^{2+}-bound to Ca^{2+}-free fura-2) in differentiated NE-4C cells was measured for a minimum of 120 seconds to establish a stable baseline level prior to determining the R_{max} and R_{min} value. The ratio values were then converted to calcium concentration using the following formula as we previously described [121].

2.3.4. Cytosolic and growth cone calcium level in differentiated NE-4C cells

On day 12, the basal 340/380 ratio value was measured for 60 seconds. We determined 60 seconds was a sufficient recording time to represent a stable baseline of calcium level based on our preliminary recordings, whereby the baseline was recorded for 2 minutes, followed by a further 10 minutes once the R_{max} was determined. The cytosolic and growth cone regions were

selected using NIS Elements software (Nikon). Mean basal calcium levels were found by calculating the mean of 340/380 ratio values obtained at each 5-second time intervals for 60 seconds. Global changes in growth cone calcium fluctuation dynamics were determined by measuring the amplitude, maximum (max) and minimum (min) 340/380 ratio values in the entire growth cone. The max and min values of the 340/380 ratios were averaged ($\Delta 340/380_{max}$ and $\Delta 340/380_{min}$) in untreated and PGE_2-treated groups. The $\Delta 340/380_{max}$ PGE_2-treated cells were compared to the untreated cells followed by the same assessment for $\Delta 340/380_{min}$. Next, the differences between the $\Delta 340/380_{max}$ and $\Delta 340/380_{min}$ ($\Delta 340/380_{max-min}$) was obtained in the PGE_2-treated cells and compared to the $\Delta 340/380_{max-min}$ in the untreated cells to determine the change in growth cone fluctuation amplitude. A minimum of 3 independent experiments were completed for each treatment group. The total number of cells and growth cones (N), and original 340/380 ratio values were used for statistical analysis.

2.3.5. Data Analysis and Statistics

Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a Tukey post-hoc comparison test. Differences were considered statistically significant at *$p<0.05$, **$p<0.01$, or ***$p<0.0001$.

2.4 Results

2.4.1. Effects of PGE_2 on cytosolic intracellular free calcium concentration

In this study we measured cytosolic and growth cone intracellular calcium concentration ($[Ca^{2+}]_i$) using ratiometric real-time calcium imaging as previously described [66, 124]. PGE_2 concentrations of 0.1, 1 and 10 μM were added on day 12 of differentiation (see methods 2.3.2.) for 3 h and 24 h exposure. The R_{min} and R_{max} values in untreated NE-4C cells were 0.33 and 4.25, respectively. These values were applied to the calculation of the 340/380 ratios and the $[Ca^{2+}]_i$ [124]. The basal cytosolic $[Ca^{2+}]_i$ for differentiated NE-4C cells (serum free media (SFM) condition) was 80.5 nanomolar (nM) (N = 270). This corresponds to values normally found in neuronal cells [78, 79]. All concentrations of PGE_2 treatment used for 3 h and 24 h on day 12 of differentiation resulted in significantly increased mean basal cytosolic $[Ca^{2+}]_i$ **(Figure 3A)**. Exposure to 0.1, 1 and 10 μM PGE_2 for 3 h increased the mean basal cytosolic $[Ca^{2+}]_i$ to 187, 184, and 134 nM, respectively. This corresponds to a significant 132% (N = 95, $p = 4.44 \times 10^{-13}$), 129% (N = 91, $p = 4.44 \times 10^{-13}$) and 66% (N = 144, $p = 4.44 \times 10^{-13}$) increase **(Figure 3A)**. Exposure to the same concentrations of PGE_2 for 24 h increased the mean basal cytosolic $[Ca^{2+}]_i$ to 173, 165, and 141 nM, respectively. This corresponds to a significant 114% (N= 79, $p = 2.63 \times 10^{-13}$), 105% (N = 76, $p = 2.63 \times 10^{-13}$) and 76% (N = 39, $p = 2.63 \times 10^{-13}$) increase in $[Ca^{2+}]_i$ **(Figure 3A)**.

2.4.2. Effects of PGE_2 on global growth cone intracellular free calcium concentration

Spontaneous $[Ca^{2+}]_i$ transients in growth cones are important for early neuronal development such as axonal outgrowth or early synapse formation [85], [132], [133]. Therefore, we measured whether PGE_2 treatment will have an effect on baseline $[Ca^{2+}]_i$ level in the whole growth cone in differentiated NE-4C cells. The mean basal growth cone $[Ca^{2+}]_i$ level for untreated differentiated NE-4C cells (SFM) was 501 nM (N = 247). Growth cone calcium levels in PGE_2 treated cells were compared to the untreated group. Similar to the cytosolic results, there was a significant increase in the average growth cone calcium levels in all PGE_2 concentration used. Upon 0.1, 1 and 10 μM PGE_2 treatment for 3 h, the basal growth cone $[Ca^{2+}]_i$ increased to 805, 1010, and 617 nM, respectively. This represents a 60% (N = 149, $p = 5.18 \times 10^{-13}$), 101% (N = 123, $p = 5.18 \times 10^{-13}$) and 23% (N = 123, $p = 4.88 \times 10^{-3}$) increase, respectively **(Figure 3B)**. Exposure to the same concentrations of PGE_2 for 24 h also increased $[Ca^{2+}]_i$ to 808, 697, and 746 nM, respectively. This corresponds to a 61% (N = 118, $p = 6.06 \times 10^{-13}$), 39% (N = 125, $p = 2.18 \times 10^{-7}$) and 49% (N = 97, $p = 2.14 \times 10^{-11}$) increase in mean basal growth cone $[Ca^{2+}]_i$ **(Figure 3B)**.

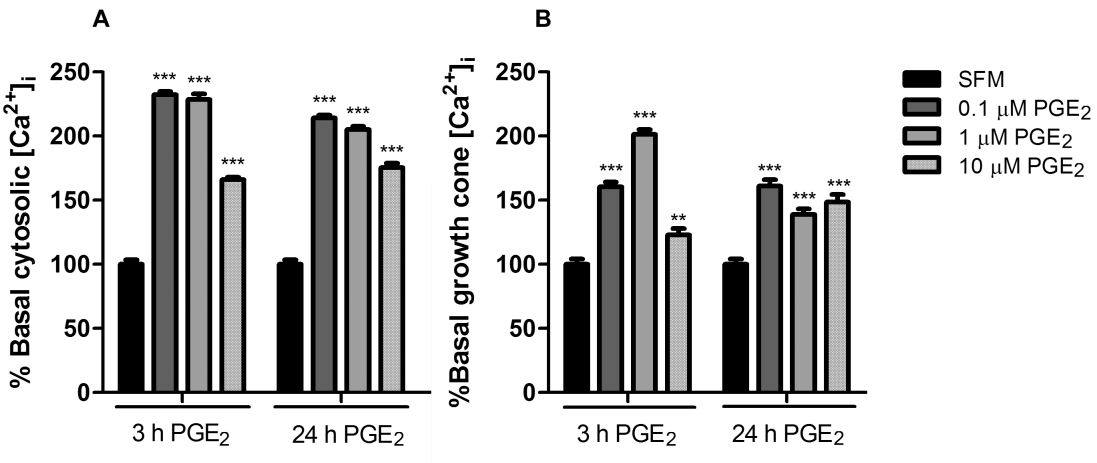

Figure 3. Exposure time dependent increase in basal (A) cytosolic and (B) growth cone $[Ca^{2+}]_i$ in response to PGE_2 exposure. **A.** Treatments with 0.1, 1 and 10 μM PGE_2 for 3 h elevated $[Ca^{2+}]_i$ by 132% (N = 95), 129% (N = 91) and 66% (N = 144); for 24 h elevated $[Ca^{2+}]_i$ by 114% (N = 79), 105% (N = 76) and 76% (N = 39). **B.** Treatments with 0.1, 1 and 10 μM PGE_2 for 3 h elevated $[Ca^{2+}]_i$ by 60% (N = 149), 101% (N = 123) and 23% (N = 123), respectively; for 24 h elevated $[Ca^{2+}]_i$ by 61% (N = 118), 39% (N = 125) and 49% (N = 97), respectively. The y-axes represents the % change in $[Ca^{2+}]_i$ (nM) and x-axes depict the time of PGE_2 exposure, with all tested concentrations grouped by exposure time. Results represent a minimum of three independent experiments. $[Ca^{2+}]_i$ = intracellular calcium concentration; PGE_2 = prostaglandin E_2; SFM = serum free media; **p<0.01; ***p<0.0001.

2.4.3. PGE_2 affects intracellular minimum and maximum $[Ca^{2+}]_i$ fluctuation in growth cones

Minimum ($[Ca^{2+}]_{i\,(min)}$) and maximum ($[Ca^{2+}]_{i\,(max)}$) spontaneous fluctuation levels in growth cones are physiologically important in neuronal development [70], [83]–[85]. In our study we found that exposure to various concentrations of PGE_2 for 3 h or 24 h adversely impacts differentiated NE-4C growth cone $[Ca^{2+}]_i$ fluctuation. **Figure 4 A** and **B** depicts individual traces of $[Ca^{2+}]_i$ fluctuation. There is a clear difference in the $[Ca^{2+}]_{i\,(min)}$ and $[Ca^{2+}]_{i\,(max)}$ values and amplitude of fluctuation with 0.1, 1 and 10 μM PGE_2 treatment as compared to the control condition (SFM). We quantified the average minimum ($[Ca^{2+}]_{i\,(min)}$) and maximum ($[Ca^{2+}]_{i\,(max)}$) fluctuation peaks across growth cones. All values of PGE_2-treated groups were compared to the $[Ca^{2+}]_{i\,(min)}$ and $[Ca^{2+}]_{i\,(max)}$ of the untreated group (SFM), which was 146 nM (N = 247) and 1479 nM (N = 247), respectively. Differentiated NE-4C cells exposed to 3 h PGE_2 show a significant increase in $[Ca^{2+}]_{i\,(min)}$ by 0.1 μM of PGE_2, whereas after 24 h PGE_2 exposure all concentrations tested resulted in significant increases. After 3 h 0.1, 1 and 10 μM PGE_2 treatments, the growth cone $[Ca^{2+}]_{i\,(min)}$ were 220 nM (N = 149, $p = 3.44 \times 10^{-13}$), 178 nM (N = 123, $p = 2.19 \times 10^{-1}$) and 147 nM (N = 123, $p = 9.90 \times 10^{-1}$), respectively. After 24 h, resulting $[Ca^{2+}]_{i\,(min)}$ increased to 223 nM (N = 118, $p = 3.02 \times 10^{-13}$), 204 nM (N = 125, 7.71×10^{-10}) and 182 nM (N = 97, 3.00×10^{-3}), respectively (Figure 4C). PGE_2 also significantly increased the $[Ca^{2+}]_{i\,(max)}$ in all conditions **(Figure 4C)**. After exposure to 0.1, 1 and 10 μM PGE_2 concentrations for 3 h the $[Ca^{2+}]_{i\,(max)}$ reached 2308 nM (N = 149, $p = 4.56 \times 10^{-17}$), 3350 nM (N = 123, $p = 5.35 \times 10^{-13}$) and 2183 nM (N = 123, $p = 1.31 \times 10^{-2}$), respectively. After 24 h exposure to the same concentration the $[Ca^{2+}]_{i\,(max)}$ were 2189 nM (N = 118, $p = 1.33 \times 10^{-9}$), 1917 nM (N = 125, $p = 7.67 \times 10^{-3}$) and 2500 nM (N = 97, $p = 2.11 \times 10^{-8}$), respectively.

Overall, we determined that application of various concentrations of PGE_2 to differentiated NE-4C cells results in significant changes in the level of $[Ca^{2+}]_{i\ (min)}$ and $[Ca^{2+}]_{i\ (max)}$ fluctuations.

2.4.4. PGE_2 treatment results in global changes of growth cone $[Ca^{2+}]_i$ amplitude

The amplitude (or range) of calcium fluctuation in growth cones is also known to play critical roles in early neuronal development [95]. We determined that the $[Ca^{2+}]_i$ amplitude in growth cones (see methods) of differentiated NE-4C cells was 1333 nM (N = 247) **(Figure 4D)**. In differentiated cells exposed to PGE_2, the calcium fluctuation amplitude was significantly increased in all concentrations used **(Figure 4D)**. The $[Ca^{2+}]_i$ amplitude after 0.1, 1 and 10 μM PGE_2 exposure for 3 h was 2088 nM (N = 149, $p = 1.00 \times 10^{-5}$), 3172 nM (N = 123, $p = 8.19 \times 10^{-13}$) and 2037 nM (N = 123, $p = 1.39 \times 10^{-2}$), which represents an increase by 82%, 137% and 54%, respectively. Similarly, the $[Ca^{2+}]_i$ amplitude after 24 h PGE_2 exposure was 1966 nM (N = 118, $p = 7.62 \times 10^{-8}$), 1714 nM (N = 125, $p = 3.88 \times 10^{-2}$) and 2318 nM (N = 97, $p = 1.95 \times 10^{-7}$), respectively, in response to the same concentrations of PGE_2. This represents 87%, 40% and 93% increase in calcium fluctuation for the corresponding PGE_2 concentrations. Overall, these results demonstrate that PGE_2 exposure has a significant effect on the range of growth cone calcium dynamics in differentiated NE-4C cells.

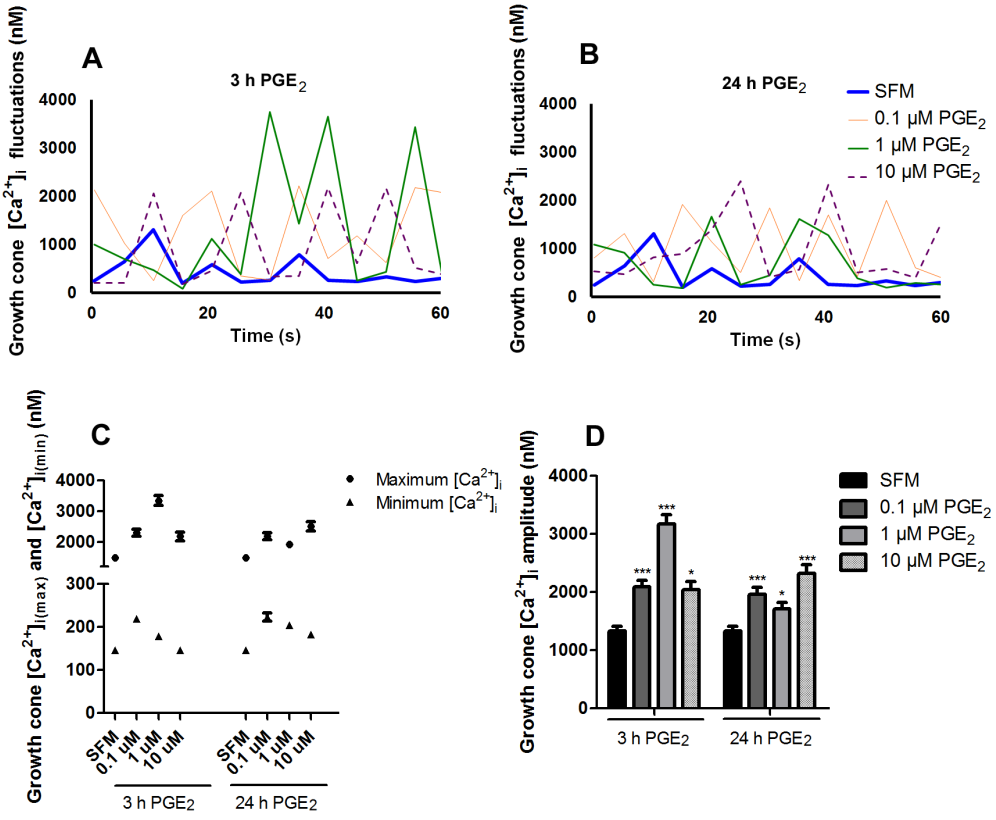

Figure 4. PGE_2 exposure time dependent change in the dynamics of growth cone fluctuation $[Ca^{2+}]_i$ levels in response to PGE_2 exposure. A, B. Traces of spontaneous intracellular $[Ca^{2+}]_i$ fluctuations in migrating growth cones after 3 h and 24 h PGE2 exposure, respectively. **C.** Minimum $[Ca^{2+}]_i$ levels increased after 3 h of 0.1 and 1 µM to 220 nM (N = 149) and 178 nM (N = 123) but did not change with 10 µM being 147 nM (N = 123), respectively, and after 24 h increased to 223 nM (N = 118), 204 nM (N = 125) and 182 nM (N = 97). Maximum $[Ca^{2+}]_i$ levels increased after 3 h to 2308 nM (N = 149), 3350 nM (N = 123) and 2183 nM (N = 125), and after 24 h to 2189 nM (N = 118), 1917 nM (N = 125) and 2500 nM (N = 97). **D.** Fluctuation amplitude increased from 0.1, 1, and 10 µM after 3 h to 2088 nM

(N = 149), 3172 nM (N = 123) and 2037 nM (N = 123), and after 24 h to 1966 nM (N = 118), 1714 nM (N = 125) and 2318 nM (N = 97), respectively. The y-axes represent the $[Ca^{2+}]_i$ (nM) and x-axes depict **A, B** the measurement time (seconds) taken at 5-second intervals, and **C, D** the time of PGE_2 exposure, with all tested concentrations grouped by exposure time. Results represent a minimum of three independent experiments. $[Ca^{2+}]_i$ = intracellular calcium concentration; PGE_2 = prostaglandin E_2; Minimum and maximum $[Ca^{2+}]_i$ are shown by triangles and circles, respectively; *p<0.05; ***p<0.0001.

2.4.5. PGE_2 treatment affects neurite extension in differentiated NE-4C cells

We also measured whether PGE_2 had an effect on neurite extension length in differentiated NE-4C cells on day 12 since neuronal outgrowth requires proper growth cone calcium dynamics [109]. The mean neurite extension length of untreated cells was 32.6 μm (N = 342). After 3 hours exposure to 0.1, 1 and 10 μM PGE_2 the extension lengths were 38.5 μm (N = 264), 24.1 μm (N = 197), and 39.8 μm (N = 210), respectively. Significantly shorter extension length was detected for the 1 μM PGE_2 treatment ($p = 0.03$) **(Figure 5)**. Interestingly, we also detected the highest $[Ca^{2+}]_i$ amplitude after 3 h treatment with the 1 μM PGE_2 concentration **(Figure 4D)**. After 24 h of PGE_2 exposure the extension lengths were 35.1 μm (N = 323), 38.2 μm (N = 377), and 52.0 μm (N = 241), respectively with significantly increased extension length by 10 μM PGE_2 ($p = 8.11 \times 10^{-10}$) **(Figure 5)**. The 10 μM PGE_2 treatment also showed the highest $[Ca^{2+}]_i$ amplitude change after 24 h **(Figure 4D)**. These results demonstrate a PGE_2 time- and concentration- dependent change in neurite extensions.

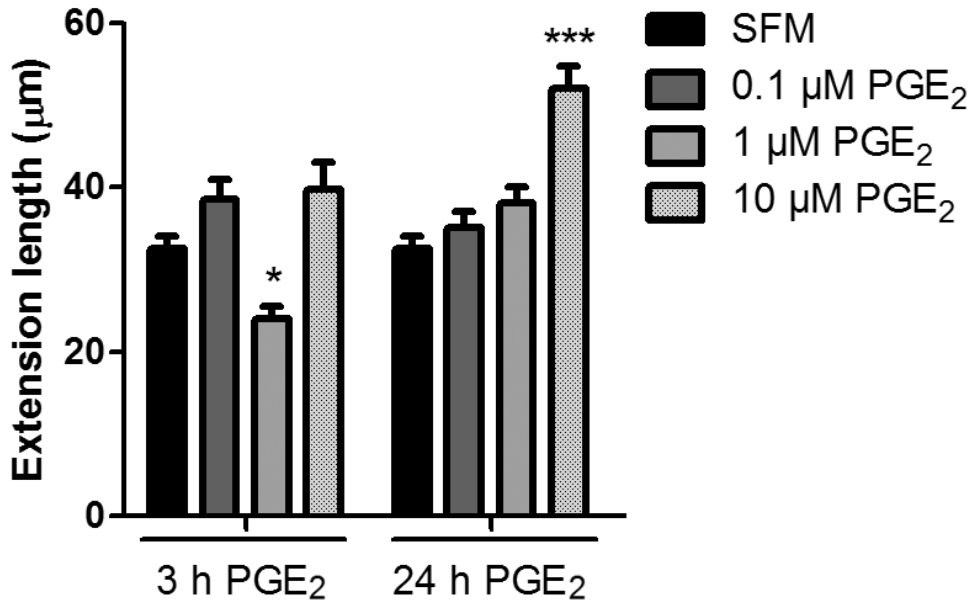

Figure 5. PGE_2 exposure time- and concentration- dependent change in neurite extension length. Mean neurite extension length in the SFM group was 32.6 μm (N = 342) and after 3 h PGE_2 decreased from 1 μM PGE_2 to 24.1 μm (N = 197), but did not change by 0.1 and 10 μM being 38.5 μm (N = 264), 39.8 μm (N = 210) respectively, and after 24 h did not change by 0.1 and 1 μM but increased by 10 μM being 35.1 μm (N = 323), 38.2 μm (N = 377), and 52.0 μm (N = 241), respectively. The y-axis represents the extension length (μm) and x-axis depicts the time of PGE_2 exposure, with all tested concentrations grouped by exposure time. Results represent a minimum of three independent experiments. PGE_2 = prostaglandin E_2; SFM = serum free media; *$p<0.05$; ***$p<0.0001$.

2.5. Discussion

This study provides molecular evidence that the lipid mediator prostaglandin E_2 can influence calcium homeostasis in differentiated neuroectodermal (NE-4C) stem cells. We show that PGE_2 can alter the cytosolic and growth cone $[Ca^{2+}]_i$ dynamics, and alter neurite extension length in a time- and concentration- dependent manner, indicating that PGE_2 may have diverse effects on the developing nervous system.

PGE_2 plays a significant role in many events during early neuronal development such as synaptic plasticity, dendritic spine formation, refining of mature neuronal connections, and cell survival and death [124]–[126]. Our previous reviews discuss the abnormalities in the PGE_2 signaling pathways due to genetic or environmental causes that leads to ASD [15], [16]. Many environmental risk factors such as infections, inflammation or misoprostol (prostaglandin E analogue and a drug used for termination of pregnancy) can affect the level of prenatal PGE_2 and result in pathologies of the nervous system [15], [16]. Our previous *in vitro* studies in mouse neuroblastoma (Neuro-2a) cells already showed that an elevated level of PGE_2 and misoprostol alter cytosolic Ca^{2+} regulation in differentiated Neuro-2a cells [40]. Defects in calcium regulation have been previously linked to neurodevelopmental disorders such as ASD [121], [122], [134] or neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and Huntington's disease [119], [135]–[137].

Considering the pivotal role of calcium in the early brain development [68], in this study we investigated the effect of PGE_2 on Ca^{2+} homeostasis in differentiated neuroectodermal (NE-4C) stem cells as a model for early neurogenesis [130]. Normally, Ca^{2+} ions are key mediators to multiple cellular processes in early neuronal development including cell growth, differentiation, synaptic activity, gene expression, activity dependent signaling, and cell death [69]–[74]. In neurons, the level of Ca^{2+} ions is very tightly regulated with a very

low basal level in the cytosol compared to extracellular concentration [85]. Any changes in frequency and amplitude of Ca^{2+} transients in the cytosol can affect gene expression [72], [73], [138], [139]. In this study we show that exposure to various concentrations of PGE_2 causes a significant increase in mean basal cytosolic $[Ca^{2+}]_i$ in differentiated NE-4C cells. Given the importance of Ca^{2+} in major developmental processes and its concentration-sensitive function, disrupted $[Ca^{2+}]_i$ due to changes in the PGE_2 level could lead to altered gene expression. In fact, we have already shown in previous studies that in NE-4C cells, higher PGE_2 concentrations affect expression of genes that belong to a major pathway involved in early development called the Wnt pathway and consequently alter Wnt-dependent cell proliferation, migration and differentiation [3].

A large body of evidence indicates that growth cone $[Ca^{2+}]_i$ has diverse roles in the control of axonal growth and guidance. During development, Ca^{2+} signals are involved in the control of elongation, orientation and arrest of growth cones in neurite extension, leading to the establishment of neuronal networks [109], [140], [141]. We show that PGE_2 significantly increases the mean basal Ca^{2+} levels globally in the growth cones of differentiated NE-4C cells for all tested concentrations. Since axon guidance via growth cone turning is heavily dependent on Ca^{2+} concentrations [85], [132], [133], it is feasible that abnormal PGE_2 signaling could contribute to underlying brain pathologies through changes in growth cone Ca^{2+} during early neuronal development.

Calcium signaling in neurons is characterized by high spatial compartmentalization, which plays very different roles in information coding depending on the neuronal region affected, such as in the entire neuron versus localized signals in the growth cone [93]. Calcium concentration in growth cones is regulated by both Ca^{2+} influx through the plasma membrane and release from intracellular Ca^{2+} stores [98], [99]. Neuronal growth cones maintain a

baseline intracellular Ca^{2+} concentration at the resting state, termed the resting $[Ca^{2+}]_i$ [100]. Calcium mediated signaling involves fluctuations of $[Ca^{2+}]_i$ above the resting level, which can be observed as Ca^{2+} fluctuations. Interestingly, we found that PGE_2 significantly increases the basal $[Ca^{2+}]_i$ fluctuations in growth cones of NE-4C cells.

Growth cone behaviours also depend on various factors of Ca^{2+} signaling including the global or local spatial distribution of Ca^{2+}, the temporal dynamics of Ca^{2+} changes, the resting $[Ca^{2+}]_i$, and effector molecules the growth cone may encounter, such as guidance cues. For example, temporal $[Ca^{2+}]_i$ signaling regulates axon outgrowth and growth cone mobility [95], and localized $[Ca^{2+}]_i$ serves as a directional cue for guidance of neurite extension [77], [105], [106] and branching [107]–[110]. Given the importance of Ca^{2+} signaling in determining growth cone behaviour, we assessed how PGE_2 affects various components of the Ca^{2+} transients.

By generating both global and local Ca^{2+} signals, a growth cone could generate different turning responses under different environmental conditions during guidance [105]. In fact, the direction of growth cone steering is regulated by local $[Ca^{2+}]_i$ elevation in addition to the baseline $[Ca^{2+}]_i$ [85], [113], [114].The magnitude of each $[Ca^{2+}]_i$ elevation can cause both attraction or repulsion cues, depending the baseline $[Ca^{2+}]_i$ [85]. In this study we found that higher level of PGE_2 caused an increase in the $[Ca^{2+}]_{i(min)}$ and $[Ca^{2+}]_{i\ (max)}$levels. PGE_2 – dependent alterations in the $[Ca^{2+}]_{i(min)}$ and $[Ca^{2+}]_{i(max)}$ fluctuations could affect activation of signaling molecules and result in abnormal growth cone turning [85].

Calcium signals usually have an oscillatory pattern, and their spontaneous spiking frequency has been shown to regulate neurite outgrowth and migration [80], [103]. It has previously been found when growth cones are exposed to stimuli that produce a large, sudden global $[Ca^{2+}]_i$ elevation, they usually slow down, stop, or retract in a Ca^{2+}-dependent manner

[100], [111], [112]. We found that all tested concentrations of PGE_2 elevated the basal amplitude of the Ca^{2+} fluctuation, whereby the greatest elevation in Ca^{2+} amplitude after 3h of PGE_2 exposure corresponded to a decrease in neurite extension length, consistent with our previous findings [40]. This is also consistent with the idea that Ca^{2+} serves as a negative regulator of neurite extension [102], [140] both in culture [102], [103], [140] and *in vivo* [95]. However, other studies have found that elevating $[Ca^{2+}]_i$ in growth cones can also promote neurite outgrowth [115], [116]. Interestingly, after 24h PGE_2 exposure, we found the greatest $[Ca^{2+}]_i$ amplitude corresponded to an increase in extension length. This discrepancy in $[Ca^{2+}]_i$ regulation of extension length may be due to differences in the resting $[Ca^{2+}]_i$ level relative to its optimal resting level [100]. Growth cones have been shown to adapt to elevated baseline $[Ca^{2+}]_i$ conditions, which suggests that the Ca^{2+}-dependent targets that define the 'optimal range' can adjust their sensitivity or be down regulated [100], [104], [117]. Therefore, it is possible that the 24h of PGE_2 exposure allowed the growth cone to adapt to its elevated baseline ($[Ca^{2+}]_{i(min)}$). Here we show that extension length was altered by PGE_2 and suggest it occurs via Ca^{2+} dynamics. Our research suggests it is that the basal amplitude $[Ca^{2+}]_i$ fluctuation also contributes to regulation of neurite extension length.

In summary, our study shows that PGE_2 induces changes in cytosolic and growth cone $[Ca^{2+}]_i$ levels and neurite extension length in differentiated NE-4C cells. We found that all tested PGE_2 concentrations and exposure times generally contributed to the same outcome of an increase in $[Ca^{2+}]_i$ levels. Moreover, neuronal extension length was modified by an increase in growth cone calcium amplitude in a PGE_2 time- and concentration-dependent manner. This study furthers our understanding of the role PGE_2 plays in neuronal cells and its importance in calcium signaling in the developing nervous system.

CHAPTER 3. PROSTAGLANDIN E2 FACILITATES SUBCELLULAR TRANSLOCATION OF THE EP4 RECEPTOR IN NEUROECTODERMAL NE-4C STEM CELLS

Ms. Ref. No.: BBREP-D-15-00191
Title: Prostaglandin E2 facilitates subcellular translocation of the EP4 receptor in neuroectodermal NE-4C stem cells
Biochemistry and Biophysics Reports

Dear Dr. Crawford,

Your submission "Prostaglandin E2 facilitates subcellular translocation of the EP4 receptor in neuroectodermal NE-4C stem cells" has been assigned manuscript number BBREP-D-15-00191.

Thank you for submitting your work to Biochemistry and Biophysics Reports.

Kind regards,

Rashika Venkataraman
Journal Manager
Biochemistry and Biophysics Reports

Note: Jennilee Davidson performed all experiments, collected and analyzed all data, and wrote the manuscript.

3.1. Abstract

Prostaglandin E2 (PGE_2) is a lipid mediator released from the phospholipid membranes that mediates important physiological functions in the nervous system via activation of four EP receptors (EP1-4). There is growing evidence for the important role of the PGE_2/EP4 signaling in the developing nervous system. Previous studies in our lab show that the expression of the EP4 receptor is significantly higher during the neurogenesis period in mouse. In mouse neuroblastoma cells the PGE_2/EP4 receptor signaling pathway plays a role in regulation of intracellular calcium via a phosphoinositide 3-kinase (PI3K)-dependent mechanism. New research indicates that the functional importance of the EP4 receptor depends on its subcellular localization. PGE_2-induced EP4 externalization to the plasma membrane of primary sensory neurons has been shown to play a role in the pain pathway. In the present study, we detected a novel PGE_2–dependent subcellular trafficking of the EP4 receptor in neuroectodermal (NE-4C) stem cells and differentiated NE-4C neuronal cells. We show that PGE_2 induces EP4 externalization from the Golgi apparatus to the plasma membrane in NE-4C stem cells. We also determined that PGE_2 enhanced EP4 translocation to the growth cones of differentiated NE-4C neuronal cells. These results demonstrate that the EP4 receptor relocation to the plasma membrane and growth cones in NE-4C cells is PGE_2 dependent. Thus the functional role of the PGE_2/EP4 pathway in the developing nervous system may depend on the subcellular localization of the EP4 receptor.

3.2. Introduction

Prostaglandin E2 (PGE_2) is a bioactive lipid derived from plasma membrane phospholipids, through enzymatic metabolism of arachidonic acid by cyclooxygenases -1 and -2 (COX -1, -2) and prostaglandin synthase [31]. PGE_2 mediates biosynthetic pathways that regulate biological functions such as sleep, fever, inflammation, and pain [48]. PGE_2 also plays a critical role in the proper development of the nervous system. It induces differentiation of neuronal cells [142] and plays a regulatory role in membrane excitability and synaptic transmission in neurons [143]. PGE_2 can also increase dendritic length and alter neuronal firing activity in the brain [144].

PGE_2 exerts it physiological function through its four cell surface G protein-coupled receptors (GPCRs) designated EP (E-Prostanoid) 1-4 with a different affinity [31], [32], [145]. Activation of EP1 receptor is associated with an increase of intracellular calcium $[Ca^{2+}]_i$, mediated by phospholipase C and inositol 1,4,5-triphosphate (IP_3) [32], [53]. EP2 and EP4 are coupled to the stimulatory G_s protein and cause an increase in cAMP through activation of adenylate cyclase [64], which in turn activates protein kinase A (PKA) and mediates phosphorylation of cAMP response element binding the protein (CREB) transcription factor [54]. Activation of a specific EP3 isoform, can both decrease and increase cyclic AMP (cAMP) and IP_3. It is also shown that the EP4 receptor signaling can operate via G_i proteins-phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathways [66], [146].

Interest in the PGE_2/EP4 pathway is increasing given its diverse capability of regulating central nervous system activity [147]. EP4 has a protective function by reducing cerebral injury and improving functional outcome after stroke [148], and in suppressing brain inflammation [149]. The EP4 receptor has been suggested to contribute to PGE_2-induced changes in body temperature [150]. Furthermore, EP4 activation can decrease the level of amyloid-beta in the

brain and improve behavioural performance in a murine model of Alzheimer's disease [151]. In sensory neurons the EP4 receptor along with the EP3C (EP3γ) mediates PGE_2-induced sensitization of sensory neurons [152]. PGE_2-prolonged sensitization of nociceptive dorsal root ganglion (DRG) neurons may also contribute to the transition from acute to chronic pain by facilitating EP4 receptor synthesis and anterograde axonal trafficking [60]. We have previously found that the PGE_2/EP4 pathway plays an inhibitory role in regulating the intracellular calcium homeostasis in mouse neuroblastoma (Neuro-2a) cells via PI3K mechanism [65]. Expression of the EP4 receptor is higher during early neurogenesis as compared to later embryonic stages in mouse embryos suggesting its importance in the developing nervous system [40].

Recent research shows that the subcellular trafficking of the EP4 receptor may have functional implications. It has been shown that PGE_2-induced EP4 externalization to the plasma membrane in DRG neurons is important for the inflammatory pain response [59]. The goal of this study was to determine whether PGE_2 can also induce EP4 receptor trafficking in neuroectodermal (NE-4C) stem cells used as an experimental model system for early neuronal development. We show that PGE_2 causes translocation of the EP4 receptor from its normal location in the Golgi apparatus [3] to the plasma membrane in undifferentiated NE-4C stem cells. This was confirmed with a specific EP4 receptor agonist. We also show for the first time that PGE_2 can enhance trafficking of the EP4 receptor to growth cones of differentiated neuronal NE-4C cells. This study shows that PGE_2 can influence the subcellular localization of the EP4 receptor in neuronal stem cells and differentiated neuronal cells.

3.3. Methods

3.3.1. Cell cultures

Mouse NE-4C cells were obtained from American Tissue Culture Collection (ATCC) and grown in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1X penicillin-streptomycin mixture (Invitrogen). Cells were maintained in an incubator containing 5% CO2 at 95% humidity at 37°C. Cells were plated on 0.01% poly-L-lysine (Sigma) coated 100mm culture plate (BD Falcon) and subcultured at a 1:10 ratio. Supplemented MEM was changed every 2 days. NE-4C cells were seeded onto culture plates containing poly-L-lysine and incubated overnight at 37°C before treatment.

3.3.2. Differentiation of NE-4C stem cells into neurons

Differentiation of NE-4C cells was induced on day 0 using Neurobasal media (NBM; supplemented with L-glutamate, 1× Pen Strep, and 1 × B-27; Invitrogen) in poly-L-lysine (Sigma, MW 70000–150000 kDa) pre-coated 100mm culture plates. By day 6, neurospheres (clusters of neural stem cells) were dissociated, seeded onto 35 mm culture plates containing poly-L-lysine coated coverslips and grown until day 12. Supplemented differentiating media was replaced every 2 days. Pou Class 5 homeobox 1 (*Pou5f1*) and Mouse BIII tubulin (*Tubb3*) were used on day 12 as stem cell and late neuronal cell markers to confirm differentiation using PCR. PCR primers are as follows: *Pou5f1* forward 5'- ctggctaagcttccaagggc-3' and reverse 5'-ccagggtctccgatttgcat-3'; *Tubb3* 5'- AGCAGCTACTTCGTGGAGTG -3' and reverse 5'- GGGCTTCCGATTCCTCGTCA -3'. As compared to day 0, which showed presence of the *Pou5f1* marker, we confirmed that on day 12 only the *Tubb3* marker was detected (data not shown).

3.3.3. Cell culture treatments

To explore the time course of PGE_2-induced EP4 externalization, we subjected NE-4C cells to 10μM PGE_2 (Sigma) for 1, 3 and 24 hours (h). The abundance of EP4 at the plasma membrane appeared after 3h, thus we selected the 3h time for the following experiments. We also subjected NE-4C cells to 1 and 10μM concentration of PGE_2 for 3h (no effect seen by 1μM). Controls contained a corresponding concentration of the PGE_2 organic solvent DMSO (dimethyl sulfoxide).

PGE_2-dependent EP4 externalization was confirmed using EP4 agonist (CAY10580; Cayman Chemicals; 10, 50, 100μM) for 3h, with representative figures presented. Specificity of this externalization was confirmed through use of EP4 antagonist (AH23848; Cayman Chemicals; 100μM) alone. To ensure sufficient EP4 receptor blocking prior to co-treatment with the agonist or PGE_2, a pre-treatment (1hour) was applied followed by co-treatment (3 hours) with CAY10580 (100μM) and PGE2 (10μM). PGE_2-induced EP4 translocation was also performed in NE-4C cells differentiated into neurons after 12 days. PGE_2 (1 and 10μM) was applied to differentiated cells for 3 and 24 hours, and compared to the untreated group (DMSO).

3.3.4. Immunocytochemistry

The cells were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (4.3 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4) for 20 minutes at 4°C and washed twice with phosphate buffered saline (PBS). Cells were then incubated with primary antibodies in PBS with 0.3% Triton-X 100 and 2% normal goat serum (NGS). Cellular localization of the EP4 receptor was determined by incubation with anti-rabbit EP4 primary

antibody (1:60; Santa Cruz Biotechnologies) along with monoclonal anti-mouse PMCA1 plasma membrane marker (1:500; Abcam), at room temperature for 2 hours. Following primary antibody incubation, cells were washed three times with PBS for 15 min and incubated with secondary antibodies in Tris-Buffered Saline and Tween 20 (TBS-Tween 20, 0.05%) and 2% NGS for 1 hour at room temperature in the dark.

Secondary antibodies used were anti-rabbit fluorescein isothiocyanate (FITC) (1:100; Jackson ImmunoResearch Laboratories) and anti-mouse Texas Red (1:200; Jackson ImmunoResearch Laboratories). Cells were then washed three times with PBS for 15 min, followed by a 10 minute incubation of 4′,6-diamidino-2-phenylindole (DAPI) (1:10000; Molecular Probes) at room temperature in the dark. Cells were washed three times with PBS for 15 min and coverslips were mounted on glass microscope slides with anti-fade mounting media (Invitrogen). The staining was visualized and captured using an Olympus Fluoview 300 Confocal Laser Scanning Microscope. Secondary antibodies were used without primary antibodies to serve as a control for specificity (not shown).

3.3.5. Immunocytochemistry quantification

NIS Elements software (Nikon) was used to measure the signal intensity of EP4 receptor at the plasma membrane in undifferentiated NE-4C stem cells. A line of 3pt width was drawn around the plasma membrane (perimeter of the cell) using the PMCA1 marker to define the perimeter. Once the plasma membrane was outlined, the region of interest (ROI) was overlaid on the EP4 image in the same location, and then the mean signal intensity was measured. The mean signal intensity accounts for the area of the ROI (line; length x width) overlaying the plasma membrane. The EP4 signal intensity is presented as a ratio in comparison to the control. Growth cone signal intensity was measured using the same

technique with ImageJ software [153]. Growth cone EP4 receptor intensity at the growth cone was taken as a mean for each treatment and compared to the mean of the control (DMSO, vehicle), and presented as a ratio value in comparison to the control. Background signals were subtracted from the signal intensity measurements for each cell and growth cone. Treatment groups were compared to the untreated group and was considered statistically significant at $p<0.05$ by ANOVA and/or a student's t-test.

3.4. Results

3.4.1. PGE_2 induces EP4 externalization to the plasma membrane in undifferentiated NE-4C stem cells.

We previously found that in NE-4C cells the EP4 receptor is normally localized in the Golgi apparatus [3]. To determine if PGE_2 can induce EP4 cell surface externalization in NE-4C cells, similar to in PGE_2-treated DRG neurons [59], undifferentiated NE-4C cells were treated with 1 and 10μM of PGE_2. Similar to untreated NE-4C cells the 1μM PGE_2-treated cells show EP4 present in the Golgi apparatus (not shown). However, with 10μM PGE_2 EP4-immunoreactivity (IR) co-localized in the peripheral region with the PMCA1 (plasma membrane Ca^{2+}-ATPase) marker, suggesting dose-dependent PGE_2-induced EP4 translocation toward the plasma membrane **(Figure 6A)**. The EP4 plasma membrane externalization in NE-4C cells became apparent after 3 hours exposure to 10μM PGE_2.

The observed PGE_2-induced EP4 externalization to the plasma membrane was inhibited by a selective EP4 antagonist, AH23848, and AH23848 alone had no effect on the EP4 trafficking **(Figure 6A)**. This indicates that the trafficking occurred through the EP4 receptor. We verified the increased abundance of EP4 receptor at the plasma membrane through

immunofluorescence quantification **(Figure 6B)**. These results show a novel PGE_2-dependent subcellular translocalization of the EP4 receptor in NE-4C stem cells.

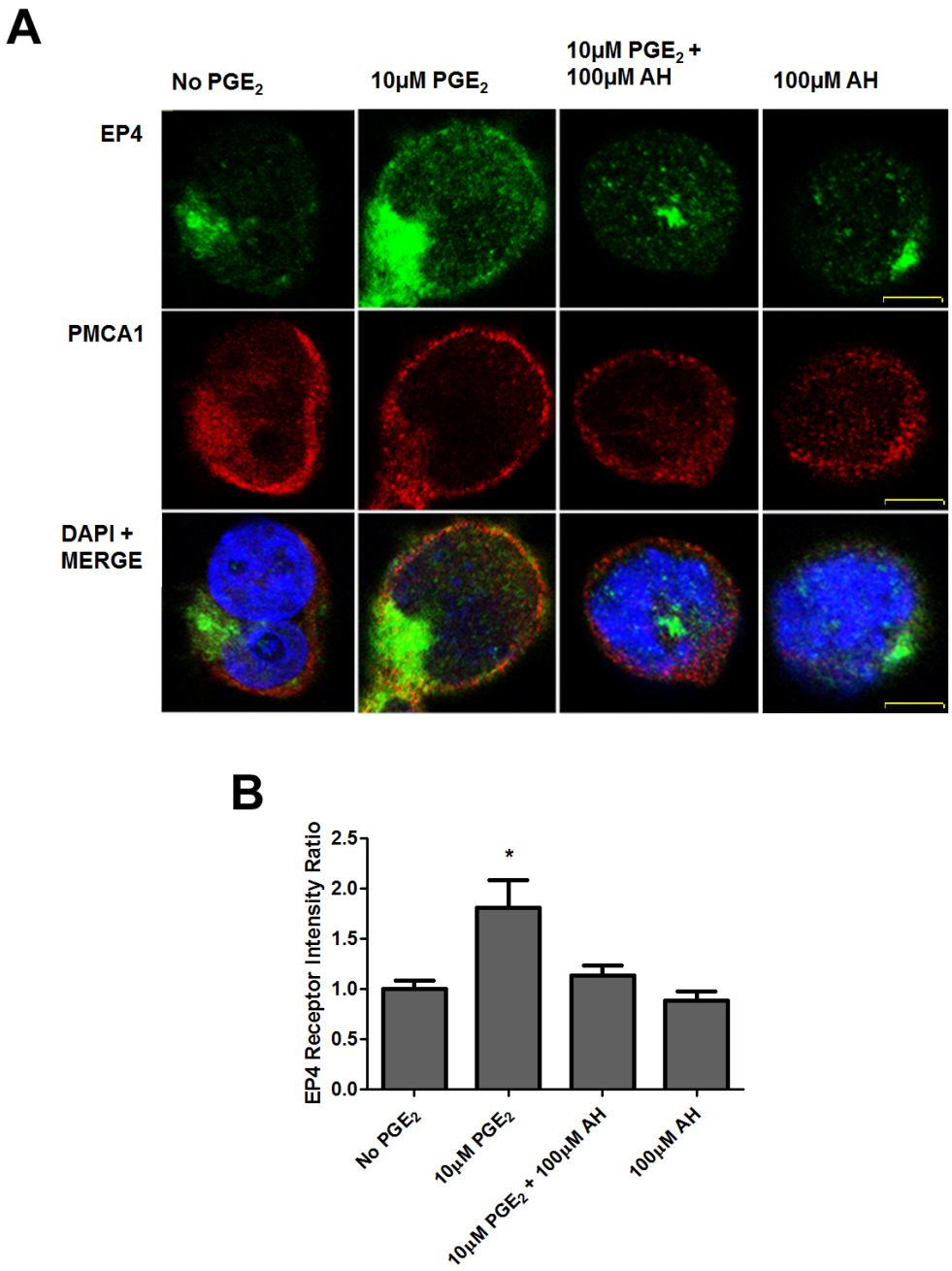

Figure 6. PGE_2–induced EP4 localization at the plasma membrane in undifferentiated NE-4C stem cells. A. Immunocytochemistry visualization of EP4 receptor. The EP4 receptor (Anti-EP4; *top panel*); plasma membrane marker (anti-PMCA1; *middle panel*); merged images (the nucleus marker DAPI; *lower panel*). EP4 localizes to the Golgi apparatus in *no PGE_2*

control and at the plasma membrane with *10μM PGE_2*. The plasma membrane localization was blocked by the EP4 antagonist (*10μM PGE_2+100μM AH*), and there was no effect with antagonist alone (*100μM AH*). The scale bar represents 10μm. **B.** Quantification of immunofluorescent EP4 receptor localization at the plasma membrane depicted as a ratio value in comparison to *no PGE_2* group (set at 1.0); 10μM PGE_2 significantly increased the EP4 at the plasma membrane ($p<0.05$). PGE_2=prostaglandin E2.

3.4.2. EP4 receptor agonist induces EP4 externalization to the plasma membrane in undifferentiated NE-4C stem cells.

The PGE_2-induced EP4 externalization in undifferentiated NE-4C cells was confirmed with a selective EP4 agonist (CAY10580). We used 10, 50 and 100μM treatment with CAY10580 for 3h [59]. Similar to PGE_2, the CAY10580 also induced EP4 externalization to the plasma membrane. All CAY10580 concentrations tested show the Golgi expression whereas the 100μM significantly increased the EP4 externalization to the plasma membrane **(Figure 7A)**. AH23848 blocked CAY10580-induced EP4 externalization **(Figure 7A)**, which was confirmed through immunofluorescence quantification **(Figure 7B)**. Overall, these results suggest that the EP4 receptor subcellular re-location from the Golgi apparatus to the plasma membrane is regulated though the EP4 signaling pathway.

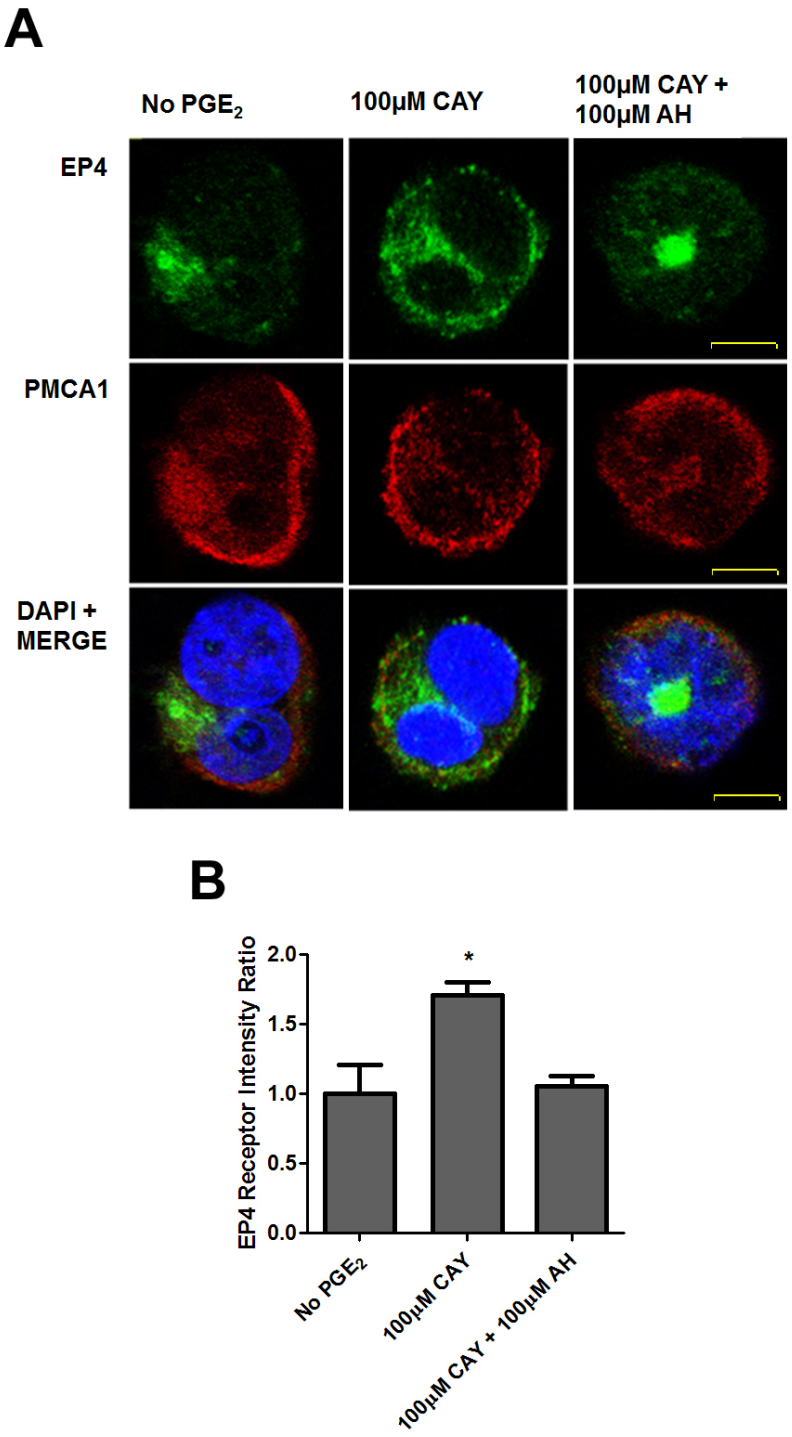

Figure 7. Agonist–induced EP4 localization at the plasma membrane in undifferentiated NE-4C stem cells. **A.** Immunocytochemistry visualization of EP4 receptor. The EP4 receptor

(Anti-EP4; *top panel*); plasma membrane marker (anti-PMCA1; *middle panel*); merged images (the nucleus marker DAPI; *lower panel*). EP4 receptor localization at the plasma membrane with EP4 agonist (*100μM CAY*) was blocked with EP4 antagonist (*100μM CAY+100μM AH*). The scale bar represents 10μm. **B.** Quantification of immunofluorescent EP4 receptor localization at the plasma membrane depicted as a ratio value in comparison to *no PGE_2* group (set at 1.0); 100μM CAY significantly increased the EP4 at the plasma membrane ($p<0.05$). PGE_2=prostaglandin E2.

3.4.3. PGE_2 dependent EP4 receptor localization in differentiated NE-4C neuronal cells.

Given the increasingly important role of EP4 in the developing nervous system, we also assessed the localization of the EP4 receptor in differentiated NE-4C neuronal cells. Similar to NE-4C stem cells, we still observe typical Golgi localization in the untreated differentiated NE-4C cells **(Figure 8)**.

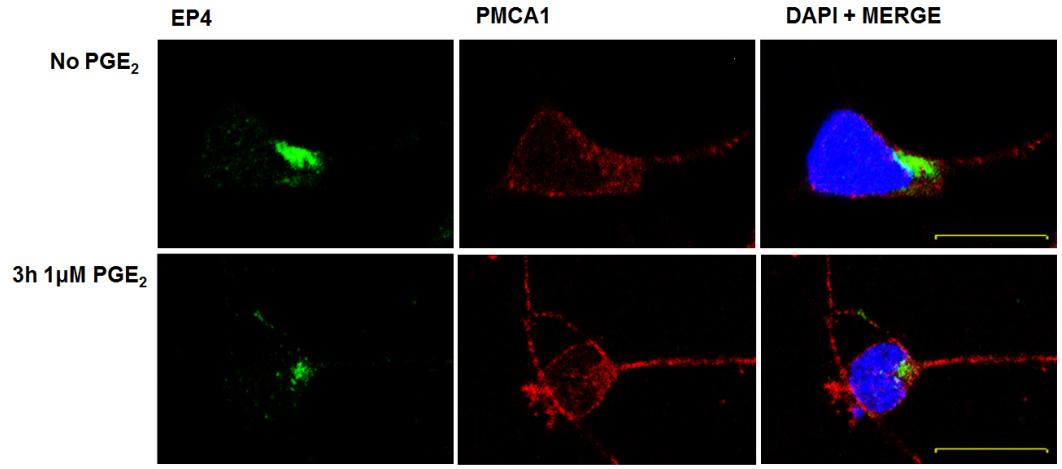

Figure 8. EP4 receptor is localized in the Golgi apparatus in differentiated NE-4C neuronal cells. The EP4 receptor (Anti-EP4; *left panel*); plasma membrane marker (anti-PMCA1; *middle panel*); merge images (the nucleus marker DAPI; *right panel*). Scale bar represents 10μm. Arrows show plasma membrane localization with PGE_2. PGE_2=prostaglandin E2.

Interestingly, we show for the first time that the endogenous EP4 receptor was also present in the growth cones of untreated differentiated NE-4C cells **(Figure 9A)**. Exposure of differentiated NE-4C cells to 1 and 10μM concentrations of PGE_2 resulted in significantly increased localization of EP4 in the growth cones after 3h and remain unchanged for the duration of 24h **(Figure 9A and B)**.

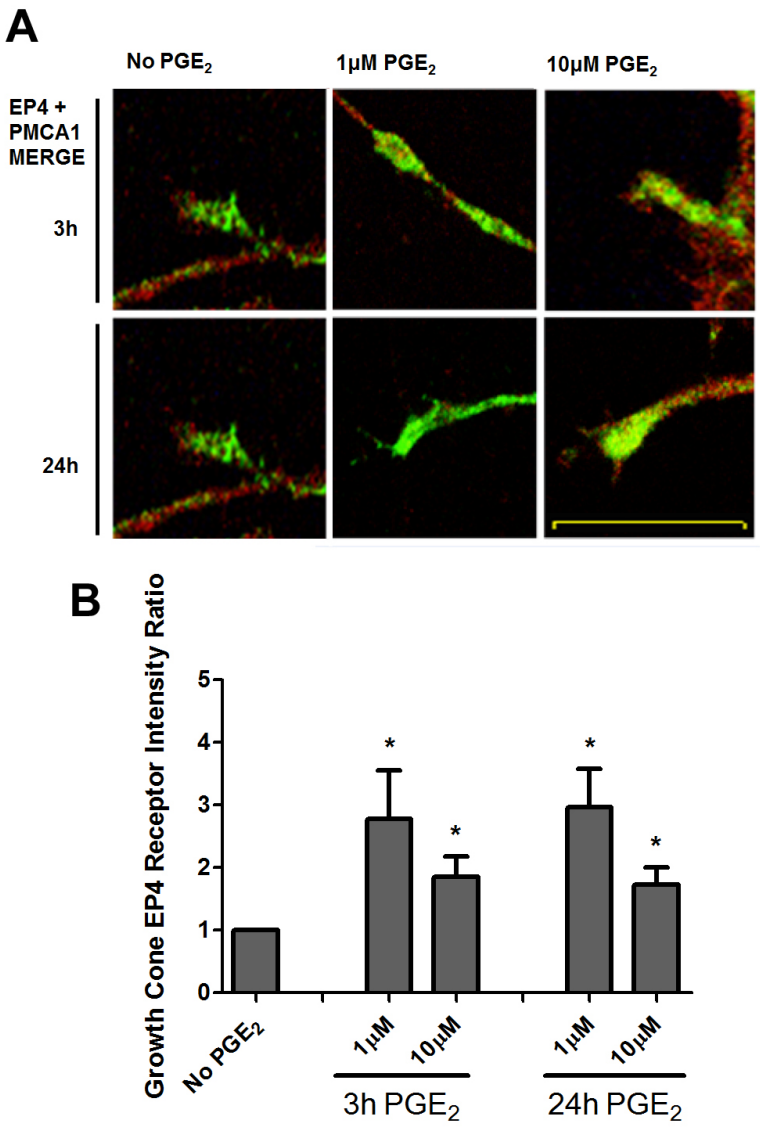

Figure 9. EP4 Receptor localization in the growth cones of differentiated NE-4C cells. A. The EP4 receptor (*Anti-EP4*) merged image with plasma membrane marker (*Anti*-PMCA1). 1μM and 10μM PGE_2 treatment for 3 hours (*top row*), 24 hours (*bottom row*). Scale bar represents 5μm. **B.** Quantification of immunofluorescent EP4 receptor localization in the growth cones depicted as a ratio value in comparison to *no PGE_2* group (set at 1.0); Both 1μM and 10μM PGE_2 significantly increased the EP4 in the growth cones after 3 and 24h ($p<0.05$). PGE_2=prostaglandin E2.

3.5. Discussion

The results of this study show that PGE_2 induces EP4 externalization from its previously characterized localization in the Golgi apparatus [3] to the plasma membrane in undifferentiated NE-4C stem cells. Interestingly, we also found that in differentiated NE-4C neuronal cells PGE_2 enhanced EP4 trafficking to the growth cones. The observed subcellular translocation of the EP4 receptor from its normal location in the Golgi apparatus to the plasma membrane and growth cones indicates that it may play an important role in early function of neuronal cells.

The functional importance of PGE_2-induced subcellular localization of the EP4 receptor in the developing nervous system is still largely unknown. It also needs to be investigated whether the EP4 signaling in various subcellular compartments is regulated by G_s and/or G_i proteins. However, there is growing evidence for the functional importance of the PGE_2/EP4 signaling in the developing nervous system. The PGE_2/EP4 pathway has been suggested to play a role in the transition from acute to chronic pain in nociceptive DRG neurons [60]. St. Jacques and Ma (2014) found that PGE_2-prolonged sensitization of DRG neurons facilitated the synthesis and anterograde axonal trafficking of EP4 receptors [60]. Our previous study shows that EP4 is involved in PGE_2-dependent regulation of intracellular calcium level through a novel PI3K inhibitory mechanism and it also reduces neurite lengths in differentiated Neuro-2a cells [65]. This is interesting because calcium ions are key mediators to multiple cellular processes in early neuronal development. For example, in neuronal growth cones calcium contributes to axonal growth and guidance, which must be strictly regulated during neuronal development [109], [140], [141]. Interestingly, we also showed that the level of the EP4 receptor is higher in the mouse embryo (embryonic stage 7, 11 and 15) as compared to the later stage E17 [40], indicating it's important role in early development.

PGE_2-EP4 activation has been found to attenuate the activation of microglia and to prevent lipid peroxidation and pro-inflammatory gene expression in a murine model of lipopolysaccharide (LPS)-induced brain inflammation [149]. Moreover, PGE_2/EP4 signaling has elicited a protective function in reducing injury and improving functional recovery after stroke via dual and independent cell-specific mechanisms of neuroprotection and enhanced vascular perfusion [148]. In contrast, genetic and pharmacologic inhibition of EP4 receptor, via EP4 deficiency or antagonist respectively, in a murine mouse model of Alzheimer's disease decreased amyloid-β levels in the brain and improved the behavioral performance of the animals [151]. EP4 receptors are the most widely expressed PGE_2 receptors in the body [147], and the various biological effects observed due to PGE_2 signaling via EP4 may be mediated by the externalization of EP4 to the plasma membrane and growth cones.

These results confirm that the EP4 externalization from the Golgi apparatus to the plasma membrane in NE-4C stem cells is PGE_2-induced. Furthermore, this study provides the first evidence that PGE_2 can also enhance the growth cone localization of the EP4 receptor in differentiating NE-4C neuronal cells. Our results show that the important role of the PGE_2/EP4 pathway in the developing nervous system may depend on the subcellular localization of the EP4 receptor.

CHAPTER 4. DISCUSSION

4.1 Discussion

The PGE_2 signaling pathway is critical in regulating many important physiological functions such as synaptic plasticity [34], [35], dendritic spine formation, refining of mature neuronal connections [41]–[43], pain transmission [44], and cell survival [45] or death [46]. Elevation of endogenous PGE_2 has been found during events such as inflammatory response and oxidative stress [49] and can be detrimental to the developing central nervous system. It is possible that heightened inflammatory response during early development may lead to nervous system defects. Our lab has previously shown that elevated levels of PGE_2 cause an increase in basal calcium levels [65] and neurite retraction [40] in Neuro-2a cells. Moreover, our lab also suggested that the EP4 receptor plays an inhibitory role in calcium homeostasis [65]. Here, I have shown that increased levels of PGE_2 for both 3 and 24h increase the basal cytosolic and growth cone calcium levels in differentiated NE-4C cells, and that the growth cone calcium fluctuations, including the maximum and minimum levels and amplitude of fluctuation are increased regardless of PGE_2 dose **(Chapter 2)**. Calcium signaling is critically regulated during development of the nervous system and any insults may adversely impact the downstream signaling that control functions such as neurite extension length. I have also shown that PGE_2 causes a time- and dose-dependent change in neurite extension length **(Chapter 2)**. Since axonal growth is regulated by calcium signalling [95], it is possible that PGE_2 altered the extension lengths via altered calcium signaling. It is possible that increased level of PGE_2 exposure from maternal infection or drug exposure during neurodevelopment may contribute to the altered calcium signaling found. If PGE_2 were to alter calcium dynamics during neurodevelopment, it is possible that it may alter the axon lengths and thus the proper wiring of

areas in the brain. In fact, hyperconnectivity of brain structures has been found in individuals with autism [154]. It would be interesting to determine if prenatal PGE_2 exposure contributed to hyperconnectivity in the brain by use of a mouse model. Furthermore, my results suggest that PGE_2 exposure since the initiation of differentiation **(Appendix A)** may not be as detrimental with respect to calcium signaling. Although calcium levels in the cytosol were differentially affected by PGE_2 dose, the chronic PGE_2 exposure did not alter calcium levels in the growth cones. These results suggest neurons may have a compensatory mechanism to adjust for the elevated levels observed after short-term exposure (3 and 24h, **Chapter 2**), which is consistent with previous literature. If altered PGE_2 signaling during development contributes to ASD, it is promising that the neurons may be able to adapt to chronic PGE_2 exposure and not be as adversely affected. However, the neurite extension length was altered in a dose-dependent manner. If PGE_2 does indeed alter extension length via calcium signaling, this suggests that elevated PGE_2 levels already contributed to the signaling that causes neurite extension prior to measuring the growth cone calcium levels on day 12. Therefore the period of neurogenesis is important for proper regulation of PGE_2 signaling. These results provide a basis of which to begin future investigations.

The EP4 receptor was of particular interest in my research given our lab's previous findings that the EP4 receptor plays an inhibitory role in calcium homeostasis [65]. The PGE_2/EP4 signaling pathway has also plays a functional role in pain signaling [59], [60]. In DRG neurons, PGE_2 induces EP4 receptor externalization to the plasma membrane to facilitate pain signaling via the EP4 receptor [59]. My results also show that PGE_2/EP4 signaling facilitates EP4 subcellular localization to the plasma membrane and growth cones in NE-4C stem cells **(Chapter 3)** and in differentiated neuronal cells **(Appendix B)**, respectively. Given its functional role in DRG neurons, it is likely that the trafficking of the EP4 receptor in NE-4C

cells also plays a functional role. It is possible that EP4 is trafficked to the plasma membrane and growth cones to regulate calcium levels. The increase in calcium levels observed in differentiated NE-4C neuronal cells was observed after 3 hours exposure, which was the same time required to observe EP4 receptor at the plasma membrane in NE-4C cells. The EP4 receptor may be trafficked to the plasma membrane and growth cones to act as a G_i in response to the increase in calcium levels. The novel findings that the EP4 receptor is localized in growth cones is also interesting since PGE_2 induced increased EP4 localization in the growth cones. The growth cone calcium signaling plays a pivotal role in axonal growth and guidance [77], [105], [106]. Thus the EP4 may be contributing the proper calcium regulation in response to the PGE_2-induced elevated calcium levels in the growth cone.

Evidence supporting the involvement of PGE_2 signaling abnormalities of the nervous system stems from the connection between the usage of the drug misoprostol during the first trimester of pregnancy, and the consequence of Mobius syndrome and autism [155]. I have contributed to another *in vivo* study that shows elevated levels of PGE_2 exposure during embryonic development effects mRNA expression of Wnt-target genes in the brain **(Appendix C)**. This *in vivo* study confirms our labs previous *in vitro* research that suggests there is crosstalk between the Wnt and PGE_2 signaling pathways during neurodevelopmental periods such as neurogenesis [3]. In this study, although the mice are genetically identical, each are encased in an amniotic sac and have separately attached placentas. Therefore, even the same maternal environmental exposure to PGE_2 can have a different impact on each pup of the pregnancy. This further emphasizes the point that environmental factors influence the Wnt-pathway during development, and thus its interaction with autism.

4.2. Limitations and future areas of study

There are many molecules involved in the PGE_2 signaling pathway, and various cellular functions are modulated by changes in PGE_2 levels. In this study only some of the effects were examined, such as calcium homeostasis and receptor localization. The present study used an increase in PGE_2 levels to investigate the functional effects of increased PGE_2 exposure on NE-4C stem cells and neuronal cells. Thus, the results only pertain to the regulation of downstream effects on calcium regulation and EP4 receptor localization under artificial conditions of increased levels of PGE_2. One should be cautious interpreting the results as there may be differences in sensitivity and viability of NE-4C stem cells and differentiated NE-4C neuronal cells and primary neuronal cells. However, similar results have been found in primary neurons (DRG) thus it is likely an effective preliminary model.

In regards to the ratiometric method of calcium imaging, the calcium level in the whole cell is measured and a distinction between the calcium homeostasis of different organelles was not made. Thus, it is only possible to determine the effect of increased PGE_2 on the calcium levels in the cell as a whole.

Additionally, the quantification of the EP4 receptor externalization to the plasma membrane in differentiated NE-4C neuronal cells similar to the NE-4C stem cells was not possible. Despite disrupting the neurospheres to obtain individual neurons for analysis, the cells were mostly still attached to other cells, making individual cell plasma membrane measurements not possible.

Calcium homeostasis was previously shown to be altered by increased levels of PGE_2 exposure in Neuro-2a cells, and that the EP4 receptor can play both an inhibitory and excitatory role [65]. Here, it was shown that PGE_2 also affects calcium levels in differentiated NE-4C neuronal cells. For future experiments, it would be interesting to determine if the EP4

receptor plays an inhibitory role in NE-4C neuronal cells as well. To determine this, the calcium imaging experiment could be repeated with the addition of a selective EP4 antagonist.

Furthermore, given the PGE_2-induced externalization of the EP4 receptor to the plasma membrane and growth cones in the NE-4C stem cells and neuronal cells, it is likely that EP4 is trafficked to play a functional role. Considering the importance of calcium signaling in the developing nervous system for regulated gene transcription, and axonal guidance and steering in the growth cones, it is possible EP4 receptor is trafficked to the plasma membrane and growth cones to modulate calcium homeostasis in response to increased PGE_2levels. Since it was found that EP4 externalization was induced via PGE_2/EP4 signaling, the localization of the EP4 receptor could be identified via ICC in experiments with an EP4 agonist and antagonist. Future research should investigate whether the calcium response is being modulated by the EP4 receptor in neuronal cells and if its localization plays a role. The evidence obtained will likely continue to provide new insights into the neural mechanisms behind neuronal development.

4.3. Conclusions

This research has provided results that suggest the PGE_2 lipid signaling pathway is critical in early neuronal development. The *in vitro* studies will help identify the effect of PGE_2 on calcium homeostasis in differentiated NE-4C neuronal cells, which possibly includes the translocation of EP4 receptor to the plasma membrane in response to altered PGE_2 levels. Comparisons made between PGE_2-induced EP4 receptor localization in NE-4C stem cells and differentiated NE-4C neuronal cells will help contribute to the identification of an EP4 receptor functional role. My contribution to the *in vivo* study helps identify which genes are differentially regulated in the brain in response to prenatal PGE_2 exposure. This confirms our

previous findings that the early development Wnt-pathway crosstalks with the PGE_2 signaling pathway, and confirms our suggestion that autism is a complex interaction between the environment and genetics. Abnormal alterations in the PGE_2 exposure may have profound effects on the sensitive period of embryogenesis severely influencing the proper development of the nervous system. These could include but are not limited to environmental, genetic, and/or immunological factors.

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APPENDICES

A.1. Appendix A

A.1.1 Calcium Imaging

To determine if PGE_2 modulates calcium mobilization in NE-4C neuronal cells, the esterified, membrane derivative 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy) ethane-N,N,N',N'-tetraacertic acid, acetoxylmethyl ester (fura-2 AM) real-time calcium imaging was used to determine intracellular free calcium ($[Ca^{2+}]_i$). Fura-2 AM is hydrolyzed by cytosolic esterases to an impermeable fura-2 form, which facilitates chelation of free $[Ca^{2+}]_i$. The ratio (R) fluorescent intensity obtained at 340 nanometer (nm) excitation (fura-2-Ca^{2+}-bound complex) to that at 380nm (Ca^{2+}-free fura-2) was used as a measure of $[Ca^{2+}]_i$. The emission wavelength for both compounds is 520nm. Using the ratiometric fura-2 AM calcium indicator, the maximum 340/380 ratio (R_{max}) and minimum 340/380 ratio (R_{min}) were determined (see methods). Ionomycin was used to determine the R_{max} to stimulate $[Ca^{2+}]_i$ release and facilitate extracellular calcium ($[Ca^{2+}]_E$) entry. Ethylene glycol tetraacetic acid (EGTA) was used to determine the R_{min}. Intracellular calcium was estimated from the following equation:

$$[Ca^{2+}]_i = B\ K_d\ (R\text{-}R_{min}) / (R_{max}\text{-}R),$$

whereby K_d is the dissociation constant for fura-2 (224nm under standard conditions), R is any given 340/380 ratio value of fluorescence measured for Ca^{2+}-free and Ca^{2+}-bound fura-2, R_{min} is the 340/380 intensity ratio in the absence of Ca^{2+} and presence of EGTA, R_{max} the 340/380 intensity ratio when the cells were exposed to ionomycin, and β is the ratio of fluorescence measured at 380nm in calcium-depleted and calcium-saturated solution [124, 163].

Untreated differentiated NE-4C neuronal cells were loaded with 5uM of the Ca2+-sensitive fura-2AM and an equal volume of 20% pluronic acid F127 (Invitrogen) to minimize compartmentalization of the dye. Cells were incubated for fura-2 AM for 45 mins at 37C, 5% CO2, washed with Hanks balanced salt solution with no phenol red ($HBSS_{red\text{-}free}$) and further incubated in fresh $HBSS_{red\text{-}free}$ at 37C, 5% CO2 for an additional 20 mins to de-esterify fura-2 AM. Cells were washed once with $HBSS_{red\text{-}free}$ and the basal 340/380 nm ratio was measured [121].

Measurements were taken in 5 sec intervals. After recording the basal level for a minimum of one minute, 4μM of the Ca^{2+} ionophore, ionomycin (Santa Cruz) was added to the media to determine the R_{max} value, and activity was recorded until a decrease in ratio was observed. Next, 15mM ethylene glycol tetraacetic acid (EGTA) was added and recorded for a further 10 minutes to determine the minimum 340/380 ratio (R_{min}) value. The R_{max} and R_{min} were recorded for untreated differentiated neuronal cells to subsequently use for the calculation of cytosolic and growth cone (differentiated only) $[Ca^{2+}]_i$.

A.2. Expanded Calcium Results

A.2.1 Determining 340/380 ratio maximum and minimum

After addition of 4μM ionomycin the R_{max} was found to be 4.249 and following the addition of 15 mM EGTA the R_{min} was found to be 0.334 **(Figure 10)**. The R_{max} and R_{min} values were then used as parameters to determine the $[Ca^{2+}]_i$ in the PGE_2 treated groups **(Figure 11)**. A minimum of three experimental replicates were completed with similar results, and the total number of individual cells was used for statistical analysis.

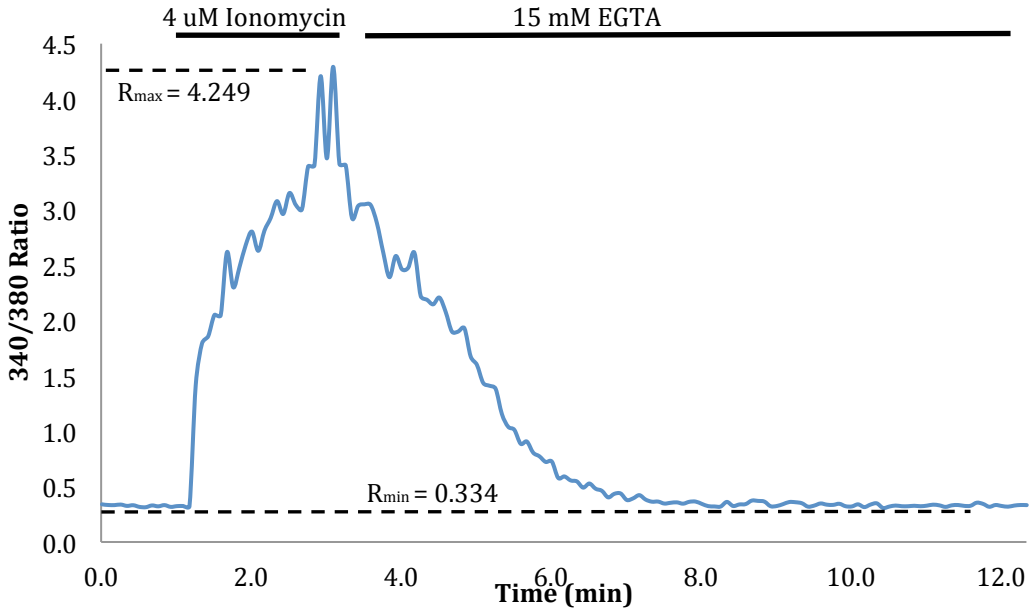

Figure 10. Obtaining R_{max} and R_{min} values. 340/380 ratio (R) tracing showing the baseline R value, the maximum R value (R_{max}) and the minimum R value (R_{min}) following the addition of 4µM ionomycin and 15mM EGTA to the medium, respectively. The baseline R value was recorded for 1 minute. Once the R_{max} value was obtained, the recording was continued for until the 12 minute time point.

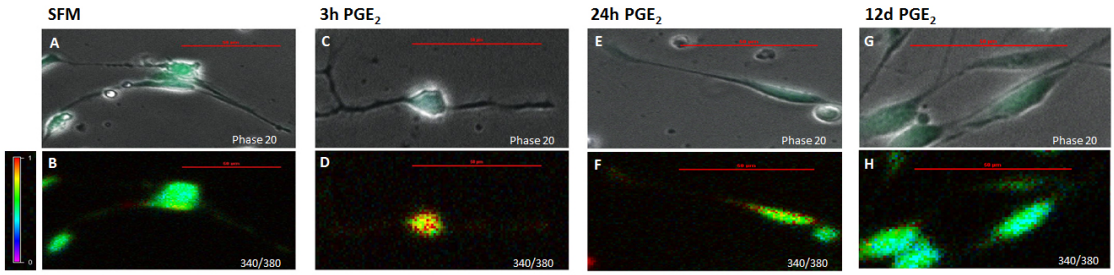

Figure 11. **Ratiometric imaging 340/380 nm ratio (R) in fura-2-AM-loaded differentiated NE-4C neuronal cells in response to PGE_2 exposure on day 12.** (*Top row*) Phase-contrast 20 X (Phase20) objective long working distance (LWD) of differentiated NE-4C cells. *(Bottom row)* 340/380 nm fluorescent image of cytosol. **A, B** serum free media (SFM), untreated cells differentiated for 12 days, **C, D** 3h PGE_2 exposure and **E, F** 24h PGE_2 exposure on day 12, **G, H** 12 days of PGE_2 exposure. Scale bar represents 50 μm.

A.2.2. Effects of PGE_2 since the initiation of differentiation on cytosolic and global growth cone intracellular free calcium concentration

When NE-4C cells were differentiated in the presence of PGE_2 from day 0 to day 12, we found that all concentrations of PGE_2 resulted in significant changes in the mean basal cytosolic $[Ca^{2+}]_i$ but no significant change was noted in the growth cone $[Ca^{2+}]_i$. Exposure to 0.1 and 10μM PGE_2 increased the mean basal cytosolic $[Ca^{2+}]_i$ by a 74.46% (N = 156, $p = 2.44 \times 10^{-13}$) and 70.7% (N = 166, $p = 2.44 \times 10^{-13}$), and 1μM PGE_2 resulted in a 17.1% (N = 166, $p = 4.37 \times 10^{-4}$) decrease in mean basal cytosolic $[Ca^{2+}]_i$ **(Figure 12A)**. The results show a dose-dependent response to PGE_2. No significant change in $[Ca^{2+}]_i$ was observed after exposure for 12 days of the same concentrations of PGE_2 (N = 152, $p = 0.29$; N = 264, $p = 0.99$; N = 137, $p = 0.18$, respectively) **(Figure 12B)**.

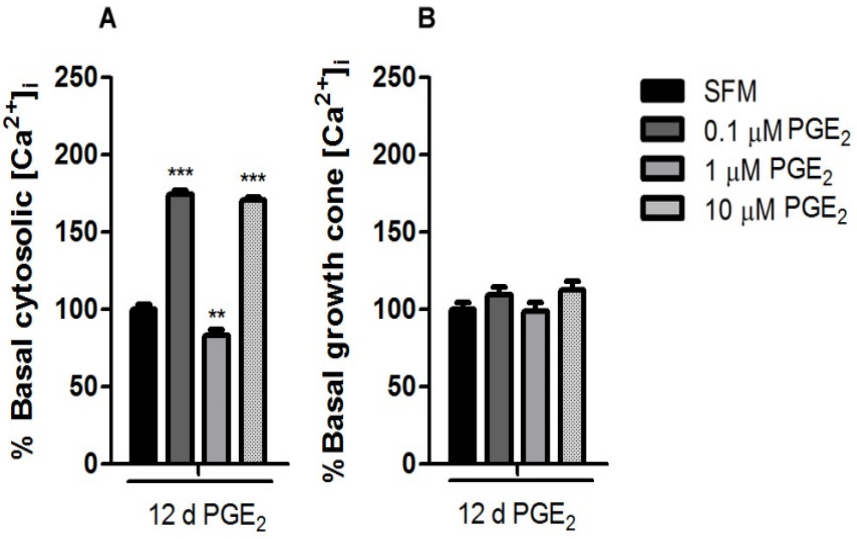

Figure 12. Exposure time dependent increase in basal (A) cytosolic and (B) growth cone $[Ca^{2+}]_i$ in response to PGE_2 exposure. A. Treatments with 0.1 and 10 μM PGE_2 for 12 days significantly increased the cytosolic $[Ca^{2+}]_i$ by 74.46% (N = 156) and 70.7% (N = 166), and 1 μM PGE_2 resulted in a 17.1% (N = 166) decrease. **B.** No significant changes in global growth cone $[Ca^{2+}]_i$ was observed after exposure for 12 days of the same concentrations of PGE_2. Calcium levels only increased by 7.67%, decreased by 4.04%, and increased by 5.94%, respectively (N = 152; N = 264; N = 137, respectively). The y-axes represents the % change in $[Ca^{2+}]_i$ (nM) and x-axes depict the time of PGE_2 exposure. Results represent a minimum of three independent experiments. $[Ca^{2+}]_i$ = intracellular calcium concentration; PGE_2 = prostaglandin E_2; SFM = serum free media; **p<0.01; ***p<0.0001.

A.2.3. Calcium dynamics in growth cones of differentiated NE-4C cells exposed to PGE_2 since the initiation of differentiation

The growth cone $[Ca^{2+}]_i$ fluctuations were also assessed **(Figure 13A).** $[Ca^{2+}]_{i\ (min)}$ levels was significantly changed from 146 nM in untreated cells to 164 nM, 132 nM and 176 nM (p = 2.01 x 10^{-2}, 2.41 x 10^{-2}, 7.52 x 10^{-4} in cells treated with 0.1, 1 and 10 μM PGE_2 concentrations, respectively **(Figure 13B)**. However, the corresponding concentrations of PGE_2 did not significantly change the $[Ca^{2+}]_{i\ (max)}$ value, which was 1479 nM in untreated cells. The $[Ca^{2+}]_{i\ (max)}$ values were 1697 nM, 1459 nM and 1673 nM (p = 8.74 x 10^{-1}, 3.09 x 10^{-1}, 7.59 x 10^{-1}, respectively) with 0.1, 1 and 10 μM PGE_2 concentrations, respectively **(Figure 13B)**.

The continuous exposure to 0.1, 1 and 10 μM PGE_2 resulted the $[Ca^{2+}]_i$ amplitude to be 1533 nM, 1325 nM and 1497 nM (p = 9.29 x 10^{-1}, 2.30 x 10^{-1}, 8.59 x 10^{-1}, respectively). This represents a 13%, 36%, and 18% increase, respectively, which was not significantly different from the amplitude found in untreated cells **(Figure 13C)**. In summary, we have shown the short-term exposure to PGE_2 induces greater changes in calcium dynamics than the continuous exposure.

To determine if chronic PGE_2 exposure affected neurite extension length, PGE_2-exposed groups were compared to untreated (SFM). Only 1μM PGE_2 exposure resulted in significantly increased extension length to 43 μm (N = 383, p<0.0001), whereas 0.1 and 10 μm PGE_2 remained unchanged at 33 μm and 36 μm, respectively (N = 195; N = 201) **(Figure 13D)**.

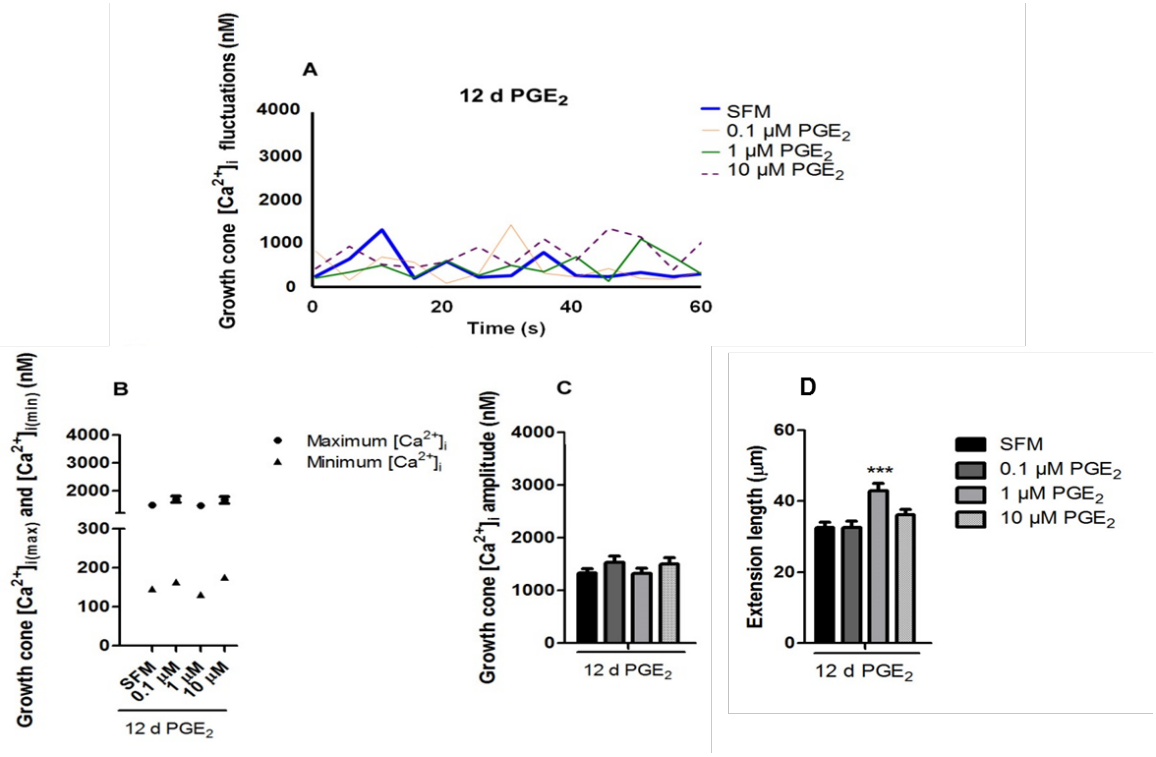

Figure 13. PGE_2 exposure time dependent change in the dynamics of growth cone fluctuation $[Ca^{2+}]_i$ levels and neurite extension length in response to PGE_2 exposure. A. Traces of spontaneous intracellular $[Ca^{2+}]_i$ fluctuations in migrating growth cones after 12 days (12 d) PGE2 exposure. **B.** Minimum $[Ca^{2+}]_i$ levels were significantly changed from 146 nM in untreated cells to 164 nM, 132 nM and 176 nM in cells treated with 0.1, 1 and 10 µM PGE_2 concentrations, respectively. However, the corresponding concentrations of PGE_2 did not significantly change the Maximum $[Ca^{2+}]_i$ levels, which was 1479 nM in untreated cells. The $[Ca^{2+}]_{i\ (max)}$ values were 1697 nM, 1459 nM and 1673 nM with 0.1, 1 and 10 µM PGE_2 concentrations, respectively. **C.** The continuous exposure to 0.1, 1 and 10 µM PGE_2 resulted the $[Ca2+]_i$ amplitude to be 1533 nM, 1325 nM and 1497 nM, respectively, which was not significantly different from the amplitude found in untreated cells. **D.** Mean neurite extension length in the SFM group was 32.6 µm (N = 342) and after 12 days (12 d) PGE_2 increased from 1 µM PGE_2 to 43 µm (N = 383, $p<0.0001$), but did not change by 0.1 and 10 µM being 33 µm

(N = 195), 36 μm (N = 201) respectively. The y-axes represent **A, B, C** the $[Ca^{2+}]_i$ (nM) and **D** extension length (μm), and x-axes depict, **A** the measurement time (seconds) taken at 5-second intervals, and **B, C, D** the time of PGE_2 exposure. Results represent a minimum of three independent experiments. $[Ca^{2+}]_i$ = intracellular calcium concentration; PGE_2 = prostaglandin E_2; SFM = serum free media. Minimum and maximum $[Ca^{2+}]_i$ are shown by triangles and circles, respectively; *p<0.05; ***p<0.0001.

A.3.1. Submitted Manuscript Documents

A.3.1. Manuscript submission proof

-------- Forwarded Message --------
Subject: MCN-15-82: Submission to MCN
Date: 17 Apr 2015 14:22:53 +0100
From: MCN
To: Dorota Crawford

MCN-15-82
TITLE: Lipid mediator prostaglandin E2 alters intracellular calcium transients in differentiated neuroectodermal stem cells

Dear Dr. Crawford,

Your submission entitled "Lipid mediator prostaglandin E2 alters intracellular calcium transients in differentiated neuroectodermal stem cells" has been assigned the following manuscript number: MCN-15-82.

You will be able to check on the progress of your paper by logging on to the Elsevier Editorial System as an author:
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Thank you for submitting your work to Molecular and Cellular Neuroscience.

Kind regards,

Administrative Support Agent
Administrative Support Agent [17-Jan-11]
Molecular and Cellular Neuroscience

==========

A.3.2. Manuscript permission

Email correspondence:
Hi Jennilee,

Thanks for asking. I have signed the form below. Please let me know if you have any further question.

Best Regards
Hongyan Li

On 5/19/2015 3:29 PM, Jennilee Davidson wrote:
19 May 2015
Jennilee Davidson
York University, 4700 Keele Street
Re: Request for Permission to Use Copyrighted Material in a Thesis

Dear Hongyan Li,
I am preparing my thesis for submission as part of the requirements of my Master's Degree in Biology.
The reason I am writing is to ask permission from you as a co-author to include the following material in my thesis: The manuscript entitled, Lipid mediator prostaglandin E2 alters intracellular calcium transients in differentiated neuroectodermal stem cells, which was submitted to the journal Molecular and Cellular Neuroscience in April 2015 for publication. This would also include any figures, and the material will be fully cited in my thesis.
In the interest of facilitating research by others, my thesis will be available on the internet for reference, study and/or copy. The electronic version of my thesis will be accessible through the York University Libraries website and catalogue, and also through various web search engines. I will be granting Library and Archives Canada a non-exclusive license to reproduce, loan, distribute, or sell single copies of my thesis by any means and in any form or format.
Could you please confirm in writing by email that these arrangements meet with your approval. You can do so by signing and completing the information below.
Thank you very much.
Sincerely,
Jennilee Davidson

I, the undersigned, hereby represent and warrant that I have authority to grant the permission requested and do grant the permission.
Signature: Hongyan Li

Name: Hongyan Li
Date: May 20, 2015

A.3.3. Submitted manuscript title page

LIPID MEDIATOR PROSTAGLANDIN E2 ALTERS INTRACELLULAR CALCIUM TRANSIENTS IN DIFFERENTIATED NEUROECTODERMAL STEM CELLS

Jennilee M Davidson[b,c], Hongyan Li[a], and Dorota A Crawford[a,b,c]

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B.1. Appendix B

B.1.1. Immunocytochemistry secondary antibody only controls

Specificity of the EP4 antibody in NE-4C stem cells was confirmed using a secondary antibody only control. Trials with omission of the EP4 antibody were run in parallel with the control (no PGE_2) and 3 hours of PGE_2 exposure **(Figure 14)**. The EP4 receptor was not visualized in the Golgi apparatus as expected without the primary antibody.

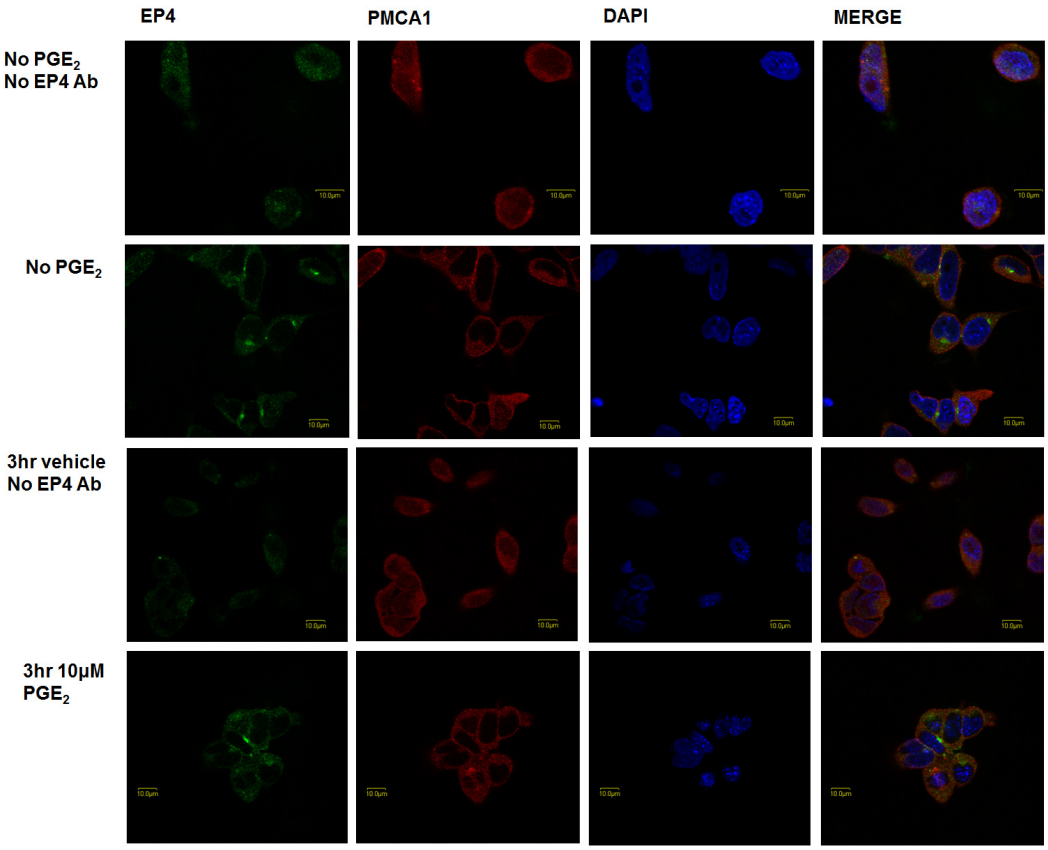

Figure 14: Immunocytochemistry visualization of EP4 receptor antibody specificity in NE-4C cells. Secondary antibody only controls *(first and third rows)* were run in parallel with no PGE_2 control *(second row)* and 3 hours of 10μM PGE_2 treatments *(fourth row)*. The EP4 receptor was not visualized in the Golgi apparatus without the EP4 antibody, confirming its specificity. EP4 receptor (FITC; green) is the first panel, PMCA1 (TR; red) is the second panel, nuclear marker (DAPI; blue) is the third panel, and merged in the last panel. The scale bar represents 10μm.

B.1.2. EP4 agonist induces EP4 receptor externalization to the plasma membrane

To see whether the EP4 receptor agonist induces EP4 externalization to the plasma membrane in NE-4C stem cells similar to after 10μM PGE_2 exposure, immunofluorescence quantification was conducted. It was confirmed that PGE_2 acts through EP4 receptor to translocate EP4 to the plasma membrane by use of EP4 agonist (CAY10580) treatments. Increasing concentrations of CAY10580 (10, 50, and 100μM) were applied for 3h to NE-4C cells and found that 100μM CAY10580 was required to significantly increase the EP4 translocation to the plasma membrane **(Figure 15)**, which is consistent with previous literature [59].

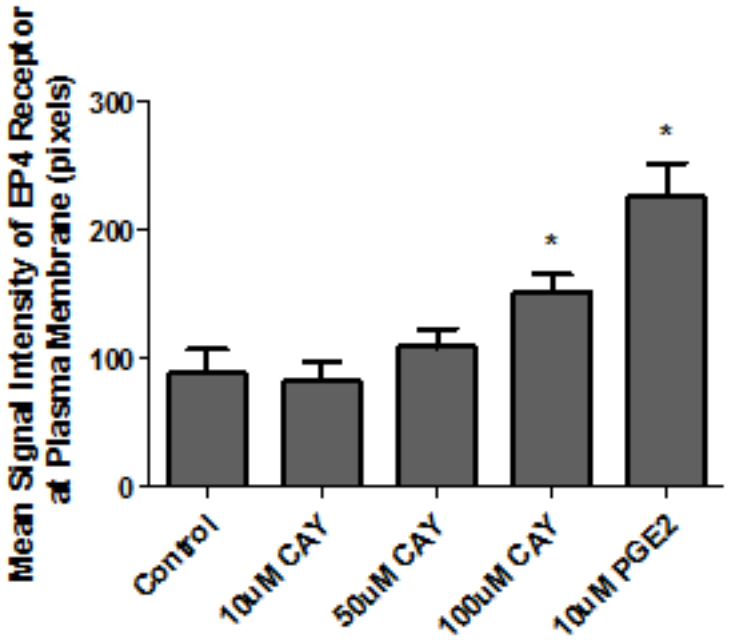

Figure 15. Immunofluorescence quantification of EP4 receptor at plasma membrane in NE-4C cells. Only 100μM CAY10580 and 10μM PGE_2 significantly increased the EP4 at the plasma membrane. All treatments were applied for 3 hours. *denotes significance ($p<0.05$) in comparison to the control (No PGE_2 exposure) by ANOVA and t-test. Quantification was conducted using NIS Elements Software (Nikon).

B.1.3. Subcellular Localization of EP4 Receptor in Differentiated NE-4C Cells

Previous research in our lab shows that differentiated NE-4C cells express increased EP4 mRNA four days after differentiation, when compared to day 0 (induction) (unpublished). Other studies have also shown that EP4 externalization has been observed in DRG neurons in response to PGE_2 [59]. Here, it was tested whether PGE_2 induces EP4 externalization in differentiated NE-4C cells. Visualizing EP4 receptor externalization to the plasma membrane, and possibly to the growth cones, would suggest a possible involvement in the altered PGE_2 signaling pathway and also a role in calcium mobilization [65].

ICC visualization results suggest that EP4 receptor localization was present in the Golgi apparatus with and without PGE_2 exposure **(Figure 16)**. Furthermore, PGE_2 induces EP4 receptor externalization to the plasma membrane **(Figure 17)**, which is consistent with EP4 externalization in NE-4C stem cells (chapter 4) and DRG neurons [59].

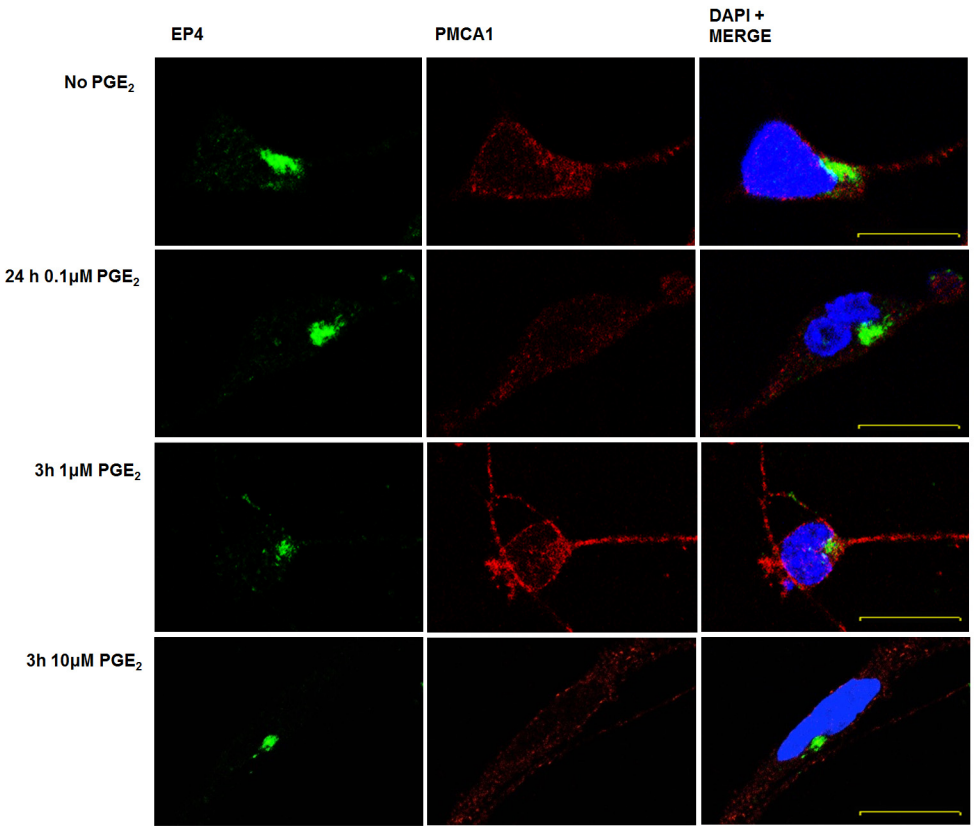

Figure 16. Immunocytochemistry visualization of EP4 Receptor in Differentiated NE-4C cells. The EP4 receptor (FITC; green) is visible in the Golgi apparatus of untreated cells *(top row)* and PGE_2 exposed cells *(second, third, bottom row)*. Day 12 neuronal cells both untreated and exposed to different concentrations and times of PGE_2 appear to have EP4 present in similar locations. Plasma membrane marker (anti-PMCAI-Texas Red; red); nucleus marker (DAPI; blue); or merged images. Scale bar represents 10μm.

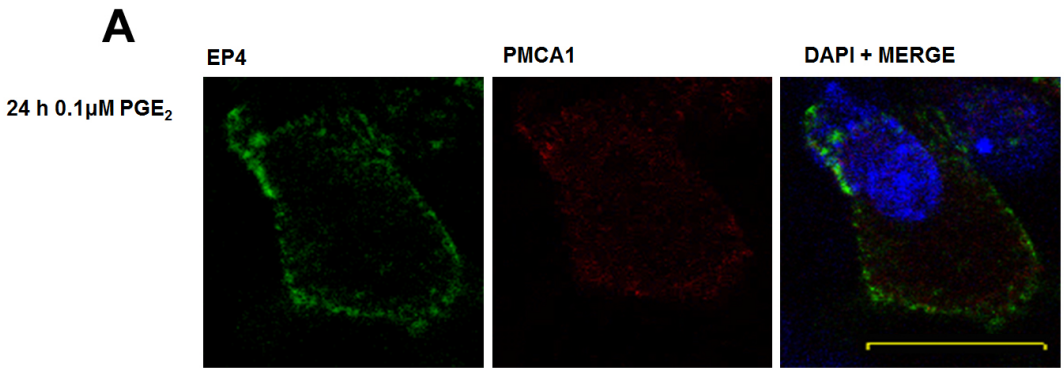

Figure 17. Immunocytochemistry visualization of EP4 receptor in differentiated neuronal NE-4C cells. The EP4 receptor (FITC; green) is visible in the plasma membrane of day 12 PGE_2-exposed neuronal cells. Plasma membrane marker (anti-PMCAI-Texas Red; red); nucleus marker (DAPI; blue, merged images). Scale bar represents 10μm.

B.2. Submitted Manuscript Documents

B.2.1. Manuscript permission

Yes. Please check if it meets your needs. Thanks for asking!

Hongyan

****
Hongyan Li PhD, CCRP
Research Associate
Dr. D.A.Crawford's Developmental Neuroscience Laboratory
****

---- Original Message ----
From: "Jennilee Davidson"
To: Hongyan Li
Sent: Mon, Jul 20, 2015, 12:40 PM
Subject: permission to use co-authored manuscript in thesis

20 July 2015
Jennilee Davidson
York University, 4700 Keele Street
Re: permission to use co-authored manuscript in thesis

Dear Hongyan Li,

I am preparing my thesis for submission as part of the requirements of my Master of Science Degree in Biology.

The reason I am writing is to ask permission from you as a co-author to include the following material in my thesis in which I was the main contributor to experiments and writing: The manuscript entitled, Prostaglandin E2 facilitates subcellular translocation of the EP4 receptor in neuroectodermal NE-4C stem cells, which was submitted to the journal Biochemical and Biophysical Reports in July 2015 for publication. This would also include any figures, and the material will be fully cited in my thesis.

In the interest of facilitating research by others, my thesis will be available on the internet for reference, study and/or copy. The electronic version of my thesis will be accessible through the York University Libraries website and catalogue, and also through various web search engines.

Could you please confirm in writing by email that these arrangements meet with your approval. You can do so by signing and completing the information below.

Thank you very much.

Sincerely,
Jennilee Davidson

I, the undersigned, hereby represent and warrant that I have authority to grant the permission requested and do grant the permission.
Signature: Hongyan Li
Name: Hongyan Li
Date: July 20, 2015

B.2.2. Submitted manuscript title page

PROSTAGLANDIN E2 FACILITATES SUBCELLULAR TRANSLOCATION OF THE EP4 RECEPTOR IN NEUROECTODERMAL NE-4C STEM CELLS

Jennilee M Davidson[b,c], Hongyan Li[a], Dorota A Crawford[a,b,c]

[a]School of Kinesiology and Health Science, York University, Toronto, ON, M3J 1P3, Canada,

[b]Neuroscience Graduate Diploma Program, York University, Toronto, ON, M3J 1P3, Canada,

[c]Department of Biology, York University, Toronto, ON, M3J 1P3, Canada

Corresponding author:
Dorota A. Crawford, Ph.D.
Associate Professor, Faculty of Health
York University

C.3. Appendix C

C.1.1. Prostaglandin signaling and Wnt signaling crosstalk in autism

Evidence supporting the involvement of COX/PGE2 signaling abnormalities of the nervous system stems from the connection between the usage of the drug misoprostol during the first trimester of pregnancy, and the consequence of Mobius syndrome and autism [155]. Previous work done in our lab has shown that PGE_2 can affect cell function through various molecular events. For example, PGE_2 or misoprostol induced alteration of calcium fluctuation in growth cones was previously shown by Tamiji and Crawford [65]. We have also shown that PGE_2 interacts with the Wnt signaling pathway in an *in vitro* (NE-4C cells) system. PGE_2 affects Wnt-dependent migration and proliferation of NE-4C cells [3].

Wnt signaling is involved with the determination of cell fates by activating transcription of various target genes [156], [157]. Moreover, our lab has recently shown that higher levels of PGE_2 can also change expression of wnt-regulated genes such as b-catenin, COX-2, cyclin D1, and Mmp9, which have been implicated in autism [3]. The Wnt signaling pathway is of particular interest because it is involved in early embryonic development and recent literature suggests that the wnt canonical pathway is involved in development of ASDs [158], [159]. Our lab recently proposed this pathway as a possible autism candidate pathway [3]. Proper signaling through wnt ligands during early embryonic and neuronal development is crucial for the outcome of the nervous system [160], and abnormal interference through the PGE_2 pathway can lead to changes of the regulation of cells and gene expression. Therefore, during pregnancy, any mechanisms at the level of genetics, the environment, or a combination, can abnormally modulate levels of PGE_2, may be detrimental to the development of the offspring during a critical sensitive window.

Wnt-target genes that are implicated in either autism or development were selected by a previous graduate student in the lab. Two of those genes that were the most downregulated, *Wnt3a* and *Fosl1*, were chosen for further analysis of the effect of PGE_2 on mRNA expression at various developmental stages.

WNT3A is a member of the Wnt protein family and may be involved in the fate decision of stem/progenitor cells [161]. *Wnt3a* can stimulate proliferation and enhance the neurogenesis of neonatal neural progenitor cultures isolated from the cerebral cortices of newborn mice [162]. However, recombinant WNT3A protein promotes differentiation of neural stem cells but has a negative effect on proliferation [163], [164]. Autism is a neurodevelopmental disorder, thus *Wnt3a*'s role in neurogenesis may be a contributing factor to the dysregulation observed in central nervous system development.

FOSL1 is a basic leucine zipper transcription factor that can distinctly regulate both cell type and stimulus-specific gene expression involved in various physiologic and pathologic processes, including *in utero* embryonic development [165]. Several studies have shown that *Fosl1* regulates gene expression involved in cell cycle progression, cell motility and invasion in several cancer cell types [41], [166]. This gene is a known target of the canonical Wnt signaling pathway. *Fosl1* may be one of the downstream genes regulated by b-catenin-dependent transcription in hippocampal neurons. The expression of *Fosl1* is very low in various adult tissues with elevated levels seen in the brain, bladder, breast, pancreas, skin and lung, but its transcription is strongly inducible by mitogens and inflammatory cytokines as well by various environmental toxicants, carcinogens and pathogens [167], [168], mainly at the transcriptional level [175]. The induction of *Fosl1* is notably delayed (peaking at 90-180 min) by various mitogenic and stressful stimuli [169].

C.1.2. Background Methods

C.1.2.1. Gene Selection

For *in vivo* studies, I will use mRNA collected from embryonic mouse brain as my experimental model. A mouse model was used since 99% of mouse genes have analogues in humans. A previous graduate student in the lab selected 44 Wnt-target genes that are implicated in either autism or developmental pathways. mRNA expression analysis using Custom TaqMan® Array Plates showed downregulation of the *Fosl1* and *Wnt3a* genes from 0.2μM dmPGE$_2$ treated mouse brain samples across different developmental stages (E16, E19, P8, adult). Based on microarray results, differentially regulated genes only with a fold change above 1.5 were further characterized using mouse as an experimental model system. To confirm the results I ran real time PCR using SYBR green reagent.

C.1.2.2. Animals

Male and female mice (C57BL/6) were obtained from Charles River Laboratories. C57BL/6 colonies are inbred and thus are considered as genetically identical. Upon arrival, they are maintained at the animal facility at York University, kept at a 12 h light/dark cycle, and provided with unlimited food and water. All protocols for animal procedures used in this study were approved by the Animal Care Committee (ACC). This was carried out by a PhD student in our lab.

C.1.2.3. Maternal Injections

Male and female mice were mated overnight, and the females were checked every morning until a vaginal plug was observed. The day of a vaginal plug was considered as

embryonic day 1 (E1), and the females were housed separately for the remaining time. On embryonic day 11 (E11), the pregnant females were weighed, and injected subcutaneously with 0.2mg/kg concentration of 16,16-dimethyl prostaglandin E_2 ($dmPGE_2$; Cayman Chemical) in saline. Controls were injected with saline only. This was carried out by a PhD student in our lab.

C.1.2.4. Sample Collection

Brain tissue and tail samples were collected from all pups of each litter, at embryonic day 16 (E16), embryonic day 19 (E19), and postnatal day 8 (P8), from maternal treatment of saline, or $dmPGE_2$. Total RNA and protein were extracted from brain tissue using the trizol (Sigma) method for further quantification of mRNA using quantitative real-time polymerase chain reaction (qRT-PCR). This was carried out by a PhD student in our lab.

C.1.3. Methods

C.1.3.1. RNA Sample Concentrations

To ensure RNA samples that had been stored in -80C freezer were still useable and to obtain more current and accurate RNA concentrations, the total RNA yield was assessed on the NanoDrop ND-1000 Spectrophotometer.

C.1.3.2. Pooling RNA Samples

To determine the mean gene of interest expression in each litter, equal concentrations of brain RNA samples from each pup, as determined by the NanoDrop ND-1000, was combined into a pooled sample.

C.1.3.3. Reverse Transcription Polymerase Chain Reaction and Gel Electrophoresis

Reverse transcription polymerase chain reaction (RT-PCR) was used to determine if the genes of interest were expressed in the wild-type and prenatally PGE_2-exposed mouse brain. First, 4µg of RNA was treated with 4µL of 10X DNAse I Buffer and ddH2O (up to a total of 20µL). The mixture was incubated at 37°C for 10 minutes, then 2µL of ethylenediaminetetraacetic acid (EDTA) was added to the mixture and incubated for an additional 10 minutes at 75°C. The treated RNA was combined with 46µM Oligo dT, 2mM (10X) dNTP mix, and incubated at 65°C for 5 minutes, then briefly put on ice. Reverse transcription (RT) was performed with M-MuLV Reverse Transcriptase (200units/µL; New England Biolabs) in accordance with the manufacturer's instructions. The reaction was mixed with 10x RT Buffer and ddH2O. The mixture was then incubated at 42°C for one hour and then at 90°C for 10 minutes to deactivate the enzyme. The success of the RT reaction was confirmed by polymerase chain reaction (PCR) with *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase) primers.

The PCR reaction contained complementary DNA, 10X Taq Reaction buffer, forward and reverse primers (10µM), dNTP mix (2.5mM), MgSO4 (20mM), Taq DNA Polymerase (5units/µL; Biobasic) and ddH2O. The thermal cycler was programmed for 1 cycle of 94°C for 30 seconds followed by 30 cycles of PCR amplification at 94°C for 30 seconds for denaturation; 55°C for 30 seconds for annealing; and 72°C for 30 seconds for primer extension, and then 1 cycle at 72°C for 5 minutes. The PCR products were then verified by gel electrophoresis. A gel contained 1.2% agarose in 1X TAE buffer with SafeView Nucleic Acid Stain. The samples were mixed with 6X nucleic acid loading buffer and loaded into the wells of the gel. Electrophoresis was conducted at 100V on a Basic Power Supply. An ultraviolet Transilluminator was utilized to visualize the size of the deoxynucleic acid (DNA) bands. A

100 base pair DNA standard ladder was used to estimate the size of the DNA bands by comparison.

C.1.3.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The expression level of the two selected genes of interest, *Wnt3a* and *Fosl1*, chosen based on functional relevance in the central nervous system and associated with ASD, were quantified using the 7500 Fast Real-Time PCR System with SYBR Green reagent (Applied Biosystems). Primers were previously designed and used by a lab member **(Table 1)**. Quantitative values were obtained using the threshold cycle (C_T) number. Raw C_T values from the experimental (PGE_2 exposure) samples were normalized using the geometric mean of the housekeeping genes *Hprt* (hypoxanthine phosphoribosyl transferase) and *Pgk1* (phosphoglycerate kinase) to obtain the ΔC_T values. The ΔC_T values of the smaples were compared with a calibrator (saline exposure) to generate the relative quantity (RQ) value, which represents the fold change expression of each target sample compared to the reference sample. Statistical analysis was conducted using ANOVA and t-test.

Table 1: qRT-PCR primer sequences for microarray genes of interest.

Gene	Primer Sequence (5' → 3')	Amplicaon Size (base pair)
Hprt	F: TCCATTCCTATGACTGTAGATTTTATCAG R: AACTTTTATGTCCCCCGTTGACT	75
Pgk1	F: CAGTTGCTGCTGAACTCAAATCTC R: GCCCACACAATCCTTCAAGAA	65
Wnt3a	F: GCACCACCGTCAGCAACAG R: GCACCACCGTCAGCAACAG	57
Fosl1	F: ACCGAAGAAAGGAGCTGACAGA R: CGATTTCTCATCCTCCAATTTGT	65

C.1.4. Results

C.1.4.1. mRNA expression of *Wnt3a* and *Fosl1* at E16, E19, and P8 brain development

The mouse E16 stage is a critical time in development for neurogenesis, E19 is one day prior to birth, and P8 is 8 days post-birth. For normal brain development, in comparison to the expression of *Wnt3a* in the adult mouse brain, the *Wnt3a* expression was significantly higher for all developmental stages tested. The highest expression was found in the earliest stage (E16) followed by E19 and P8; with RQ values of 16.6 ($p<0.005$), 14.3 ($p<0.005$) and 8.3 ($p=0.005$), respectively **(Figure 18A).**

For normal brain development, in comparison to the adult *Fosl1* brain expression, *Fosl1* was significantly decreased in all developmental stages tested. The RQ values for E16, E19, and P8 were 0.3 ($p<0.005$), 0.6 ($p<0.05$), 0.3($p<0.001$), respectively **(Figure 18B)**.

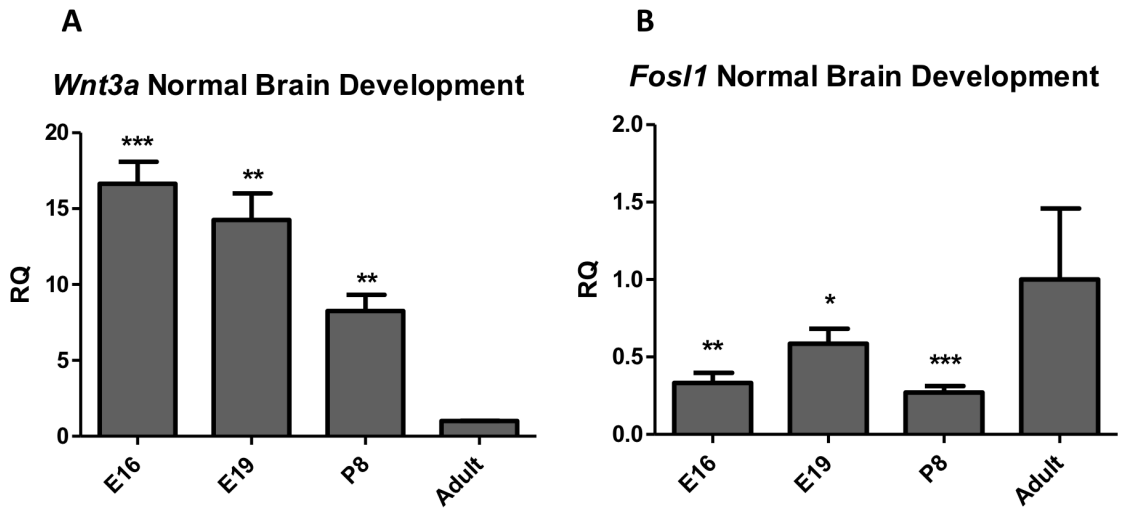

Figure 18. mRNA RQ values of normal brain development for three developmental stages tested. A. *Wnt3a* mRNA RQ values were significantly increased in all developmental stages tested but was greatest at E16, followed by E19 and P8, respectively. **B.** *Fosl1* mRNA RQ values were significantly downregulated in normal brain development for all stages tested. P8 was the most downregulated followed by E16, then E19. * denotes $p<0.05$, ** $p<0.01$, ***$p<0.001$.

C.1.4.2. Pre-natal PGE_2 exposure effects mRNA expression of *Wnt3a* at E16, E19, and P8 brain development

At stage E16 **(Figure 19A)**, in litter 1 there were 6 pups with RQ values of 1.7, 2.9, 2.3, 1.3, 1.2 and 1.1, respectively (none were significantly different than control), and the mean of litter 1 was 1.8. Of 8 pups in litter 2 the mean RQ for *Wnt3a* expression was 1.2, and all pups, except for Pup 4 and 8, had higher *Wnt3a* expression with RQ values of 1.8, 1.8, 1.4, 0.9, 1.2, 1.1, 1.0 and 0.6, respectively. Similarly, in the third litter the mean RQ increased to 2.9, whereby all 7 pups had increased *Wnt3a* expression with RQ values of 1.3, 6.1, 1.8, 4.7, 2.7, 1.8 and 1.7, respectively. Only pup 2 and pup 4 were significantly greater ($p<0.05$ and $p<0.05$, respectively). Overall, the mean *Wnt3a* expression at E16 for all three litters was increased with a mean RQ value of 1.9 ($p<0.005$) **(Figure 19D)**.

At stage E19 **(Figure 19B)**, in the first litter the mean RQ was 1.1, whereby half of the pups were increased, while the other half had decreased expression. The RQ values were 0.7, 2.2, 1.6, 0.8, 1.6 and 0.9, respectively. Three were significantly increased: pup 2 ($p<0.05$), pup 3 ($p<0.05$), pup 5 ($p<0.05$). Pup 1 was significantly decreased ($p<0.005$). In the second litter the mean RQ was 1.5, and all of the pups had increased expression with RQ values of 1.2, 1.5, 1.6, 1.3, 1.6, 1.8, 1.8 and 1.1, respectively. Half of this litter was significantly increased: pup 3 ($p<0.05$), 5 ($p<0.005$), 6 ($p<0.005$), 8 ($p<0.05$).In the third litter the mean RQ value was increased to 1.3. Similarly to the first litter, three pups from the third E19 litter had increased *Wnt3a* expression with RQ values of 1.6, 1.1 and 1.2; two pups had decreased expression with RQ values of 0.8 and 0.8; and one pup was unchanged with a RQ value of 1.0. The overall expression of *Wnt3a* (all three litters combined) was increased with a mean RQ value of 1.3 ($p<0.005$) in comparison to the adult **(Figure 19D)**.

At stage P8 **(Figure 19C)** interestingly, not all three litters had increased *Wnt3a* expression; two litters had increased expression while the third litter had decreased expression. The first litter had an increased mean RQ of 1.5 whereby individual pups had RQ values of 1.0, 2.2, 1.5, respectively where only pup 2 had significantly increased expression ($p<0.0005$). The second litter had an overall increase in expression with an RQ value of 2.5. All 9 pups in litter 2 had increased expression with RQ values of 2.8, 2.8, 2.4, 1.7, 1.8, 2.0, 2.9, 3.3, and 2.6, respectively ($p<0.05$). The third litter had decreased expression with a mean RQ value of 0.4. Five of seven pups in the third litter were significantly decreased with RQ values of 0.2 ($p<0.005$), 0.1 ($p<0.0005$), 0.2 ($p<0.000005$), 0.2 ($p<0.00005$), 0.6, 0.7 and 0.5 ($p<0.005$), respectively. The overall mean RQ value was 1.6 ($p<0.05$) **(Figure 19D)**.

Overall the mean trends from each developmental stage appear to be increased in *Wnt3a* brain expression **(Figure 19D)**.

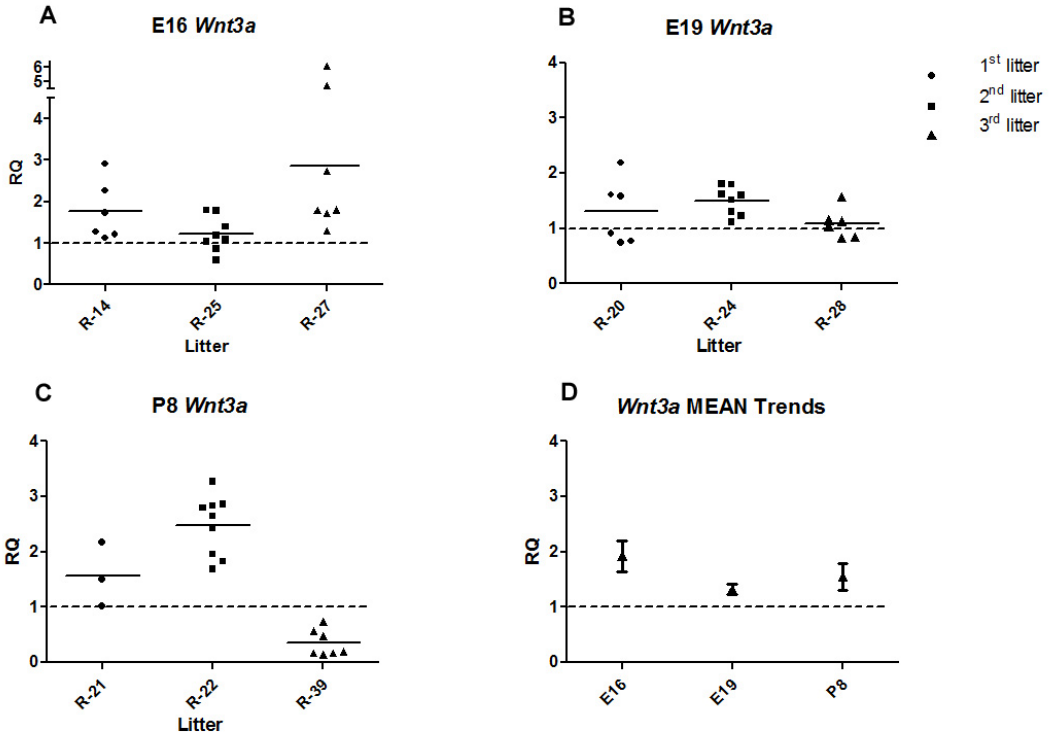

Figure 19. *Wnt3a* mRNA RQ values for three developmental stages tested. Individual pups and the pooled sample RQ values were normalized to the control (saline; RQ = 1) group. **A.** The *Wnt3a* mRNA expression from E16 appears to be upregulated in most litters, upregulated in E19, but not as great as in the earlier developmental stage, **C** and two litters upregulated and one litter downregulated at P8, and **D** the mean trends from all litters combined at each developmental stage appear to be upregulated.

C.1.4.3. Pre-natal PGE_2 exposure effects mRNA expression of *Fosl1* at E16, E19, and P8 brain development

At E16 **(Figure 20A)**, in litter 1 the mean RQ value was 1.0, and only 2 of 6 pups had increased *Fosl1* expression with RQ values of 1.4, 0.8, 1.8, 1.0, 0.7 and 0.5, respectively. Of 8 pups in the second litter, the mean litter RQ was 0.6, and all had decreased expression with RQ values of 0.4, 0.4, 0.8, 0.6, 0.5, 0.5, 0.6, and 0.6, respectively. The third litter had a mean RQ of 1.1, with all 7 pups having similarly unchanged RQ values of 1.2, 1.3, 1.4, 1.2, 1.0, 0.9, and 0.7, respectively. The mean *Fosl1* expression at E16 for all three litters was decreased with a mean RQ value of 0.87 (p=0.13) **(Figure 20D)**.

At stage E19 **(Figure 20B)**, *Fosl1* expression was too low to detect for one litter, but of the other two, there was an overall mean RQ value of 0.9 (p=0.06) in comparison to the adult **(Figure 20D)**. At E19 **(Figure 20B)**, the first litter had 6 pups and a mean RQ of 0.9, whereby 4 were decreased and 2 were increased. Individual pups had RQ values of 0.67, 0.2, 0.9, 0.8, 0.9, and 1.1, respectively. The second litter Fosl1 expression was too low to detect. The third litter had 6 pups: pup 6 had an RQ value of 19 and was not included in the mean values for overall E19 Fosl1 expression or for the third litter mean RQ. Most of the pups had decreased expression with RQ values of 0.8, 0.7, 0.6, 1.0, and 1.1, respectively.

At stage P8 **(Figure 20C)**, the first litter had a mean RQ of 1.2 and individual pups had RQ values of 1.2, 1.2, and 1.2. In the second litter the mean RQ was 1.0, but 3 of 9 were increased and the remainder had decreased expression. The RQ values were 0.6, 0.9, 0.8, 0.8, 1.3, 0.6, 2.3, 0.8, and 1.2, respectively. The third litter had a mean RQ of 1.5 with only 1 pup having decreased expression, and the remainders were increased. RQ values were 1.0, 0.9, 1.2, 2.8, 1.2, 2.1, and 1.5, respectively. The overall Fosl1 expression was not changed with an RQ value of 1.2 (p=0.08) **(Figure 20D)**.

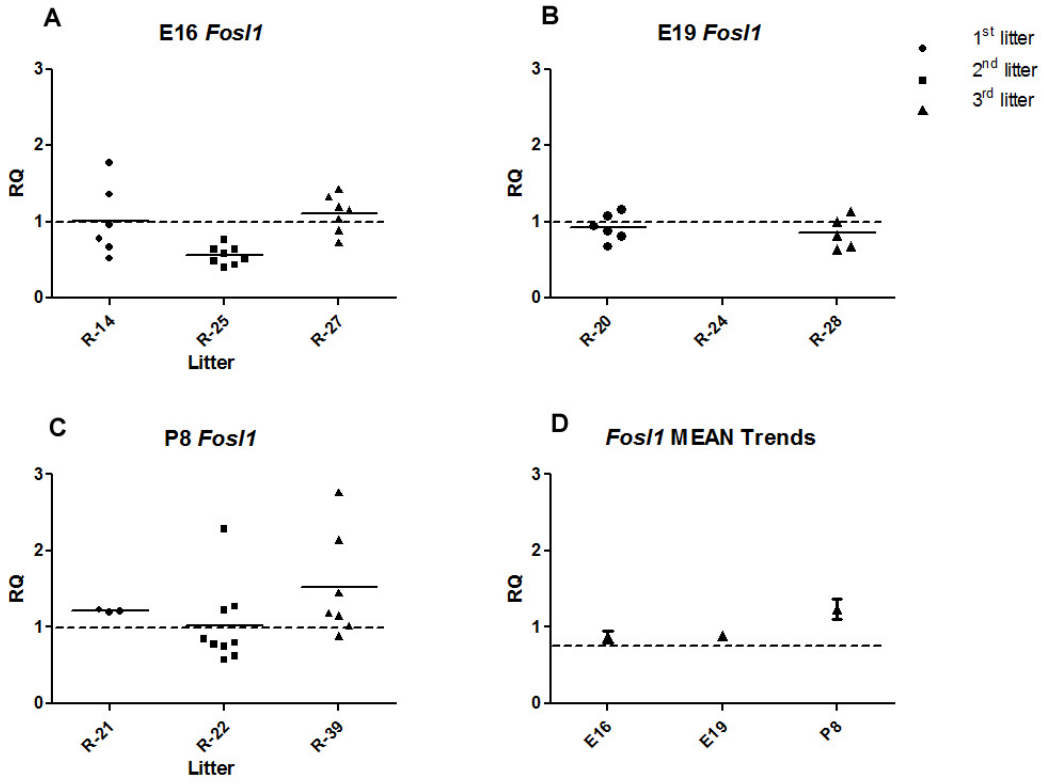

Figure 20. *Fosl1* mRNA RQ values for three developmental stages tested. Individual pups and the pooled sample RQ values were normalized to the control (saline; RQ = 1) group. **A.** The *Fosl1* mRNA expression from E16 appears to be unchanged except for the second litter, **B** unchanged in E19, **C** and some litters upregulated at P8, and **D** the mean trends from all litters combined at each developmental stage.

C.1.5. Discussion

This research shows that genetically identical mice offspring can have variable mRNA expression of Wnt-target genes after prenatal exposure to PGE_2. This further emphasizes the point that environmental factors influence the Wnt-pathway during development, and thus its interaction with autism.

My current results in the mouse brain show that maternal exposure to PGE_2 during critical prenatal development result in differential regulation of Wnt-target genes in the genetically identical offspring at various developmental stages. I show that the exposure to PGE_2 effects the expression of two genes *Wnt3a* and *Fosl1*.

In human twin studies, it has been established that there is a genetic component to the etiology of ASD by testing the concordance rates between monozygotic and dizygotic twins. The concordance is 32-88% in monozygotic twins, compared to 0-31% in dizygotic twins [170]–[173]. However, even in monozygotic twins, the severity of symptoms observed in each child may be different [174]. Monozygotic twins that share the same placenta (monochorionic) are more likely to have high concordance compared to monozygotic-dichorionic twins [175]. In our study, although the mice are genetically identical, each are encased in an amniotic sac and have separately attached placentas. Therefore, even the same maternal environmental exposure can have a different impact on each pup of the pregnancy.

There is also evidence that misoprostol is not neurodevelopmentally toxic at birth. Misoprostol was injected in mice on postnatal day 7, the approximate developmental stage in mice of human birth, and no significant effects of exposure were found for any measure of development or behavioural endpoints [176]. However, this confirms our findings that the PGE_2 signaling pathway plays a critical role during neurodevelopment prenatally.

C.1.6. Conclusion

Overall, this study contributes to the first *in vivo* evidence in the mouse for the interaction of two critical developmental pathways PGE_2 and Wnt. This research identifies the expression levels of Wnt-target genes, *Fosl1* and *Wnt3a*, in the brain after prenatal exposure to PGE_2. Assessing different developmental stages will help us better understand the influence of PGE_2 on the Wnt signaling pathway during critical periods of development. This study furthers our knowledge that a maternal increase in PGE_2, during a vulnerable window in brain development can affect the expression of genes crucial to the development of the nervous system, and potentially contribute to pathology of neurodevelopmental disorders such as autism. Based on the previous literature and our current findings, PGE_2 should be considered an autism candidate signaling pathway.