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Dean, S.L.*, **Knutson, J.F.***, Krebs-Kraft, D.L., & McCarthy, M.M. (2012) Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. *European Journal of Neuroscience*. (35)1218-1229. *co-first author

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- Hoffman, J.F.**, Wright, C.L., & McCarthy, M.M. (2013) A postnatal sensitive period in cerebellar development where disruption of a prostaglandin E2 mediated estradiol signaling pathway may be a risk factor for Autism Spectrum Disorder. **Baltimore Chapter of Society for Neuroscience Abstracts**. Baltimore, MD.
- Hoffman, J.F.**, Wright, C.L., & McCarthy, M.M. (2013) A postnatal sensitive period in cerebellar development where disruption of a prostaglandin E2 mediated estradiol signaling pathway may be a risk factor for Autism Spectrum Disorder. *Society for Neuroscience Abstracts*. San Diego, CA.
- Knutson, J.F.** & McCarthy, M.M. (2012) Neonatal disruption of the PGE2-E2 signaling pathway in the cerebellum affects normal Purkinje cell development and sex-specific behaviors relevant to Autism Spectrum Disorder. *Organization for the Study of Sex Differences Abstracts*. Baltimore, MD.
- Knutson, J.F.** & McCarthy, M.M. (2011) A sensitive period in prostaglandin-estradiol mediated signaling in cerebellar development. *Society for Neuroscience Abstracts*. Washington, DC.
- Knutson, J.F.**, Dean, S.L., & McCarthy, M.M. (2011) Effects of PGE2 and COX1/2 inhibitors on cerebellar Purkinje cell morphology are seen during, but not before, the second week of development in the rat. *Baltimore Chapter of Society for Neuroscience Abstracts*. Baltimore, MD.
- Knutson, J.F.**, Dean, S.L., & McCarthy, M.M. (2010) Effects of PGE2 and COX1/2 inhibitors on cerebellar Purkinje cell morphology are seen during, but not before, the second week of development in the rat. *Society for Neuroscience Abstracts*. San Diego, CA.
- Knutson, J.F.**, Dean, S.L., & McCarthy, M.M. (2010) Early treatment with PGE2 and COX-inhibitors affect dendritic branching of cerebellar Purkinje cells with implications for sex differences in social and exploratory behavior. *University of Maryland, Baltimore 13th Program in Neuroscience Annual Retreat*. Baltimore, MD.

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Abstract

Title of Dissertation: Identification and Characterization of a Sensitive Period in Cerebellar Development

Jessica Hoffman, Doctor of Philosophy, 2014

Dissertation Director: Margaret M. McCarthy, Professor, Chair, Department of Pharmacology, Program in Neuroscience

The cerebellum, a brain region long established to have a role in motor control, has begun to be appreciated for its involvement in sensory perception and higher cognitive functions. Our previous research has found evidence for a prostaglandin E2 (PGE2)-estradiol signaling pathway in the rat cerebellum, and decreasing PGE2 synthesis in the second postnatal week results in excessive growth of Purkinje cell dendritic trees and abnormal sensory and social behavior. Here we confirm that this signaling pathway has a role in Purkinje cell development by demonstrating that during the second postnatal week, inducing an increase in PGE2 synthesis by treatment with lipopolysaccharide (LPS) increases aromatase activity and estradiol synthesis and stunts Purkinje cell dendrite growth. Additionally, we report that inflammation induced by LPS or mimicked by directly injecting PGE2 disrupts social and cognitive behaviors without affecting normal motor control. Specifically, in males and females, both LPS and PGE2 increase total object exploration, which we interpret as increased perseverance of interest with objects. LPS and PGE2 also decrease social play behavior, but only in males. These changes in Purkinje cell development and in behavior can be prevented with the

aromatase inhibitor, formestane, suggesting a link between estradiol, cerebellar development, and its role in higher cognitive functioning. Further, these biochemical, morphological, and behavioral changes are only seen when inflammation occurs within the second postnatal week, not in the first or third.

Autism spectrum disorder and Schizophrenia are neurodevelopmental diseases with complex and poorly understood genetic and environmental components. Both have been associated with cerebellar pathology and early life inflammation, particularly the developmental period between *in utero* and early childhood – a very similar time frame to the sensitive period we describe. Our findings suggest that the second postnatal week of life in the rat is a developmental period in which fever and inflammation may increase the risk for neurodevelopmental disorders by altering cerebellar development.

Identification and Characterization of a Sensitive Period
in Cerebellar Development

by
Jessica Hoffman

Dissertation submitted to the faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2014

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**To my husband, my (0,0,0,0), my better half. I love you.
To our furkids, the cheapest (and cutest) therapy a girl could hope for.
To my parents and my family for always being there, always believing in me.
To my Ya-Yas, for a true sisterhood. And cookies!**

**Without all of you I would not be who I am today.
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I would also like to thank my thesis committee: Dr. Robert Koos, Dr. Asaf Keller, Dr. Jessica Mong, and Dr. Todd Gould, for their suggestions and advice throughout the development of my thesis. Without their support I would not have been able to achieve the clarity in my writing that exists now. Many thanks to my colleagues, past and present: Desiree Krebs-Kraft, Jaylyn Waddell, Mike Bowers, Chris Wright, Katy Lenz, Nina Banerjee, Kathryn Argue, Pedro Paredes-Ramos, Miguel Pérez Pouchoulén, Lidia Fuenzalida, Shannon Dean, Bridget Nugent, Kathy Kight, Lindsay Pickett, Jonathan Van Ryzin, Sara Stockman, Melissa Konopko, Clint Roby, Ashley Whitmarsh, Shalon Edwards, and Kevin Barnett, for their continual source of help, training, support, and friendship. Thanks especially to Shannon Dean for beginning this project and for much of my initial training. Thanks to Clint Roby, for your patience in training me and your wealth of knowledge with all things PCR. Thanks to Chris Wright, for your integral part in this story and always pushing me to be a better scientist.

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List of Abbreviations

<u>Abbreviation</u>	<u>Full Term</u>
3 β HSD	3 β -hydroxysteroid dehydrogenase
ADHD	attention hyperactivity disorder
ANOVA	analysis of variance
ArKO	aromatase knock-out
ASD	autism spectrum disorder
BDNF	brain-derived neurotrophic factor
Ca ⁺⁺	calcium
CACNA1C	calcium channel, voltage dependent, L type, alpha 1C subunit
cAMP	cyclic adenoside 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
COX1	cyclooxygenase 1
COX2	cyclooxygenase 2
CYP27B1	cytochrome p450 27B1
DNA	deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of mental disorders
DTNBP1	dystrobrevin-binding protein
E	embryonic day
E2	estradiol
EP1-4	E-prostanoid receptors 1-4 (for PGE2)
ER α	estrogen receptor α

ER β	estrogen receptor β
F.M.	Foramen Magnum
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
i.p.	intraperitoneal
IACUC	Institutional Care and Use Committee
IAD	integrative grayscale pixel area density
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MECP2	methyl CpG binding protein 2
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
NICHHD	National Institute of Child Health and Human Development
NRG1	neuregulin 1
NSAID	non-steriodogenic anti-inflammatory drugs
P450scc	cytochrome P450 side chain cleavage
PBS	phosphate-buffered solution
PET	positron emission tomography
PGE2	prostaglandin E2
PGs	prostaglandins

PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PLC- β	phospholipase C- β
PN	postnatal day
POA	preoptic area
PTEN	phosphatase with tensin homology
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RORA/ROR α	retinoic acid-related orphan receptor-alpha
RT-PCR	real-time polymerase chain reaction
s.c.	subcutaneous
StAR	steroidogenic acute regulatory protein
TBS	tris-buffered solution
TLR4	toll-like receptor 4
ZNF804A	zinc finger binding protein 804A

Chapter 1:

General Introduction

The cerebellum, a brain region long established in a role for motor control, has begun to be appreciated for its involvement in sensory perception and higher cognitive functions. Additionally, pathologies in cerebellar development have begun to be associated with neurodevelopmental diseases such as autism and schizophrenia. While the exact causes are unknown, these types of diseases have been strongly associated with early life environmental insults at a time when cerebellar development is the most dynamic. We will begin with a discussion of cerebellar anatomy and development, describe its function and pathology in relation to neurodevelopmental diseases, and finish with a discussion of inflammation and hormones in the cerebellum and their relevance to these diseases.

Normal Cerebellar Anatomy and Development

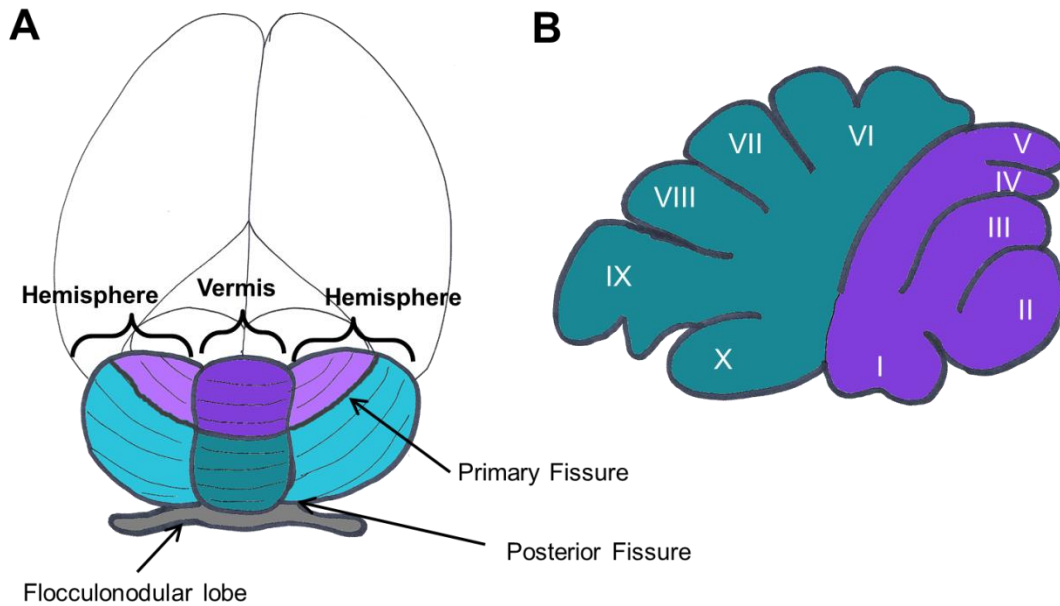
Structure and cell types

The term cerebellum means “little brain” in Latin, and earned the name for its multiple folds in the cerebellar cortex and small, seemingly separate structure; visually a smaller version of the cerebrum. The cerebellum is located below the cerebral hemispheres directly dorsal to the pons and classified as part of the metencephalon. Visually three lobes can be distinguished in a rostral-to-caudal direction: the anterior lobe (above the primary fissure), the posterior lobe (below the primary fissure and above the posterior fissure), and the flocculonodular lobe (below the posterior fissure). The

floculonodular node, the most primitive part of the cerebellum, is involved in regulating balance and eye movements and rarely implicated in neurocognitive disorders. The anterior and posterior lobes are further divided into the vermis and lateral hemispheres (Figure 1.1A). The anterior and posterior vermis together are known as the spinocerebellum, a midline structure that receives proprioceptive information from the trunk of the body (spinocerebellar tract and trigeminal nerve) as well as auditory and visual information to fine-tune body and limb movements (Kawato, 1987). The spinocerebellum is involved in sensory processing and discrimination (Parsons, 1997) and processing emotions (Snider, 1950) in addition to the classical role in posture modulation. The lateral zone (hemispheres) constitutes the cerebrocerebellum and receives input exclusively from the cerebral cortex (especially the parietal lobe) via the pontine nuclei and sends output mainly to the ventrolateral thalamus (Ghez, 1985). This region is involved in planning motor action and fluid coordination of mental activity (Kern, 2002).

The vermis and hemispheres are further subdivided into ten lobules numbered I through X (Figure 1.1B). Lobules I-V are in the anterior lobe and VI-X in the posterior lobe, and these lobules have distinct functional significance. Lobules IV and V are most active during working memory tasks (Desmond, 1997) while VI and VII are both strongly involved in sensation (Crispino, 1984) and emotion (Stoodley, 2009).

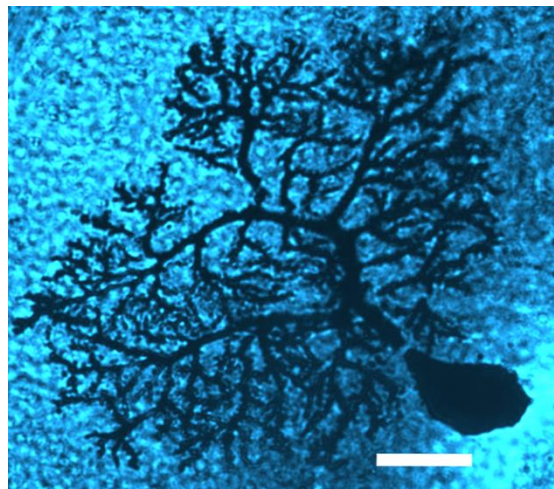
Figure 1.1: A visual representation of the rat cerebellum. A) Dorsal view of the surface of the cerebellum and its location relative to the rest of the brain. The anterior (purple) and posterior (teal) lobes are labeled, as is the vermis (dark colors) and the lateral hemispheres (light colors). B) A sagittal section of the cerebellum subdivided into its lobules (I-X).



Within the cerebellum there are multiple cell types. Purkinje cells are by far the largest cell type. They are characterized by a large soma, a complex, spiny monodendritic tree, and are located in a monolayer between the granule layer and the molecular layer. Their somas align along a single layer, called the Purkinje cell monolayer (Figure 1.2 and 1.3). Their dendritic arbors are two-dimensional, aligning parallel with each other along a sagittal orientation in the cerebellum. Purkinje cells are GABAergic (inhibitory) cells, and are the only cells in the cerebellar cortex that send efferents out of the cortex and on to the deep nuclei and the rest of the brain (Altman, 1972a). They project to each other as well as receive inputs from several other cell types. Climbing fibers originating from the inferior olive connect with Purkinje cell dendritic trees in a 1:1 cell-to-cell ratio, but form thousands of synaptic connections, providing a strong excitatory input. Parallel fibers originating from cerebellar granule cells connect

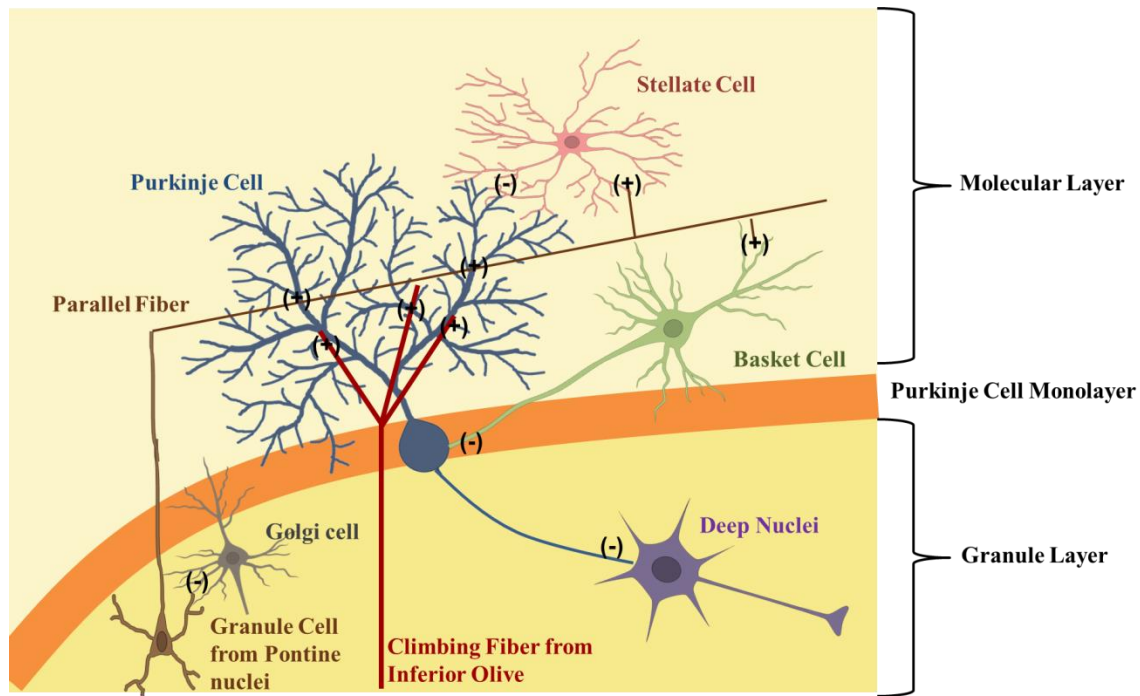
with Purkinje cell dendritic trees in a 1:200,000 cell-to-cell ratio, but only form a few synaptic connections per tree, resulting in a weak excitatory input (for review see Purves, Neuroscience 4th edition). There is an estimated 10^{11} granule cells in the human cerebellum (Bezzi, 2001), and these tiny cells receive their input from the mossy fibers of the pons and spinal cord. Climbing fibers will make synaptic connections with only a single Purkinje cell, but each parallel fiber runs parallel to the field of Purkinje cell dendritic trees and will synapse onto multiple different Purkinje cells. Synapses, from Greek *synaptein* "to clasp," are small structures between neurons that allow them to pass an electrical or chemical signal from one cell to the next, allowing communication between cells and networks in the brain. Spinophilin is a protein enriched in dendritic spines integral to their proper functioning (Feng, 2000). Analysis of spinophilin by methods such as western blot provides a reliable semiquantitative indicator of the number of spines in many brain regions (Amateau, 2004; Schwarz, 2008).

Figure 1.2: A representative photo of a Purkinje cell. Stained with Golgi stain and counterstained with Methylene Blue.40X, scale bar 25 μ m.



Within the cerebellum there are also three main types of inhibitory neurons: Golgi cells, basket cells, and stellate cells (Altman, 1972b). Golgi cells send inhibitory input to granule cells and receive their input from mossy fibers and granule cells themselves in a feed forward and feedback inhibition circuit. Basket cells are located in the molecular layer above the Purkinje cell monolayer and form inhibitory synapses onto the soma of Purkinje cells. Stellate cells form inhibitory synapses on Purkinje cell dendritic trees. Both basket and stellate cells receive excitatory input from parallel fibers, allowing granule cells to excite a central array of Purkinje cells while also providing direct inhibition to neighboring Purkinje cells (Takayama, 2005) (Figure 1.3).

Figure 1.3: A representative schematic of the synaptic connections with the Purkinje cell. Cerebellar granule cells receive excitatory input from the pontine nuclei and are inhibited by golgi cells. They send weakly excitatory inputs to Purkinje cells via parallel fibers, as well as excitatory inputs to basket and stellate cells. Purkinje cells also receive strongly excitatory input from climbing fibers originating in the inferior olive. Basket and stellate cells inhibit Purkinje cells through connections with the soma and dendrites, respectively. Purkinje cells then become the sole output of the cerebellar cortex, utilizing inhibitory synapses on cells in the deep nucleus. These cells then send inhibitory input to the inferior olive and other brain regions. Excitatory inputs are indicated with (+) and inhibitory inputs with (-).



Neurodevelopment

The cerebellum develops late relative to other brain regions, deriving from the metencephalon primarily; a small rostral portion develops from the mesencephalon (Hallonet, 1990). In rats the neurons of the deep nuclei of the cerebellum are born between embryonic day 13 and 14 (E13 and 14) in a region called the cerebellar rhombic lip, composed of the roof plate of the fourth ventricle and neuroepithelium caudal to it (Altman, 1978). In general, cells in the hemispheres are born before those in the vermis. Starting on E16 the external granule layer (EGL), eventual birth place of the granule cells, spreads across the surface of the developing cerebellum (Altman, 1972c). Golgi cells are born from precursors in the external granule layer starting on E19 and continuing on to postnatal day 3 (PN3) (Uzman, 1960). Purkinje cells are born between E14 and 16 from precursor cells in the same region and migrate through the cerebellar parenchyma and reelin-expressing granule cells (Carletti, 2008). Reelin serves as a stop

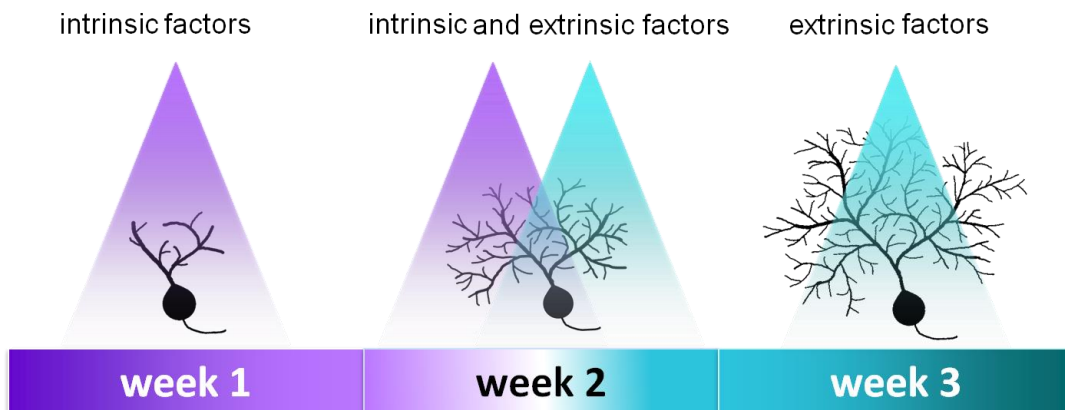
signal for Purkinje cell migration, and Purkinje cell somas align into the Purkinje cell monolayer just prior to the animal's birth at PN0 (Altman, 1972a). Between E20 and E21 fissures appear in the cerebellar cortex, with white matter organizing into discrete trunks of future lobules (Altman, 1997). Basket cells are primarily born from precursors located in the white matter on PN6-7 and begin to take on mature morphology by the middle of the second week of life; stellate cells are born on PN8-11 and mature during the third postnatal week (Zhang, 1996).

In the rodent the second postnatal week is the period of the most dynamic and significant cytoarchitectural changes, particularly for Purkinje Cells. Around day of birth (postnatal day 0 or PN0) settled Purkinje cells sprout multiple primary dendrites which are innervated by multiple climbing fibers, and between PN3 and PN6 these dendrites are pruned, leaving a single primary dendrite. Between PN6 and PN10 the single primary dendrite begins to grow and branch dramatically, synapsing with parallel fibers around PN12. By PN13 the dendritic tree has reached its adult width and is innervated by a single climbing fiber, and by PN30 the final adult height of the Purkinje cells is reached (Altman, 1972a; Crepel, 1976).

The first postnatal week of Purkinje cell development is cell autonomous. Dendrite development is most strongly directed by intrinsic factors, involving preprogrammed timing of specific transcription factors for developmental events such as neurogenesis, migration, differentiation, axonal and dendritic growth and synaptogenesis (de Luca, 2009; Paul, 2012). Spine formation occurs independently of a presynaptic connection. The third postnatal week, by contrast, is influenced less by intrinsic factors and more strongly by extrinsic factors such as afferents from nearby cells, trophic factors,

and hormones (Woodward, 1977; Sotelo, 2009). Spines make connections and are preserved, while many of the initial spines are excessive and lost. Stress, infection, injury, pharmaceutical drugs and diet are also more influential during this time. The second postnatal week is both the most dynamic period of Purkinje cell change and a developmental window equally sensitive to both intrinsic and extrinsic factors (Figure 1.4).

Figure 1.4: Sensitive periods in Purkinje dendrite development. In the rat cerebellum, development of Purkinje cell morphology is driven predominantly by intrinsic factors during the first postnatal week, giving way to predominantly extrinsic drivers in the third postnatal week. The second week is thus a period more at risk for external stimuli to affect Purkinje cell development.



Knowledge of how rat brain development compares with human brain development is vague, at best. Different regions and cell types develop at different times and with varying speeds – while we understand the exact days of developmental milestones for specific cell types in the rat, we do not have access to the human brain in the same level of detail. Thus far we can say that *in general*, the rat postnatal days 1-3 correlate with human gestational age 23-32 weeks, where the brains of both species

undergo developmental milestones such as immune system development, blood-brain barrier establishment, and oligodendrocyte maturation (Craig, 2003; Lodygensky, 2010; Holsapple, 2003; Engelhardt, 2003). Rat postnatal days 7-10 *in general* correlate with human gestational age 36-40 weeks (a full term infant), defined by developmental milestones of rapid increase in brain growth, gliogenesis, increased axonal and dendritic density, and consolidation of the immune system (Catalani, 2002; Kriegstein, 2009; Cowan, 1979, Bockhorst, 2008; Craig, 2003; Holsapple, 2003). Rat postnatal days 20-21 *in general* correlate with human age 2-3 years old, defined by brain development reaching approximately 95% of its adult weight, a peak in synaptic density and myelination rate, and major neurotransmitter and receptor changes (Dobbing, 1973, 1979; Dekaban, 1987; Geidd, 1999; Huttenlocher, 1979; Keshavan, 2002; Hedner, 1986). Rat postnatal days 25-35 *in general* correlate with human age 4-11 years old (preadolescence), a time where specialization of prefrontal cortex structures occurs (Tsujimoto, 2008; Sowell, 1999). Rats PN35-49 *generally* correlate with human age 12-18 years old (adolescence), and rats PN60+ *generally* correlate with humans 20 years or older (adults) (Huttenlocher, 1979; Lidow, 1991; Giedd, 1999; Baloch, 2009; Lebel, 2012). However, these markers are only a guideline. In order to accurately compare specific sensitive periods in brain development between species, more detailed comparisons need to be examined.

As noted above, in the rat, the cerebellum develops later than the rest of the brain, with the first three postnatal weeks being the most important to its development. One meta study by Clancy (2003) created a timetable comparing key developmental events across multiple mammalian species. It shows that the peak neurogenesis for the inferior

olivary nucleus for humans is gestational day 40, peak neurogenesis for Purkinje cells is at human gestational day 47, and neurogenesis for the deep cerebellar nuclei is at human gestational day 47.2. This does not speak to the maturation of Purkinje cells or the rest of the cerebellum. Most of the studies comparing human brain development to other mammals focus on the hippocampus or frontal cortex, and the cerebellum is often not described.

While we can make the general statement that in rats the second and third postnatal week (PN7-21) correspond to human full term birth to toddler (gestational day 36 to 2-3 years), there is missing information regarding specific timing of developmental events and the factors that influence them. Thus it is important to examine additional markers of development, such as changes in gene expression, in both rats and humans to describe more accurate correlations between these two developmental timelines.

Cerebellar Function and Pathology

Function

Initially the cerebellum was believed to be separate from the brain. Although several scientists gave detailed accounts of its anatomy, it was not until the early 19th century that its function began to be understood. In 1809 Luigi Rolando discovered that cerebellar damage results in motor disturbances, and Jean Pierre Flourens' experiments on cerebellar lesions in animals revealed that the animals could still move, but their fine movement was disrupted (lack of coordination, awkward movements, imbalance) (Ito, 2002). Motor function was confirmed and widely accepted by the 20th century, in part thanks to clinical observations of World War I soldiers with cerebellar lesions (Holmes,

2007). The cerebellum does not initiate movement, but it contributes to coordination, precision, and accurate timing to fine-tune motor responses. It is also necessary for several types of motor learning, most notably adjusting to changes in sensorimotor input (Ghez, 1985; Fine, 2002).

Although best known for its role in motor control, the cerebellum has begun to be appreciated for its multi-faceted role in non-motor functions. Functional magnetic resonance imaging (fMRI) is used to study changes in brain activity during a given task. For a long time, activity in the cerebellum during these tasks was ignored as simply activation of regions required to move the muscles (eyes, mouth, vocal chords, etc.) required for motor planning or execution of task. One of the first hints that the cerebellum had a cognitive role was the discovery that during an fMRI session, different areas of the cerebellum showed activity during a task requiring verb generation related to a given noun as compared to areas that showed activity when simply reading or repeating nouns (Petersen, 1989). Examinations of humans with lesions of the cerebellum revealed that in addition to motor deficits, many patients also exhibited impairments in many non-motor functions such as attention, procedural memory, working memory, language, and visual-spatial processing (Schmahmann, 1998; Timmann, 2007), further suggesting a role for the cerebellum in higher functions. Results from human lesion studies are varied, however, due to large variation in the location and degree of lesions, making it difficult to pinpoint which areas of the cerebellum are associated with which functions. Further, evidence that the cerebellum has a role in normal cognitive development is also beginning to emerge: children with a history of cerebellar tumors exhibit a variety of intellectual, emotional, and educational impairments (Cantelmi, 2008), and as we will

outline later, cerebellar pathologies have been associated with disorders such as autism and schizophrenia. The precise nature and mechanism of these functions remains poorly understood.

Output of the cerebellum targets multiple non-motor areas of the prefrontal and posterior parietal cortex in addition to cortical motor areas (Strick, 2009). The visual, auditory, and somatosensory cortices project to the cerebellum via the pontine nuclei. Association cortices, regions of the cerebral surface responsible for coordination of input from primary sensory cortices and planned behavioral output, project to the cerebellum via the superior colliculus. These indirect connections between sensory input and behavioral output make the cerebellum ideally situated to participate in sensory modulation and the integration of multisensory information (Parsons, 1997; Kern, 2002). The cerebellum's bidirectional connection with the limbic lobe, amygdala, septal nuclei, thalamus and hypothalamus all point to a role for the cerebellum in emotion processing, something also suggested by the blunting of emotional affect observed following lesions to the cerebellar vermis (Konarski, 2005). The corticothalamic- cerebellar-cortical circuit has also been suggested to have a role in more cognitive tasks such as coordinating thought and execution of motor movement (Andreasen, 1996) and working memory (Bellebaum, 2007). Additionally, indirect connections to the medial prefrontal cortex (Hayhow, 2013; Rogers, 2013), an area implicated in social play behavior (van Kirkhof, 2013), together with connections to the amygdala, an area involved in processing memory and emotional reactions (Bzdok, 2012), suggests a potential role for the cerebellum in social interaction (Berntson, 1980; Tavano, 2007).

Cerebellum and neuropsychiatric diseases

Typical pathologies of the cerebellum are motor-related, and will have different symptoms depending on which regions are damaged. Common motor pathologies include loss of equilibrium, altered walking gait, and difficulty balancing (Ghez, 1985). Errors in skilled voluntary and planned movements are also common, and can include 1) hypotonia - decreased muscle tone, 2) dysarthria - problems with speech articulation, 3) dysmetria - problems judging distances or ranges of movement, 4) dysdiadochokinesia - inability to perform rapid alternating movements, and 5) tremors (Schmitz, 2007). Depending on the location and extent of cerebellar damage, these motor disruptions can be localized to a portion of the body or limb or more wide-ranging. It is becoming more and more apparent, however, that beyond disrupted motor control pathologies of the cerebellum have also been closely associated with neuropsychiatric diseases, including Schizophrenia, Autism Spectrum Disorder (ASD), and Attention Hyperactivity Disorder (ADHD).

Schizophrenia generally manifests in the late teens or early twenties. It is a neurodevelopmental disease characterized by both positive symptoms (sensory hallucinations, delusions, disorganized speech, disorganized behavior, or catatonia) and negative symptoms (flattening of affect, poverty of speech, lack of social interest, general apathy, and general inattentiveness). Three chromosomal regions have surfaced as strong linkage targets for genetic susceptibility loci for schizophrenia: chromosome 6 (6p24–22), chromosome 1(1q21–22), and chromosome 13 (13q32–34). Specific genes in these regions include Neuregulin 1 (NRG1), dystrobrevin-binding protein 1 (DTNBP1), zinc finger binding protein 804A (ZNF804A), and the calcium channel, voltage-

dependent, L type, alpha 1C subunit (CACNA1C) (O'Donovan, 2003; O'Donovan, 2009). Although there is a strong genetic component in schizophrenia, there are also environmental influences and the exact cause is unknown. Environmental insults can include exposure to toxins, drug use, and prenatal stressors (van Os, 2009), specifically early life inflammation (Clarke, 2009; Li, 2009; reviewed in Fan, 2007).

The cerebellum has been implicated in schizophrenia both histologically and functionally. Cerebellar atrophy has been reported in schizophrenic patients (Weinberger, 1979) and levels of synaptic proteins in the cerebellum are reduced in schizophrenic patients relative to controls (Mukaetova-Ladinska, 2002). Functional magnetic resonance imaging (fMRI) scans during a Wisconsin Card Sorting Task indicate schizophrenic patients have reduced cerebellar activation along with reduced cognitive flexibility (Riehmman, 2001).

Another neurodevelopmental disorder with genetic and environmental components, ASD is characterized by inappropriate social interactions, impairments in social communication, repetitive and stereotyped behaviors, resistance to change, and abnormal responses to sensory stimuli. ASD is generally diagnosed between 18 months and three years of age, and is 3-4 fold higher in males than females (Berg, 2009). Several genetic mutations have been associated with autism spectrum disorder but these only account for a small fraction of ASD cases. Rett syndrome is a neurodevelopmental disorder characterized by normal initial development that slows and reverses, leading to the loss of the purposeful use of hands and controlled movements. Children in early stages of Rett syndrome exhibit autistic-like behaviors, and for this reason it was categorized within autism spectrum disorder until its removal in the Diagnostic and

Statistical Manual of Mental Disorders, 5th edition (DSM-V). The genetic cause of Rett syndrome is spontaneous mutations in methyl CpG binding protein 2 (MECP2).

Although Rett syndrome is considered distinct from ASD, increased MECP2 expression has been found in the cerebella of autistic patients (Zhubi, 2014). In addition to MECP2, a few other genetic candidates for ASD appear promising. Genetic polymorphisms of cytochrome p450 27B1 (CYP27B1), necessary for proper vitamin D metabolism and thus normal neuronal growth and development, have been implicated in autism spectrum disorders (Currenti, 2009). Significant down-regulation of glial glutamate transporters, GLT1 and GLAST, leads to autistic-like and schizophrenic-like behaviors in a mouse model as well (Tanaka, 2009).

Although there are many genetic suspects for risk of autism, environmental factors seem to be a stronger influence. Exposure to mercury, cadmium, nickel, trichloroethylene, and vinyl chloride increase the rate of *de novo* mutations, and an increase in pre-conception exposure appears to be associated with increased risk for autism (Kinney, 2009; Aschner, 2009). Early life inflammation has been strongly linked to increased risk in autism as well (Torres, 2003; Cohly, 2005; Persico, 2006). With diagnosis applying to a spectrum of disorders, it's no surprise that symptoms and pathologies are widely varied, though cerebellar pathology is one of the most consistent findings. MRI studies revealed that some autistic patients have reduced cerebellar volume, particularly in the posterior vermis (Courchesne, 1987, 1988; Murakami, 1989; Levitt, 1999). Postmortem studies revealed that some autistic patients have a reduced number of Purkinje cells (Courchesne, 1991) and granule cells (Heh, 1989; Palmen, 2004). Functional differences exist in the cerebella, too: during fMRI, autistic patients

have more activation in their cerebella during motor tasks, but less activation during attentional tasks compared to unaffected individuals (Allen, 2003; Hadjikhani, 2007).

ADHD is a milder neurodevelopmental disorder, characterized by hyperactivity, impulsivity, and attention deficits. It is generally diagnosed in young children and commonly comorbid with autism (Hofvander, 2009). ADHD has a stronger genetic component than either schizophrenia or autism. Genes associated with this condition include those involved in dopamine neurotransmission (DAT1, DRD4, DRD5, TAAR1, MAOA, COMT, and DBH) (Kebir, 2009; Berry, 2007; Sotnikova, 2009). Environmental factors, including prenatal exposure to alcohol or tobacco smoke, appear to play a smaller role in ADHD (Burger, 2011; Abbot, 2012). Children with ADHD have been found to have smaller cerebella than controls, with a correlation between the magnitude of volume reduction and severity of attentional deficits based on clinical ADHD ratings (Schneider, 2006). Patients with ADHD also have reduced blood flow to the cerebellum, evidenced by PET scan (Kim, 2002).

All three of these neurodevelopmental disorders are diagnosed relatively early in a person's life, usually from childhood to late teens, and all three have both environmental and genetic risk factors. These disorders exist along a spectrum with variations in severity, symptoms, and potential causes. Since exact causes are currently unknown, treatments are not well established or always successful.

Inflammation, Hormones, and the Cerebellum

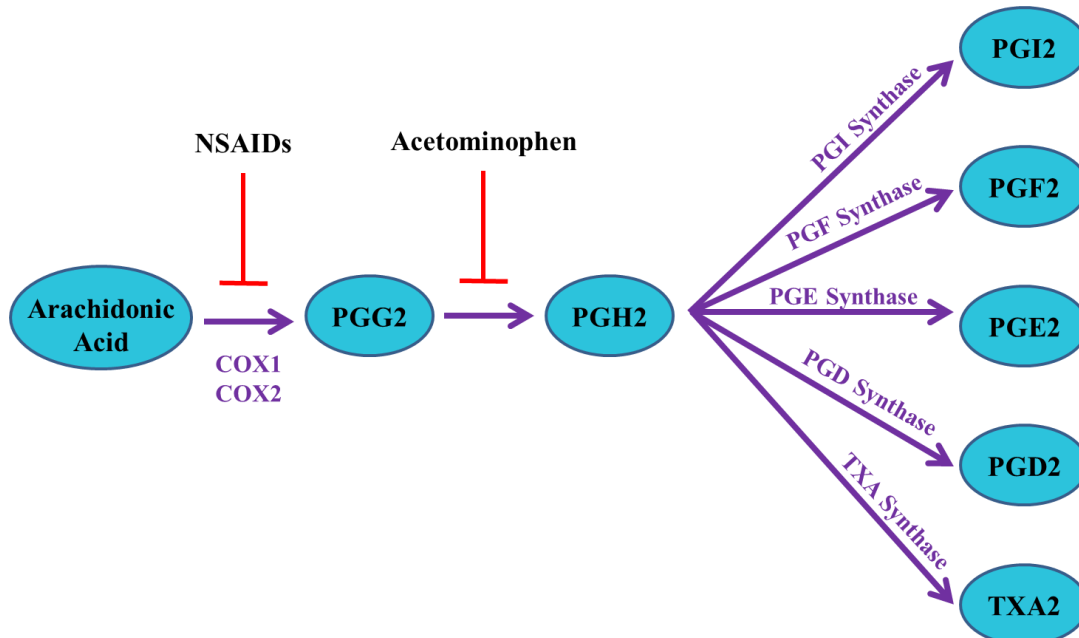
Neuroinflammation and pro-inflammatory molecules

Lipopolysaccharides (LPS), also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond, and found in the outer membrane of Gram-negative bacteria, such as *E. coli* (Rietschel, 1994; Kentner, 2010). The toxic activity of LPS was first discovered and termed "endotoxin" by Richard Friedrich Johannes Pfeiffer. Endotoxins are released only after destruction of the bacterial cell wall, while exotoxins are actively released by live bacteria into the environment (Subash, 2009). LPS acts by binding the CD14/TLR4/MD2 receptor complex in many cell types, but especially in monocytes, dendritic cells, macrophages and B cells. This elicits a strong immune response by promoting the secretion of pro-inflammatory cytokines, nitric oxide, and eicosanoids (Abbas, 2006), as well as a fever response because of its actions as an exogenous pyrogen. Scientists have been using LPS in research for several years because it safely mimics a bacterial infection, inducing inflammation without actually making the animal sick. Humans are much more sensitive to LPS than other animals (e.g., mice). A dose of 1 µg/kg induces septic shock in humans, but mice will tolerate a dose up to a thousand times higher (Warren, 2010). In rats, doses of LPS between 1 µg/kg and 250 µg/kg have been shown to exhibit sickness behaviors, changes in the hypothalamic-pituitary adrenal axis, and immune system responses similar to those in humans (Romanovsky, 2005; Bison, 2009).

Prostaglandins (PGs) are membrane bound lipid-derived compounds produced throughout the body and have a wide variety of effects, including regulation of inflammation, fever, cell growth, and hormone levels (Randy, 2005). They were first

described in 1935 as an unknown substance contained in human seminal fluid that acted as a powerful depressor of blood pressure (Goldblatt, 1935). The name was coined a year later based on the (incorrect) assumption that these compounds were produced by the prostate. Arachidonic acid is converted to the precursor prostaglandin G₂ (PGG₂) by cyclooxygenase isoenzymes 1 and 2 (COX1/COX2) (Rouzer, 2009; Hla, 1992). PGG₂ is quickly converted to PGH₂, which is then converted by different PG-synthases to one of eight compounds in the prostaglandin family. The most common are PGI₂, PGF₂, PGE₂, PGD₂, and TXA₂. Synthesis of all the prostanoids can be disrupted either by inhibiting the COX1/2 enzymes with non-steroidogenic anti-inflammatory drugs (NSAIDs) (Helleberg, 1981), which include aspirin, ibuprofen, naproxen, indomethacin, and nimesulide, or by the non-NSAID anti-fever medication acetaminophen (Figure 1.5).

Figure 1.5: A simplified diagram of prostanoid synthesis from arachidonic acid. Pertinent enzymes are highlighted (purple arrows). Prostaglandin synthesis can be disrupted by inhibiting the enzymes COX1 and COX2 with compounds such as NSAIDs; common over the counter examples include aspirin, ibuprofen and naproxen, while indomethacin and nimesulide are commonly prescribed. Acetaminophen can also disrupt prostanoid synthesis by inhibiting the conversion of PGG₂ to PGH₂.

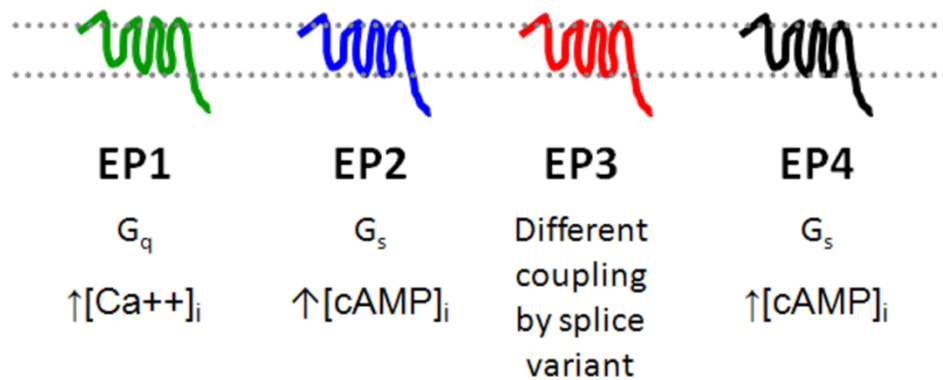


PGE2 is elevated throughout the brain following inflammation, rising immediately in the hypothalamus to initiate the febrile response. This can be mimicked in animal models by administration of isolated LPS, which activates the Tpl2 receptor and increases COX2 expression (Elioupoulos, 2002). In addition to mediation of the fever response, prostaglandins can also influence other normal brain function, including hippocampal long-term potentiation (Yamagata, 1993) and learning (Shaw, 2003). Further, prostaglandins play a fundamental role in the development of the male preoptic area, a critical brain region for the control of sexual behavior by increasing dendritic spine density in males during early postnatal development after activation by estradiol (Amateau, 2002, 2004; Todd, 2005; Burks, 2007; Wright, 2008).

PGE2 can bind to at least four receptor subtypes, EP1-4, all of which are G-protein coupled receptors (GPCRs) (Figure 1.6). GPCRs are a class of membrane receptors with seven transmembrane domains that activate G-proteins (proteins with GTPase activity). Different subtypes of G-protein activate different enzymes, producing a variety of intracellular second messengers, which in turn activate intracellular kinase proteins. EP2 and EP4 are the two PGE2 receptors with the most similar second messenger signaling and pharmacological activity. Both are coupled to a G_s protein which activates adenylyl cyclase to produce cyclic adenoside 3',5'-monophosphate (cAMP), activating protein kinase A downstream (Regan, 2003). EP2 is capable of inducing a greater cAMP response than EP4, due in part to desensitization of EP4 and a greater affinity of EP2 for PGE2 than EP4 (Fujino, 2003; Bastepe, 1999). Additionally, EP4 can couple with phosphatidylinositol 3-kinase (PI3 kinase), which activates protein kinase B- α (Akt) and modulates mitogen activated protein kinases (MAP kinase) (Fujino,

2003). EP1 couples to G_q , which activates phospholipase C- β (PLC- β). Its downstream pathway results in increased cellular concentrations of calcium (Ca^{++}). EP1 also has the lowest affinity for PGE2 (Sugimoto, 2007). EP3 is the most complex of the PGE2 receptors, capable of binding several G-proteins. When coupled to G_i , it inhibits adenylyl cyclase and decreases cytosolic cAMP, recruiting protein kinase C (Hatae, 2002a). When coupled to G_s , however, it actually activates adenylyl cyclase, increases cAMP, and recruits protein kinase A (Ito, 2000). EP3 can also couple with $G_{12/13}$, modulating small GTPases of the rho kinase family, which are important for neurite outgrowth and retraction (Aoki, 1999). To further complicate things, EP3 and EP4 can also interact through protein cross-talk and reinforce EP4- G_s signaling rather than inhibiting it.

Figure 1.6: PGE2 receptors and their downstream second messengers. EP1 acts through G_q to increase intracellular concentrations of Ca^{++} . EP2 and EP4 both act through G_s to increase intracellular cAMP. EP3 actions vary by splice variant; it can act through G_i to decrease intracellular cAMP, G_s to increase intracellular cAMP, or $G_{12/13}$ to modulate members of the Rho family.



Introduction to neuroendocrinology

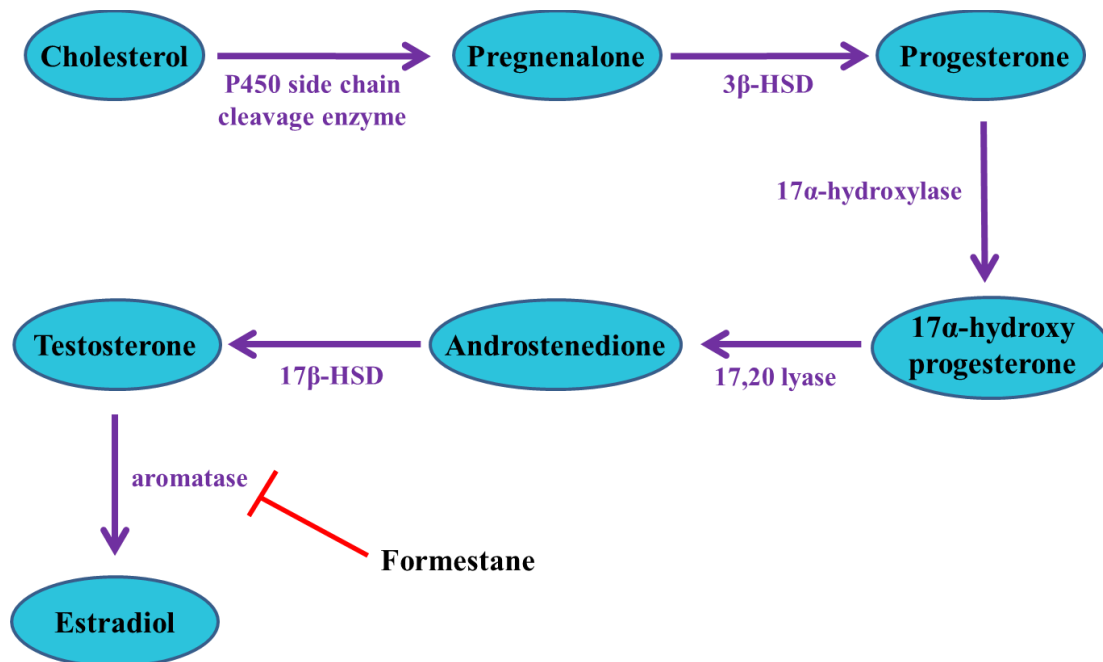
In the mid-1700s it was proposed that the function of endocrine organs, described by anatomists in third century BC, was to produce and secrete something of importance

to male development. John Hunter in 1792 and Arnold Berthold in 1849 performed ground breaking experiments using castration of chickens to demonstrate this idea experimentally. When castrated, a male chicken's comb atrophies, but if the testes are replaced by implantation elsewhere in the body, the atrophy is rescued. In the 1900s anatomists began to understand that the endocrine and brain systems were connected, and the significance of this interaction is still being explored today. They discovered that nerve fibers terminate around secretory cells in endocrine organs, which could be stimulated to induce hormone release, and further that these hormones in the circulatory system could then affect parts of the nervous system. Testosterone, the main steroid hormone produced by male testes, is capable of masculinizing female guinea pig pups when injected into pregnant dams. Not only did it masculinize their external genitalia, but it also eliminated female sexual behavior in adulthood and instead induced male sexual behavior when testosterone was administered to the females in adulthood (Phoenix, 1959). This introduced the concept of two separate endocrine actions on the brain: 1) organizational effects, which happen during early development and shape the nervous system to determine what behaviors the animal will be capable of as an adult, and 2) activational effects, which occur in adulthood and require hormones to be present to induce these behaviors. The process of sexual differentiation involves testosterone synthesized from the testes and released into the bloodstream during late gestation and again shortly after birth, acting at a distance in the brain. While in humans testosterone is the masculinizing hormone, in some animals such as rats it is actually estradiol that is responsible for masculinization. During early development, the surge of testosterone is enzymatically converted to high levels of estradiol which masculinizes the brain, and

female rats are protected from this mechanism by the estrogen-binding protein alpha-fetoprotein (McEwen, 1977b; Krey, 1979; Goy, 1980; Carter, 2002).

Estradiol, progesterone, and testosterone are all steroid hormones synthesized from cholesterol. Steroidogenesis begins when cholesterol is transferred from the outer mitochondrial membrane to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) and then cleaved to form pregnenolone, precursor of the steroid hormones, by cytochrome P450 side chain cleavage (P450_{scc}). Aromatase is the enzyme responsible for converting testosterone to estradiol (E2) (Naftolin, 1994) (Figure 1.7).

Figure 1.7: A simplified diagram of estradiol synthesis from cholesterol. Pertinent enzymes are highlighted (purple arrows). Estradiol synthesis can be disrupted by inhibiting the enzyme aromatase with compounds such as formestane.



These hormones are best known for their role in sexual behavior and fertility in adult humans and animals. However, they are also active players in the normal development of the central nervous system (for a detailed review see McEwen, 2002). Estradiol changes dendritic spine number and dendrite branching in brain regions such as the male hypothalamus (Schwarz, McCarthy 2008), and is involved in the formation and breakdown of excitatory synapses in the hippocampus (Woolley, 1990). Estrogens are also neuroprotective; they protect nerve cells from excitotoxins and free radicals (McEwen, 1999a; Lee, 2001). Estrogens have effects in a wide range of brain regions and systems, including the basal forebrain cholinergic system, the hippocampus and cerebral cortex, the caudate-putamen, midbrain raphe and brainstem locus coeruleus, and the spinal cord. Through these systems estrogen affects mood, locomotor activity, pain sensitivity, vulnerability to epilepsy, and attentional mechanisms and cognition (for further review, see McEwen, 1999b).

Over the past decade or so, understanding of the mechanisms of estradiol action and the biological processes it is involved with has greatly expanded (McCarthy, 2004; Boulware, 2005; Zhang, 2006). Estradiol has two receptors, ER α and ER β , each encoded by unique genes, with structural and functional domains characteristic of the steroid/thyroid hormone superfamily of nuclear receptors (Gugiere, 1998). ER α and ER β share a high degree of homology in some regions (DNA and ligand-binding domains), and a low degree of homology in other regions (N-terminus). While both receptors share an overlap in ligand affinity and interactions with DNA gene targets, differences in peripheral and brain tissue localization patterns suggest different roles for the two

receptors. In fact, in many cases the receptors act in opposition, with ER β inhibiting ER α -mediated transcription (Matthews, 2006; Jin, 2008).

There are four basic mechanisms of estrogen receptor signaling (for review see Hall, 2001). The first is classical ligand-dependent: upon binding to 17 β -estradiol or related ligands the receptors form homodimers or heterodimers with each other that interact with specific DNA sequences called estrogen responsive elements (EREs) to up- or down-regulate gene transcription and alter subsequent tissue responses. This mechanism is the slowest, taking several hours to days to see changes. The second mechanism of estrogen receptor signaling is ligand-independent, activating mitogen-activated protein kinase (MAP kinase) pathways; in addition to transcriptional activation, this method can also activate other cellular processes that are regulated by the kinase/phosphatase balance (Mannella, 2006; Moriarty, 2006). The MAP kinase activation pathway can occur within seconds to minutes. The third mechanism is ERE-independent signaling in which agonist-bound ER complexes are capable of regulating gene transcription of genes that do not contain an ERE in their promotor region. This occurs through alternative response elements such as AP-1 and their association with other DNA-bound transcription factors, Fos and Jun (Kushner, 2000). The fourth mechanism of estrogen receptor signaling is cell-surface, or nongenomic, signaling. Estrogens activate putative membrane-associated binding sites and intracellular signal transduction pathways that generate rapid tissue responses. Within milliseconds, estradiol is capable of modulating membrane voltage and electrochemical properties of a cell, directly affecting synaptic plasticity (Malyala, 2005; Wu, 2005). Further, estradiol is extremely stable within the body, so its ability to activate signaling pathways can remain

for hours and act upon multiple systems within the brain, making the exact actions of changing estradiol levels more difficult to pinpoint.

Prostaglandins, hormones, and cerebellar development

PGE2 receptors (EP1–4) are expressed in a variety of regions in the developing brain, including the cerebellum (Kaufmann et al., 1997; Tai et al., 1998). While PGE2 and its receptors in the preoptic area have a role in the febrile response, the function of PGE2 receptors in these other brain regions is not currently well known. We are beginning to understand the role of PGE2 in the cerebellum, however. Previously we've shown PGE2 to be involved in Purkinje cell development (Dean, 2012a; Dean, 2012b). We found that during the second postnatal week PGE2 is capable of driving an increase in aromatase activity, resulting in increased estradiol content and stunting Purkinje cell dendrite development. The tentative PGE2-E2 signaling pathway is outlined in Dean (2012b) and discussed in further detail in Chapters 1 and 7. Further, steroid hormones have been implicated in autism (James, 2008; Auyeung, 2009, Dean, 2008, 2012a, 2012b) and a relationship between PGE2 and estradiol in the brain has been established (Amateau, 2002, 2004; Wright, 2008, Dean, 2012a; Dean 2012b).

Estradiol can be synthesized *de novo* in the cerebellum during early development in both males and females. The cerebellum expresses mRNA for both the StAR and P450scc enzymes for conversion of cholesterol to steroid precursors (Lavaque, 2006), as well as aromatase for conversion of testosterone to estradiol (Tsutsui, 2008). The current literature examining developmental expression of these enzymes in the cerebellum is limited in scope and not in complete agreement with the results we present here

(discussed in Chapter 4). Current literature says that in males, mRNA for aromatase peaks between PN5 and 10 and then falls thereafter. In females the peak is smaller in magnitude and mRNA levels do not begin to fall until after PN20. In both male and female rats, real-time polymerase chain reaction (RT-PCR) shows this mRNA expression occurs specifically in Purkinje cells (Sakamoto, 2003). RT-PCR analysis showed that the expression of 3 β HSD mRNA in the cerebellum of males and females is higher at 7–14 days of age than at other times (Ukena, 1999). Estradiol levels have been reported to be higher in the cerebellum than in the plasma during the first week of life (5.92 pmol/g versus 1.97 pmol/g on PN5) but not thereafter and no difference between the sexes has been found, although the PN10 time point was not examined (Sakamoto 2003).

Both estradiol receptors are also present in the cerebellum. ER α protein is detected as early as PN3 in scattered Purkinje cells in both sexes (Perez, 2003). ER α mRNA levels in the cerebellum are high during the first three postnatal weeks (PN7, PN14, and PN21) with a peak in the second postnatal week (PN14) and regardless of sex. In adulthood expression is almost negligible (Ikeda, 2006). ER β protein begins to appear in the Purkinje layer at the end of the second week, concentrated first in the soma and spreading out to more distal ends of the dendritic tree. In adults it's expressed in the cytoplasm of most Purkinje cells as well as some cells in the granule cell layer (Price, 2000). ER β mRNA expression is low through most of the first postnatal week, peaks on PN10, then falls to adult levels (Jakab, 2001), but according to Ikeda (2006) ER β mRNA is actually not significantly different from adult levels at any of the first three postnatal weeks (PN7, 14, 21) regardless of sex. ER β protein is also seen in immature, migrating

glia during the first week of life, but not in adults (Price, 2000). No sex differences have yet been reported in ER α or ER β expression levels or their distribution in the cerebellum.

The current understanding of neonatal expression of the synthesis of prostaglandin, estradiol, and their receptors in the cerebellum is patchy and incomplete. Many of the neonatal time points examined are not directly comparable, focusing either on a few days concentrated in the first week of life, or a single time point for each of the first three postnatal weeks. Further, most studies look at only one or two genes in the same animals at a time and do not provide a comprehensive picture of how these genes may be changing in relation to each other, especially since males and females are not always compared. A methodical examination of all these prostaglandin and estradiol components in both males and females across an expanded postnatal timeline is necessary to understand their role in cerebellar development of the rat.

Goals of Dissertation and Experimental Approach

While evidence from our laboratory and others indicate that endogenous prostaglandins play a role in normal cerebellar development and behavior, the exact signaling mechanism is not fully worked out. The second postnatal week in the rat cerebellum is the most dynamic, and subject to both intrinsic and extrinsic factors that could affect development. However, it is unknown whether the second postnatal week represents a sensitive period for PGE₂-E₂ signaling implicated in cerebellar development and ultimately behavior. There is growing evidence that early life inflammation is a risk factor for neurodevelopmental diseases such as schizophrenia and autism. There is also a strong connection between these diseases and pathology of the cerebellum. Our model of

early life LPS exposure altering cerebellar pathology and behavior in the rat provides an interesting bridge between these two findings. Given the highly dynamic developmental period of the cerebellum during the second postnatal week, it is also possible that the timing of the LPS exposure lends itself to a greater risk of developing these disorders.

The experiments here were designed to achieve five major goals:

- 1) To confirm previous findings and further characterize the normal PGE2-E2 signaling pathway in normal cerebellar development in relation to exogenous inflammatory insults.
- 2) To determine whether a specific sensitive period around the second postnatal week exists for exogenous influence on the PGE2-E2 pathway in the cerebellum.
- 3) To examine changes in the expression of pathway components across time to identify which, if any, are involved in the initiation and termination of the sensitive period.
- 4) To characterize and then prevent specific behavioral deficits relevant to neurodevelopmental disorders following perturbation of the PGE2-E2 pathway mediating the sensitive period in cerebellar development.
- 5) To relate the sensitive period of the PGE2-E2 pathway of the rat to the timing of human development.

To achieve these goals, we examined the Sprague Dawley rat across the first three postnatal weeks of life for both normal changes in components of the signaling pathway and reactions to its perturbations. We mimicked an inflammatory insult either by treating pups with LPS or PGE2 and examined changes in the biochemical pathway, Purkinje cell

morphology, and a battery of behavioral tasks. We attempted to prevent behavioral deficits by preventing perturbations in the signaling pathway caused by this inflammation either with traditional anti-inflammatory drugs (nimesulide) or aromatase inhibitors (formestane).

Specifically, we predicted that systemic LPS would increase PGE2 production and have similar effects on the biochemical signaling pathway as we'd seen previously with PGE2. We hypothesized that the second postnatal week (PN7-14) in the rat cerebellum is a sensitive period for exogenous influence on the PGE2-E2 pathway. From this we predicted that LPS would increase PGE2 and E2 levels when treated in week 2, but not when treated in weeks 1 or 3, and decrease Purkinje cell dendrite length only when treated in week 2, but not when treated in weeks 1 or 3, and that these effects can be prevented. Further, we hypothesized that one or more components of the PGE2-E2 signaling pathway in the rat cerebellum exhibit significant changes in expression and/or activity over the course of the first three postnatal weeks of life. We predicted that mRNA expression of at least one gene would increase or decrease in such a way that it could frame the sensitive period we found in week 2 (PN7-14), and from this discovered four key candidates for driving the sensitive period: COX2, EP4, Aromatase, and ER α . Finally, we hypothesized that abnormalities in Purkinje cell development from exogenous insults only during the sensitive window in postnatal week 2 would correlate with behavioral impairments relevant to neurodevelopmental disorders such as autism spectrum disorder and schizophrenia. We predicted PGE2 and LPS would both result in behavioral impairments and correlate with changes in Purkinje cell morphology. We further predicted that these impairments could be prevented by blocking the LPS-induced

increase in E2 synthesis. Finally we predicted these morphological and behavioral impairments would be limited to the same week 2 sensitive period of the PGE2 – E2 pathway.

Chapter 2: General Methods

Animals

All animal use was conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and accepted standards of humane animal care. Female Sprague Dawley rats were mated in our animal facility, and pregnancy confirmed by vaginal smear, or ordered by pregnancy date at least 3 days before giving birth (Charles River Laboratories, Wilmington, MA). Pregnant females were isolated and allowed to deliver normally. Animals were maintained on a 12:12 reverse light cycle and had *ad libitum* food and water. Cages were checked daily for the presence of pups in order to determine day of birth (PN0). Dams generally delivered early in the morning. Litters were culled to no more than 12 pups in order to ensure sufficient resources for the health of both the dam and pups. In cases of animals reared for behavior, with 4-5 litters in a cohort, animals were divided evenly across sex and cross-fostered to avoid confounding variables of maternal rearing. Pups remained with their dams until they were euthanized or reached postnatal day 21 (PN21), at which point they were weaned. Weaned pups of the same sex were housed in groups of 2-3 in a cage.

Injection methods and timing

In general, treatment paradigms fell within one of three time frames: 1) **week 1** – between birth (PN0) and postnatal day 6 (PN6); 2) **week 2** – between PN7 and PN13; or 3) **week 3** – between PN14 and PN20. Tissue was either collected after a short time course, at the end of each week (PN7, PN14, or PN21), or they were reared for behavioral

testing and collected afterward. Exact timing and frequency of each injection paradigm varies by experiment and is addressed in the methods section of later chapters.

Lipopolysaccharide (LPS) from *E. coli* was suspended in pyrogen-free saline and given to pups via intraperitoneal (i.p.) injection at a dose of 200 µg/kg rat weight unless otherwise stated. This form of injection required no anesthesia.

Prostaglandin E2 (PGE2) was suspended in pyrogen-free saline at a concentration of 2.5 µg/ 1 µl and given to pups via an intracranial injection through the Foramen Magnum (F.M.) at a volume no larger than 1.0 µl per injection. Pups under PN7 were cryoanesthetized by being placed on aluminum foil on ice in a refrigerator for 10-20 minutes. These pups were kept on a cold pack covered in foil to keep them anesthetized during the injection. Pups older than PN7 were anesthetized briefly in a chamber of isoflurane for 2-3 minutes until no longer responsive to tail-pinch prior to injection. All pups were placed on a stereotaxic apparatus to angle the head at the ideal position for injection into the Foramen Magnum. No incision was made as the Foramen Magnum is easily felt as a soft spot at the base of the skull. A 1.0 µl Hamilton syringe with a fixed 30 g needle was attached to the stereotaxic apparatus and lowered until the skin and skull were pierced by the needle and then further lowered to stop just below the skull. Test injections with ink found this injection site to bathe the cerebellum. A volume of 1.0 µl containing drug or vehicle was injected over a 30 second period, the needle left in place for an additional 30-45 seconds and then retracted. The animal was warmed under a heat lamp and when fully active, returned to the dam. This procedure can be performed twice in one day with no undue harm to the pup. Entire duration away from the dam was less than 1 hr. In our laboratory, survival of this procedure is nearly 100%.

Nimesulide was suspended in inert sesame oil at a concentration of 5 mg/ml and injected subcutaneously (s.c.) in the scruff of the back at a volume no larger than 0.1 ml. This form of injection required no anesthesia.

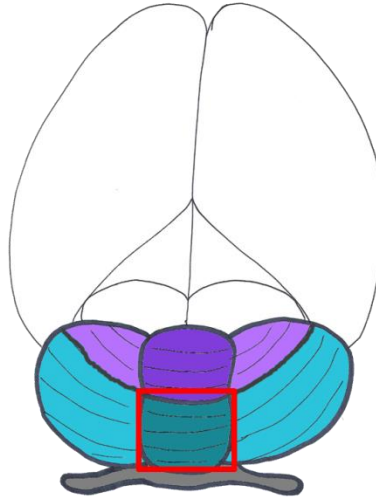
Formestane was suspended in inert sesame oil at a concentration of 100 µg / 0.1 ml oil and injected subcutaneously at a volume no larger than 0.1 ml. This form of injection required no anesthesia.

All animals received a single injection of India Ink to the pad of a paw for group identification.

Dissection and collection of cerebellar tissue

For animal tissue, animals were euthanized by lethal overdose with a pentobarbital sodium solution (Fatal Plus, Vortech Pharmaceuticals). Animals were then decapitated and the skull cap was removed using a sharp pair of scissors. The whole brain was removed from the skull and the cerebellum separated from the cortex and brain stem via razor blade. The cerebellum was microdissected on a cold block. The posterior vermis was identified by the raised midline region of the cerebellum caudal to the primary fissure and was separated from the rest of the cerebellum by making a cut at the primary fissure and two more cuts on either side to separate the vermis from the lobes of the lateral hemisphere on either side (Figure 2.1). Tissue was immediately flash frozen and stored at -80°C until homogenization. Unless otherwise stated, homogenization was performed using a Bullet Blender (Next Advance, Averill Park, NY) and 1.0 mm Zirconium Oxide beads and examined visually to ensure no solid pieces remained.

Figure 2.1: A visual representation dissection of the rat posterior vermis. Figure modified slightly from Chapter 1 - anterior lobes in purple, posterior lobes in teal, further subdivided into the vermis (dark colors) and the lateral hemispheres (light colors). The area collected for analysis is highlighted in the red box.



Protein Quantification

Sample preparation for protein quantification

Collected tissue was homogenized in cold RIPA buffer (50 mM Tris-HCl, 1% Na-deoxycholate, 0.25% NP-40, 150 mM NaCl, 1 mM EDTA) and protease and phosphatase inhibitors (1 μ g/ml of aprotinin, leupeptin, and pepstatin; 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 3000xg. The supernatant was collected and protein concentration determined by Bradford assay.

Bradford assay

Known quantities of bovine serum albumin were used to create a protein standard curve for analysis. After vortexing, 5 μ l of each sample was removed and diluted in 95 μ l of distilled water. 20 μ l of each standard or diluted sample was pipetted in triplicate onto

a microplate and 150 μl of Bradford reagent was added to each well. Absorbance was read at 595 nm wavelength, and protein concentration of the samples was determined based on known values obtained from the standard curve. The upper and lower limits of detection for the assay were 8 to 0.5 μg protein per μl of undiluted sample, respectively. Most samples fell in the range of 2 to 5 $\mu\text{g}/\mu\text{l}$.

Western blot

Using the Bradford Assay to determine total protein concentration of a sample, 10 μg of protein were loaded in a 20 μl volume of water for electrophoresis on an SDS-polyacrylamide gel (8-16%) and transferred onto a polyvinylidenedifluoride membrane. Membranes were incubated for one hour at room temperature with gentle shaking in a 5% non-fat milk and tris-buffered solution (TBS) to block nonspecific binding. Membranes were then incubated with a primary antibody in 2.5% milk overnight at 4°C on a shaker. Following a 30 minute incubation with an HRP-linked secondary antibody, immunoreactive bands were then detected using chemiluminescence (ECL-Cell Signaling) and exposed to film (Hyperfilm-ECL; Amersham Pharmacia Biotech).

The integrative grayscale pixel area-density (IAD) was captured using a CCD camera, and analyzed using NIH Image. Integrative density is the sum of pixel values minus a background value for each pixel within a bounded area, and increased dark value or size of the band correlates to increased protein amount and higher integrated density measurements. This allows for semiquantitative analysis of protein levels; absolute value of protein present cannot be determined, but comparisons between bands within an experiment give an accurate indication of differences in relative expression. Whenever

possible an entire experiment was fit onto a single immunoblot membrane. However, if more than one membrane was required, samples were evenly distributed by treatment group across the membranes, and contained a subset of the same samples on each membrane to allow for a correction factor using the IAD of these “control” samples.

To correct for variations in protein loading, following film exposure membranes were rinsed and incubated up to two minutes with 0.1% Ponceau S in 5% acetic acid to visualize a band of accepted housekeeping proteins. Blots were then washed to remove background and IAD of these ponceau bands was measured for each lane. Target protein IADs were then normalized to the ponceau IADs as a loading control before further analysis.

Estradiol Quantification

Sample preparation for estradiol extraction

Tissue was collected, homogenized, and assayed for total protein concentration via Bradford assay as described previously. An aliquot of 325 μ l homogenized tissue sample was placed in Teflon-capped glass extraction tubes, 4 ml of dry-ice chilled diethyl ether was added, and rotated horizontally for 30 min. Tubes were then placed vertically and allowed to sit upright for 15 min for phase separation. The aqueous phase was frozen in isopentane on dry ice and the organic phase decanted off into glass culture tubes and ether was allowed to evaporate overnight.

Estradiol extraction and analysis

Following complete ether evaporation 2 ml of 5% methanol in water was added to each tube, then gently agitated for 1 hour; steroids previously extracted into the ether were visible as a flaky white precipitate. Each sample was added to a previously conditioned octadecyl C18 minicolumn according to manufacturer's instructions (Amersham Biosciences). Columns were then washed sequentially with 10 ml 40% and 85% methanol to remove sulfate esters and non-conjugated esters. A final 10 ml of 100% methanol was then passed through the columns and collected. Samples were maintained at 37°C to allow methanol to evaporate. Samples were reconstituted in 500µl PBS and stored at -80°C until ready to ship for analysis.

Samples were sent to the Center for Research in Reproduction (University of Virginia, Charlottesville, VA) and analyzed by radioactive immunoassay in duplicate. The sensitivity of the assay was at least 4.7 pg/mg protein, and the intra- and interassay coefficients of variation were less than 6.2 and 15.0%, respectively, for all experiments. All samples had some detectable level of E2, and many were run at a dilution of 1:10 to fit within the detectable range. Reported estradiol values for each sample were then normalized to that sample's protein concentration before further analysis.

PGE2 Quantification

Sample preparation for PGE2 extraction

Tissue was collected, homogenized, and assayed for total protein concentration via Bradford assay as described previously. An aliquot of 100 µl for each sample was transferred to a clean microcentrifuge tube, acidified with 2M HCl to pH3.5, and sat on

ice for 15 minutes. Samples were then centrifuged 2 min (3000 x g) to remove precipitate, and poured onto previously conditioned octadecyl C18 minicolumns according to manufacturer's instructions (Amersham Biosciences). Columns were then washed sequentially with 10 ml water, 10 ml 15% ethanol in water, and 10 ml hexane. Samples were finally eluted into a 12 ml polypropylene tube with 2 ml ethyl acetate and left in an exhaust hood for 2 days to evaporate.

PGE2 extraction and analysis

PGE2 concentration was determined by a PGE2 enzyme immunoassay kit (Arbor Assays). Briefly, a standard curve was created using a known concentration of PGE2. Evaporated samples were reconstituted in 500 μ l assay buffer and 100 μ l of standard or sample was added to a microplate in triplicate. DetectX PGE2 Conjugate and DetectX PGE2 Antibody were added to each well and allowed to mix for 2 hours. After 4 washes with a wash buffer, plates were incubated 30 minutes with TMB substrate, and the reaction stopped with a Stop Solution. Absorbance was read at 450 nm wavelength, and PGE2 concentration of the samples was determined based on known values obtained from the standard curve. The upper and lower limits of detection for the assay were 1000 pg to 31.25 pg PGE2 per μ l of undiluted sample, respectively. Most samples fell within this range and then corrected for the 1:5 dilution in Assay Buffer. PGE2 values for each sample were then normalized to that sample's protein concentration before further analysis.

Aromatase Activity Quantification

Rats were killed by rapid decapitation for tissue collection because the assay of aromatase activity is potentially affected by anesthesia. Whole fresh cerebella were collected in 300 μ l of ice-cold TEK buffer (150 mM KCl, 10 mM Tris-Hepes, pH7.2) and immediately homogenized. Protein concentration was determined via Bradford assay and results reported normalized to protein concentration. Aromatase activity was quantified by measuring tritiated water production by a previously validated method using some modifications (Baillien and Balthazart, 1997). Specifically, 50 μ l of cerebellar homogenate from each animal in triplicate was placed in 1.5 ml tubes. Total volume was brought to 200 μ l, and homogenate was incubated for 24 hours at 37°C with 125 nM 1 β -H³-androstenedione and 2.4 mM NADPH. One sample per triplicate was treated with the aromatase inhibitor letrozol at 5 μ M. The reaction was stopped by cooling the samples in an ice bath and adding 400 μ l of ice-cold 10% trichloroacetic acid containing 2% activated charcoal. The tritiated water produced was purified by centrifugation and column chromatography. Briefly, tubes were centrifuged at 1200 x g for 15 min. Supernatant was applied to small columns made of Pasteur pipettes plugged with glass beads and filled (3 cm high) with a Dowex cation exchange resin AG 50 W-X4, 100-200 mesh (Biorad, Richmond, CA). Columns were eluted with 3 x 0.6 ml distilled water and effluents were collected in scintillation vials. 10 ml Ecoscint A (National Diagnostics, Atlanta, GA) was added and vials counted for 4 min on a LKB Wallac 1218 Rackbeta Liquid Scintillation Counter. Blanks were produced in triplicate by diluting 200 μ l of TEK buffer, 400 μ l trichloroacetic acid, and 1800 μ l water, and average values of the blanks were subtracted from the average value of the samples. A t-test was run to ensure

that the average value of the samples treated with the aromatase inhibitor letrozol was significantly lower than the samples without letrozol.

RT-PCR

RNA extraction

Tissue was collected and flash frozen, stored at -80°C as described above. Tissue was suspended in 1 ml Qiazol reagent at 4°C and homogenized with 1.0mm zirconia/silica beads. Next 200 µl chloroform was added to each tube, shaken, left at room temperature several minutes, and centrifuged at 12,000 x g, 4°C for 15 minutes to separate the phases. The clear supernatant was carefully removed into a new RNase free tube and combined with 600 µl 70% ethanol and mixed thoroughly. RNA was extracted using RNAeasy Mini Kits (Qiagen #74106/74104). Briefly, volumes were loaded onto the mini columns and spun through, discarding the flow through. Columns were washed with RW1, again discarding flow through, incubated for 15 minutes with DNase (Qiagen #79254), and washed with RW1 and RPE, each time discarding flow through. RNA was finally collected by eluting with 40 µl of water. RNA was assessed for purity and concentration by direct application of 1.0 µl in a Nanodrop spectrometer (Wilmington, DE), absorbance read at 260-280 nm and concentrations calculated using the RNA protocol. Samples were diluted to a final concentration of 200-500 ng/µl. Acceptable 280/260 nm purity ratios were between 1.76 and 2.05, and 260/230 ratios were above 0.5 to indicate the removal of organic solvents.

Creation of cDNA by reverse transcription

Single stranded complementary DNA (cDNA) was generated from extracted RNA using the ABI High Capacity cDNA Reverse Transcription Kit #4368814 (Foster City, CA). Volumes were calculated from RNA concentrations to provide 1 µg RNA in 10 µl water, and reverse transcription reagents were added in the following volumes: 2.0 µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2.0 µl of 10X RT Random Primers, 1.0 µl of Multiscribe Reverse Transcriptase, 1.0 µl of RNase Inhibitor, and 3.2 µl of deionized water. The reaction was carried out as follows: 10 minutes primer incubation at 25°C, 120 minutes extension at 37°C, and 5 minutes RT inactivation at 85°C. cDNA was stored at -20°C until use.

Relative real-time PCR

Each of the test samples were diluted 1:3 unless otherwise noted. Next, quantification standards were made by combining all the samples from an experiment in equal amounts; this was the 1:1 dilution. 1:3 serial dilutions were made stopping at 1:243, creating a set of standards (concentrations 1:1, 1:3, 1:9, 1:27, 1:81, 1:243) to create a standard curve and a threshold for detection of amplification product. The volumes of reagents for real-time PCR reactions were as follows: 5 µl of cDNA, 1 µl of 10 µM each forward and reverse primers, 10 µl 2X SYBR green Master Mix (New England Biolabs, Ipswich, MA), 3 µl water. Standards and samples were run in triplicate on a 384 well plate, using an ABI ViiA7 with 384 well block. Reaction protocol was as follows: 1) 10 min at 95°C start, 2) 10 sec at 94°C melt step, 3) 15 sec primer incubation step at varying temperatures depending on amplicon, 4) 15 sec at 72°C extension step, 5) 1 sec at 79°C or

80°C read fluorescence step, 6) cycle from step 2 to step 5 repeating a total of 40 times, 7) 10 min at 72°C product stabilization step, and 8) a melting curve. The melting curve was performed by starting at 65°C and increasing the temperature to 94°C in increments of 0.2°C in 1 sec intervals, stopping between intervals for a fluorescence read. The primer incubation temperatures can be found in Table 2.1. We utilized a SYBR green relative quantification method with a standard curve. In short, the SYBR green fluorophore binds to the minor groove of the double stranded DNA helix, and amplification of DNA during a PCR reaction can be detected after the extension step. Differences in starting quantities of DNA can be analyzed by first selecting a threshold fluorescence level where the standard curve has a threshold to cycle slope of -0.301 (or $\log_{10}2$) and a high degree of correlation ($R>0.98$). Next, the cycle threshold of each unknown sample was compared against the standard curve to quantify the amount of starting material in genomic equivalents. The starting material of the 1:1 standard is arbitrarily defined as 1 genomic equivalent (GE) and the starting material of all samples are expressed relative to this 1:1 standard. To normalize for variations in the amount of DNA loaded, each sample was also run with a reference gene of GAPDH and samples were normalized to this GE. All results are expressed as a ratio of the target gene GE over corresponding GAPDH GE.

To ensure the PCR reaction amplified the target product, data from the melting curve portion of the reaction was examined, comparing the melting temperature predicted for each amplicon by the primer design program to the actual melting temperature.

Primer Design

Primers were designed using Primer Express from Applied Biosystems. Details for each rat primer used can be found in Table 2.1. A few notes on the primers: EP1 has 2 splice variants; EP3 has 3 splice variants (all at 3' end); Aromatase has 2 splice variants; ER α has 4 splice variants; and ER β has 2 splice variants; the primers used for each gene were common to all variants for that gene and thus amplified all variants together. Further, since GAPDH was used as mRNA loading control to normalize all genes, it was run at the same melting temperature as each target gene. Efficiency for GAPDH ranged from 96-98% at 56°C to 96-99% at 60°C. The majority of the amplifications were run at or close to 60°C.

Table 2.1: PCR Primers – rat

Gene	Pubmed Accession #	Forward Primer and start position Reverse Primer and start position	Amplicon length	Primer Incubation Temp (°C)
COX1 (<i>PTGS1</i>)	NM 017043.3	F 5'-AAAGAACCCAATGTCCAGCAAG; 2555 bp R 5'-GGCTCCCAACCAAAATCGTAG; 2706 bp	152 bp	60.8
COX2 (<i>PTGS2</i>)	NM 017232.3	F 5'-TTCCAGTATCAGAACCGCATTG; 1069 bp R 5'-CAGCAAGTCCGTGTTCAAGGA; 1219	151 bp	60.8
Microsomal PGE-synthase (<i>PGES-1</i>)	NM 021583.2	F 5'-GCAACGACATGGAGACGATCT; 263 bp R 5'-CATGGAGAAAACAGGCGAACTG; 465 bp	203 bp	58
EP1 (<i>PTGER1</i>)	NM 013100	F 5'-AAGGCAGTGACAGGTGAAGTG; 453 bp R 5'-CGTCATGGAGAAGATAGGCAGC; 722 bp	270 bp	58.7
EP2 (<i>PTGER2</i>)	NM 031088	F 5'-CTTGCTCTTCTGTTCTTGCCG; 537 bp R 5'-GCTTCTTTTGCTCCGAAGCTG; 741 bp	205 bp	60.8
EP3 (<i>PTGER3</i>)	NM 012704	F 5'-TTGTGTCGCGCAGCTATAGAC; 231 bp R 5'-TCCGAACACTGTCATGGTCAG; 429 bp	209 bp	60
EP4 (<i>PTGER4</i>)	NM 032076	F 5'-TGCTCAGAGAGTCGGAGGACAAT; 1276 bp R 5'-GAAATTCGCAAAGTTCTCAGCG; 1490 bp	215 bp	60
Aromatase (<i>CYP19A1</i>)	NM_017085.2	F 5'-AACGTGAATCAGTGATATTGGAAATG; 1495 bp R 5'-CGTACAGAGTGACGGACATGG; 1561 bp	67 bp	56
ER α (<i>ESR1</i>)	NM_012689.1	F 5'-GAAAGCGGGATACGAAAAGA; 977 bp R 5'-TCTGACGCTTGTGCTTCAACA; 1035 bp	59 bp	60
ER β (<i>ESR2</i>)	NM_012754.1	F 5'-TCTGTCCAGCCACGAATCAG; 1035 bp R 5'-GGCCTGGCAGCTTTTACG; 1093 bp	59 bp	60
GAPDH (<i>Gapdh</i>)	NM_017008.4	F 5'-TGGTGAAGGTCGGTGTGAACGG; 77 bp R 5'-TCACAAGAGAAGGCAGCCCTGGT; 146 bp	70 bp	56-60.8

Purkinje Cell Reconstruction

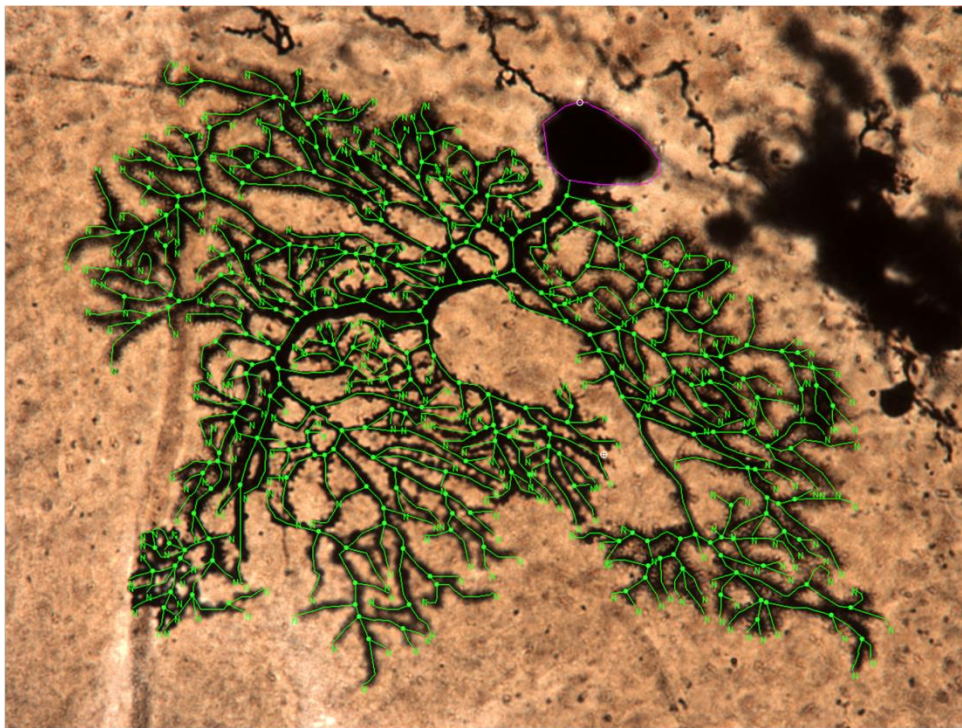
Animals were overdosed with Fatal Plus as for general tissue collection, but instead whole brains were removed and placed in 30 ml of Golgi-Cox solution (1:1 solution of 5% $K_2Cr_2O_7$ and 5% $HgCl_2$ added to 5% K_2CrO_4 in a 4:10 ratio). Brains in solution were stored in the dark at room temperature. Golgi solution was renewed once a week. PN7 brains remained in solution for 12 days, PN14 brains for 16 days, and PN21 or older brains for 21 days before being placed in a 30% sucrose solution for 3 days, cut on a cryostat (Leica CM3050S) at 100 μm , and mounted on 2% gelatin-subbed glass slides. Sections were allowed to dry for a week, then developed in 33% ammonium hydroxide. Sections were counterstained with Methylene blue to distinguish anatomical landmarks. Variations in timing and concentration of Methylene blue resulted in a range of image colors from brown to green to blue, but the neurons were all stained black and background color had no impact on the ability to reconstruct dendritic trees.

Reconstruction of cellular morphology was performed under a Nikon 60X objective using the NeuroLucida system (Nikon Eclipse E600 microscope, MicroBrightField CX900 CCD camera, MicroBrightField, Inc, Colchester, VT). Purkinje cells were identified by their large soma, complex, spiny monodendritic tree, and location in a monolayer between the granule layer and the molecular layer. The soma was traced as a reference location, and the entire dendritic tree was reconstructed from soma to distal ends (Figure 2.2). The NeuroLucida program calculated the sum total of dendrite length traced. Neurons entangled with each other or with a disruption such as a crack in the tissue were excluded. Three - five Purkinje cells from each animal were traced and averaged together for that animal. Individual animals were treated as the subjects for

statistical examination, with 5-6 animals per sex/treatment and combined across sex if there was no significant sex difference.

Images were cropped or digitally enhanced to emphasize visual contrast between cellular morphology and background for the purposes of reprinting, but any changes were made to the entire image and had no impact on reconstruction.

Figure 2.2: A representative trace of a Purkinje cell using the NeuroLucida program.



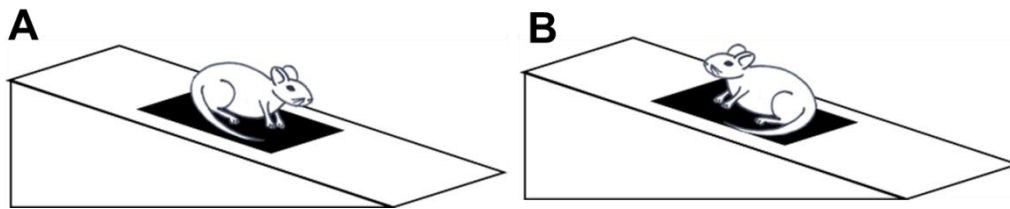
Animal Behavior

Negative geotaxis

The negative geotaxis apparatus is a 6'' wide, 12'' long Plexiglas ramp with an incline of 30° and a rubber pad to prevent slipping. At PN13 rat pups were placed on the center of the pad with their nose pointed down the incline, and the latency to turn 180°

and face up the incline quantified. This is a natural instinct for pups of this age which they perform quickly – generally within 20 seconds. This test examines normal motor control to ensure manipulations of the cerebellum are not affecting general motor ability (Figure 2.3).

Figure 2.3: Representative diagram of the negative geotaxis task – measuring the amount of time it takes for the rat to move from position A) to position B) on the ramp.



Wire hang

The wire hang task utilizes a 2mm gauge, 120cm long wire suspended 60cm above the ground with sufficient padding under the wire to prevent injury when the animal drops. At PN19 rat pups had both front paws placed on the wire, and once they grip, a 2 minute maximum hang time was started and the animals scored for how long they hung on before they dropped. If they have not let go by the 2 minute maximum, they were removed from the wire and returned to their home cage. At this age rat pups have strong grip strength and the ability to maintain such a grip, thereby providing assessment of a different aspect of motor control.

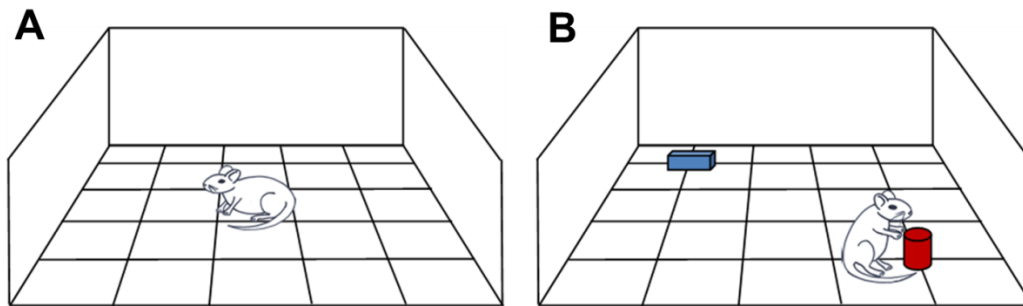
Open field

The open field is a 96cm by 80cm Plexiglas rectangle with walls 42cm high. The floor is marked off in a 16 by 16cm² grid. Red lights were used in the room to reduce anxiety. Animals were placed in the open field and their total distance traveled and number of crosses into the center of the field scored. This test examines normal motor activity. Animals were assessed at PN25 and PN60 as part of the novel object process (Figure 2.4a).

Novel object

The novel object task utilizes the open field arena. On day 1, rats were placed in the open field arena under red light conditions and allowed to explore for 10 min (see open field task). On day 2, rats were placed back in the open field arena with two identical objects (small plastic 30 mL scintillation vials wrapped in lab tape) in opposite corners and allowed to explore for 5 min. No recording occurred during this day. On day 3, rats were placed back in the open field arena with one familiar object (a vial from day 2) and a novel object (small coverslip box) and allowed to explore for 5 min. This exploration was video-recorded, and a double-blind investigator later scored time investigating each object. Percent Total Time Exploring was calculated by combining the time spent exploring each object and divided by the total time in the arena. Normal animals will spend more time investigating the novel object because they “remember” the familiar one. “Young” Novel Object task occurred on PN25, 26, and 27. “Adult” Novel Object task occurred on PN60, 61, and 62 (Figure 2.4b).

Figure 2.4: Representative diagram of the A) Open Field arena and B) Novel Object setup.



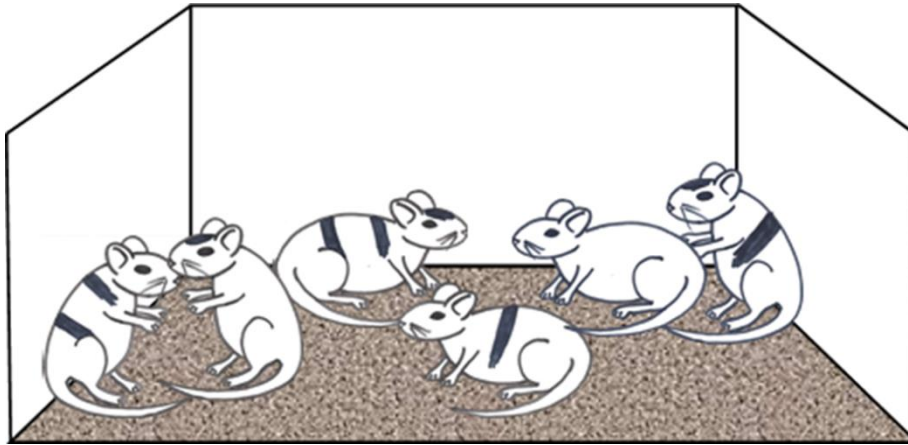
Social play

The testing arena is a black Plexiglas box (49cm L x 37cm W x 24cm H) with a front wall of clear Plexiglas and a fitted Plexiglas lid with holes and lined with an inch of soft bedding layering the bottom. Rats were assigned to non-homecage mate play groups of 5-6 rats of mixed sex and treatment. Testing occurred between PN28 and PN38 and was filmed on as many days as possible (minimum of 5). Play sessions begin a minimum of one half hour past lights off: rats were marked on their fur and tails with nontoxic Sharpie marker for identification purposes, placed in the testing arena for 3 minutes of unrecorded habituation, and then recorded for 10 minutes prior to their return to their home cages. The process was repeated for all play groups to be recorded that day.

Sharpie patterns allow the investigator to follow an individual rat throughout the video but do not indicate sex or treatment, allowing for double-blind scoring of play activity. For each rat, the entire 10 minute session is scored for the frequency of five events: 1) chasing, 2) pouncing, 3) wrestling, 4) pinning, and 5) boxing and added together for a total number of play events for that session. All session play totals for each

animal were averaged, and individual animals were treated as the subjects for statistical examination, with 6-10 animals per sex/treatment group (Figure 2.5).

Figure 2.5: Representative diagram of the social play setup.



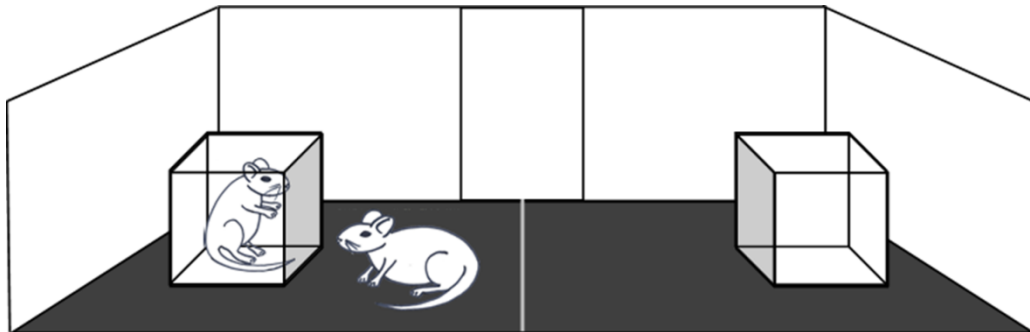
Social choice

The social choice chamber is a black Plexiglas rectangle measuring 90cm L x 40cm W x 30cm H. The box is divided in half by a line and a removable starting door creates a small center starting arena measuring 40cm L x 16cm W, and two removable vented clear Plexiglas cubes 20cm³ were used for the investigation of a stranger rat. Red lights were used in the room to reduce anxiety.

The rat was placed in the small starting arena for 1 min, then the doors were removed and the rat was allowed to explore the empty arena for 5 min. The walls were then replaced and the rat placed back in the starting arena and the top covered so it couldn't see. The removable cubes were then placed equidistant from either side of the small starting arena; one cube with an age- and sex-matched stranger rat, and the other empty. The walls were then removed and the rat allowed to explore the entire arena for 5

min, after which the doors were replaced and the rat put back and covered in the center arena. The location of the stranger rat was then switched to the other side, and the test repeated. All exploration was video-recorded, and a double-blind investigator later scored time investigating each cube. Investigation times were scored as % time investigating the stranger rat vs total time exploring both cubes. Fifty percent would indicate no preference between the two (social or non-social), a higher percentage would indicate greater interest in social interaction (expected for normal animals), and a lower percentage would indicate a reduced social interest, or potential social avoidance. Additionally, the % time spent investigating both cubes over total time in the arena was calculated to examine exploratory behavior (Figure 2.6).

Figure 2.6: Representative diagram of the social choice setup.



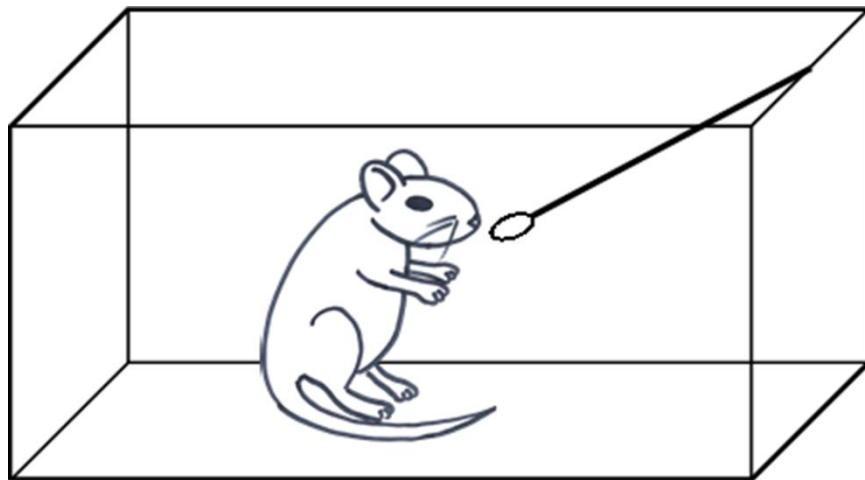
Q-tip olfactory test

Rats were placed in a cage identical to their home-cage with fresh bedding and a wire cage cover. A Q-tip scented with a particular odor was attached to the cover such that it angled down into the cage to allow the rat to investigate it for 3 min. After this time, the Q-tip was removed, and replaced after a 3-min wait period. The animal was then

allowed to investigate for another 3 min, after which the procedure is repeated, ending with a total of 3 presentations. The socially relevant odor used was a swabbing from the animal's home-cage and the home-cage of stranger rats. The non-socially relevant odor used was citrus. The order of presentation was randomized. All presentations were video-recorded, and a double-blind investigator later scored time investigating each Q-tip.

Normal animals will habituate to the same odor presented repeatedly – they spend less time investigating the same odor with each subsequent presentation. Additionally, normal animals will spend a much greater amount of time investigating socially relevant odors compared to non-socially relevant odors. This is an important test because a rat's social interaction is greatly shaped by odor cues, and it is important to see if an animal is able to detect odors normally (as seen by habituation) and whether social cues are more significant to them. Deficits in this task could indicate a confounding variable in other social tasks (Figure 2.7).

Figure 2.7: Representative diagram of the Q-tip olfactory task setup.



Chapter 3:

Characterizing the normal Prostaglandin E2 – Estradiol signaling pathway in normal cerebellar development

Introduction

Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond, and found in the outer membrane of Gram-negative bacteria, such as *E. coli* (Rietschel, 1994; Kentner, 2010). LPS acts by binding the CD14/TLR4/MD2 receptor complex in many cell types, but especially in monocytes, dendritic cells, macrophages and B cells. This elicits a strong immune response by promoting the secretion of pro-inflammatory cytokines, nitric oxide, and eicosanoids (Abbas, 2006), as well as a fever response because of its actions as an exogenous pyrogen. LPS also activates the Tpl2 receptor and increases cyclooxygenase isoenzyme 2 (COX2) expression (Eliouopoulos, 2002), resulting in an increase in prostanoid synthesis. Prostaglandins (PGs) are membrane bound lipid-derived compounds produced throughout the body and have a wide variety of effects, including regulation of inflammation, fever, cell growth, and hormone levels (Randy, 2005). Arachidonic acid is converted to the precursor prostaglandin G₂ (PGG₂) by COX1 and COX2 (Rouzer, 2009; Hla, 1992). PGG₂ is quickly converted to PGH₂, which is then converted by different PG-synthases to one of eight compounds in the prostaglandin family. The most common are PGI₂, PGF₂, PGE₂, PGD₂, and TXA₂. Synthesis of all the prostanoids can be disrupted by inhibiting the COX1/2 enzymes with non-steroidogenic anti-inflammatory drugs (NSAIDs) (Helleberg, 1981).

PGE2 is elevated throughout the brain following inflammation, rising immediately in the hypothalamus to initiate the febrile response. In addition to mediation of the fever response, prostaglandins can also influence other normal brain function, including hippocampal long-term potentiation (Yamagata, 1993) and learning (Shaw, 2003). PGE2 is capable of binding to at least four receptor subtypes, EP1-4, all of which are G-protein coupled receptors (GPCRs) (Figure 1.6). GPCRs are a class of membrane receptors with seven transmembrane domains that activate G-proteins (proteins with GTPase activity). EP1 acts through G_q to increase intracellular concentrations of Ca^{++} . EP2 acts through G_s to increase intracellular cAMP. EP3 actions vary by splice variant; it can act through G_i to decrease intracellular cAMP, G_s to increase intracellular cAMP, or $G_{12/13}$ to modulate members of the Rho family. EP4 acts through G_s to increase intracellular cAMP.

We previously described a signaling pathway in the rat cerebellum in which PGE2 stimulates activity of the steroidogenic enzyme aromatase, thereby increasing the conversion of testosterone to estradiol (Dean, 2012b). Nimesulide is a COX-2 specific inhibitor that prevents production of PGE2. When given systemically to a rat during the second postnatal week, it results in a decreased level of cerebellar aromatase activity, decreased cerebellar estradiol content, and increased size of Purkinje cell dendritic trees. Decreasing estradiol content by blocking its synthesis using the aromatase inhibitor formestane also results in increased size of Purkinje cell dendritic trees. Other fever-reducing drugs, indomethacin and acetaminophen, reduced levels of spinophilin, a proxy for dendritic size. Spinophilin, a protein enriched in dendritic spines and integral to their proper functioning (Feng, 2000), is a reliable semiquantitative indicator of the number of

spines in many brain regions (Amateau, 2004; Schwarz, 2008). Previously we determined that changes in Purkinje cell morphology were not a matter of changes in spine density, but rather changes in overall dendrite length (Dean, 2012a). Additionally, we confirmed that quantification of spinophilin by western blot is a faster method of examining changes in dendrite size than cellular reconstruction by Golgi-Cox staining. In summary, treatments that result in a decrease of estradiol synthesis or levels produce oversized Purkinje cells.

Most of our previous work focused on effects of reduction in PGE2 and estradiol synthesis on Purkinje cell morphology. The opposite manipulation, inducing an increase in estradiol production, was only briefly examined. Systemic injections of estradiol stunted Purkinje cell dendrite growth. Direct injection of PGE2 to the cerebellum resulted in an increase in aromatase activity and a decrease in spinophilin. This suggested PGE2 was increasing estradiol content and stunting Purkinje cell dendrite growth, but these measures were never directly assessed. Since we are interested in real-world situations that could naturally result in an endogenous increase in inflammatory PGE2, we therefore decided to use lipopolysaccharide (LPS) to stimulate endogenous PGE2 synthesis. We also examined the PGE2-E2 signaling pathway in more detail, focusing on alterations to the pathway that result in increased estradiol. We hypothesized that manipulations resulting in increased estradiol would stunt Purkinje cell dendritic growth. Further, we also predicted that the stunting of Purkinje cell growth could be prevented by inhibiting these exogenous influences.

Methods

Rats and treatment paradigms

Sprague Dawley rats mated in our facility were allowed to deliver normally under standard laboratory conditions. All manipulations were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Treatment timing varies by experiment, so each figure's paradigm is summarized in Tables 3.1-3.5.

Table 3.1: Treatment paradigm for Figure 3.1. The top row, regarding the aromatase activity assay paradigm, refers to Figure 3.1A. The bottom row, regarding the estradiol content assay, refers to Figure 3.1B.

Age of Injection	Treatment Groups	Age of Tissue Collection	Assay
PN10	1) Vehicle (saline, i.p. or oil, s.c.) 2) LPS (200 µg/kg), i.p. 3) Nimesulide (0.5 mg/inj), s.c. 4) Nim + LPS (1 hour separation)	16 hours post injection	Aromatase Activity
PN10	1) Vehicle (saline, i.p. or oil, s.c.) 2) LPS (200 µg/kg), i.p. 3) Nimesulide (0.5 mg/inj), s.c. 4) Nim + LPS (1 hour separation)	24 hours post injection	Estradiol Content

Table 3.2: Treatment paradigm for Figure 3.2 (top row) and Figure 3.3 (bottom row).

Age of Injections	Treatment Groups	Age of Tissue Collection	Assay
PN10, 12	1) Vehicle (saline), i.p. or s.c. 2) PGE2 (2.5 µg/inj), i.p.	PN14	Purkinje cell dendrite length
PN10, 12 (LPS)	1) Vehicle (saline, i.p. or oil, s.c.) 2) LPS (200 µg/kg), i.p.	PN14	Purkinje cell dendrite length
PN10-14 daily (Form)	3) Formestane (100 µg/inj), s.c. 4) Form + LPS (1 hour separation)		

Measuring aromatase activity (Figure 3.1)

Male and female pups were treated according to the paradigm in Table 3.1 (top row); 5-6 males and 5-6 females per group. The cerebellar posterior vermis was collected from each animal and assayed for aromatase activity as described in Chapter 2. In brief, whole fresh cerebella were collected in ice-cold TEK and immediately homogenized. Protein concentration was determined via Bradford assay as previously described. Aromatase activity was quantified by measuring tritiated water production by a previously validated method using some modifications (Baillien and Balthazart, 1997). Aliquots of cerebellar homogenate from each animal were run in triplicate; homogenate was incubated for 24 hours at 37°C with 1β3-H³-androstenedione and NADPH. One sample per triplicate was treated with the aromatase inhibitor letrozol. The reaction was stopped by an ice bath and adding ice-cold 10% trichloroacetic acid containing 2% activated charcoal. The tritiated water produced was purified by centrifugation and column chromatography. Columns were eluted with distilled water and effluents were collected in scintillation vials. Ecoscint A (National Diagnostics, Atlanta, GA) was added and vials counted for 4 min on a LKB Wallac 1218 Rackbeta Liquid Scintillation Counter. Blanks were produced in triplicate by diluting TEK buffer, trichloroacetic acid, and water, and average values of the blanks were subtracted from the average value of the samples. A t-test was run to ensure that the average value of the samples treated with the aromatase inhibitor letrozol was significantly lower than the samples without letrozol. Aromatase activity values were normalized to their total protein concentration from the Bradford Assay before further analysis.

Measuring estradiol content (Figure 3.1)

Male and female pups were treated according to the paradigm in Table 3.1 (bottom row); 6-8 males and 6-8 females per group. The cerebellar posterior vermis was collected from each animal and assayed for estradiol content as described fully in Chapter 2. In brief, tissue was homogenized in RIPA buffer and assayed for total protein concentration via Bradford assay as described previously. An aliquot was then mixed thoroughly with diethyl ether and steroids separated out by separating and collecting the aqueous phase from the organic phase. Estradiol was extracted through a series of methanol washes using octadecyl C18 minicolumn, eluted with 100% methanol, evaporated to powder, and reconstituted in PBS. Samples were sent to the Center for Research in Reproduction (University of Virginia, Charlottesville, VA) and analyzed by radioactive immunoassay in duplicate. Reported estradiol values for each sample were then normalized to their total protein concentration from the Bradford Assay before further analysis.

Measuring Purkinje cell dendritic length (Figures 3.2, 3.3)

Male and female pups were treated according to the paradigm in Table 3.2; 5-6 males and 5-6 females per group. The whole brain was collected from each animal and assayed for Purkinje cell dendrite length as described fully in Chapter 2. In brief, brains were placed in a Golgi-Cox solution (16 days) followed by a sucrose solution prior to cerebelli being cut into 100 μm sagittal sections. Tissue was developed in 33% ammonium hydroxide and counterstained with Methylene blue to distinguish anatomical landmarks. Variations in timing and concentration of Methylene blue resulted in a range

of image colors from brown to green to blue, but the neurons were all stained black and background color had no impact on the ability to reconstruct dendritic trees.

Reconstruction of cellular morphology was performed under a Nikon 60X objective using the NeuroLucida system (Nikon Eclipse E600 microscope, MicroBrightField CX900 CCD camera, MicroBrightField, Inc, Colchester, VT). Purkinje cells were identified by their morphology and location. The soma was traced as a reference location and the entire dendritic tree was reconstructed from soma to distal ends, after which the NeuroLucida program calculated the sum total of dendrite length traced. For analysis of spine density, all visible spines were marked along the dendrite tracing and a sum total number of spines per tree calculated. Spine Density was determined by dividing the total number of spines for the tree by the total dendrite length of the tree and reported as number of spines per 1 μ l length of dendrite. Spines were operationally defined as protrusions less than 5 μ m off the dendritic tree in accordance with established criteria (Amateau, 2004). Three - five Purkinje cells from each animal were traced and averaged together for that animal. Individual animals were treated as the subjects for statistical examination, with 5-6 animals per sex/treatment and combined across sex as there was no significant sex difference.

Statistical analysis

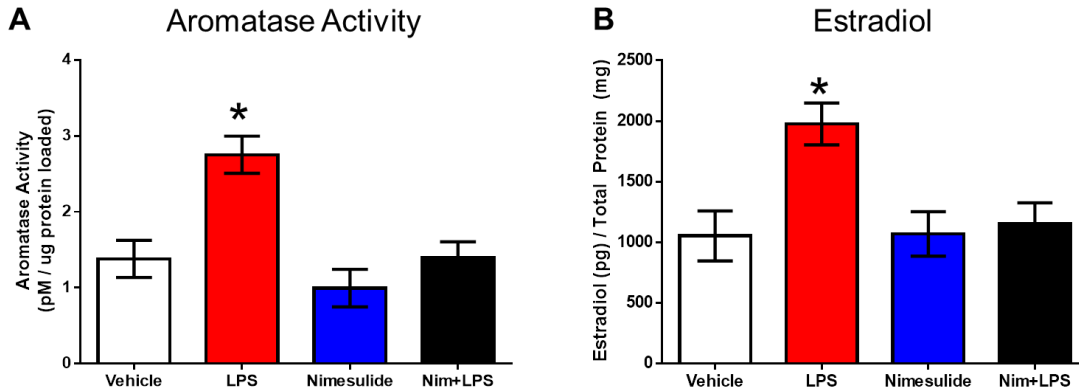
Group means for biochemical or morphological measures were compared via two-way ANOVA to determine if there was a significant effect of sex. No sex differences existed in any treatment group, and all groups were collapsed across sex and analyzed by one-way ANOVA with Tukey's post-hoc multiple comparison test to determine

significance between groups. In cases where only two groups were compared, we used a students t-test. All statistical tests used a value of $p < 0.05$ as the criterion for significance.

Results

As predicted, LPS treatment significantly increased aromatase activity in the cerebellar vermis of neonatal rat pups over vehicle treatment (Fig 3.1A, ANOVA; $F_{3,35}=10.39$, $*p < 0.0001$; Tukey's post-test, vehicle vs LPS: $t=4.206$, $p < 0.001$). COX2 inhibition by nimesulide had no effect on aromatase activity levels compared to vehicle ($t=1.147$, $p=0.7769$), but when administered prior to LPS, significantly blocked the LPS-induced increase in aromatase activity ($t=3.942$, $p < 0.01$). Consistent with increased aromatase activity, LPS also significantly increased cerebellar estradiol content at the later collection time (Fig 3.1B, ANOVA; $F_{3,48}=5.265$, $p < 0.01$; Tukey's post-test, vehicle vs LPS: $t=3.545$, $p < 0.01$) and this too was blocked when nimesulide was administered prior to LPS (LPS vs Nim+LPS: $t=2.586$, $p < 0.05$) with no effect of nimesulide alone (vehicle vs nimesulide, $t=0.066$, $p=0.999$).

Figure 3.1: LPS treatment increased aromatase activity and estradiol, and this effect was blocked by pretreatment with the COX inhibitor, nimesulide. Male and female pups treated on PN10 with vehicle, LPS (200 $\mu\text{g}/\text{kg}$, ip), nimesulide (0.5 mg per injection, s.c.), or a combination (nimesulide + LPS, one hour in between injections) and the cerebellar vermis was collected 16 hours later for measurement of aromatase activity (A) or 24 hours later for measurement of estradiol content (B), collapsed across sex. **A)** LPS increased aromatase activity, which was blocked by pretreatment with nimesulide (ANOVA; $F_{3,35}=10.39$, $p < 0.0001$; Bonferroni post-test, vehicle vs LPS: $t=4.206$, $*p < 0.001$; vehicle vs nimesulide, $t=1.147$, $p=0.7769$; LPS vs Nim+LPS: $t=3.942$, $p < 0.01$). **B)** LPS increased estradiol content, which was blocked by pretreatment with nimesulide (ANOVA; $F_{3,48}=5.265$, $*p < 0.01$; Bonferroni post-test, vehicle vs LPS: $t=3.545$, $p < 0.01$; vehicle vs nimesulide, $t=0.066$, $p=0.999$; LPS vs Nim+LPS: $t=2.586$, $p < 0.05$).



In order to further explore the impact of elevated PGE2 on Purkinje cell morphology, we examined changes in total dendrite length using cellular reconstruction. Treatment with PGE2 decreased the overall length of Purkinje cell dendritic trees (Fig 3.2A, t-test, $t_{12}=3.970$; $*p<0.05$) with no effect on spine density (Fig 3.2B, t-test, $t_{12}=1.020$; $p=0.33$). Representative pictures of Purkinje cells from each treatment group are presented in Figure 3.2C-D. Previous work indicates PGE2 increases E2 levels by driving an increasing aromatase activity and this increase in E2 stunts Purkinje cells. LPS increases PGE2 synthesis, and since we established that LPS also drives an increase in aromatase activity and E2 levels, we predicted that LPS would also stunt Purkinje cell dendrite growth. To confirm a causal relationship between elevated LPS-induced inflammation and stunted Purkinje neuron development via aromatase activity, we sought to block the downstream effects of LPS with the aromatase inhibitor formestane. Inflammation by LPS significantly impaired Purkinje neuron dendritic development as predicted (Fig 3.3A, ANOVA $F_{3, 42}=11.0$, $*p<0.001$; Bonferroni post-test, vehicle vs LPS: $t=4.323$, $p<0.001$), but this effect was blocked under conditions of aromatase inhibition by the drug formestane (LPS vs formestane+LPS: $t=2.789$, $p<0.05$) with no effects of formestane alone (vehicle vs formestane: $t=0.3013$, $p=0.7659$). Previously, we

reported that treatment with formestane alone increases Purkinje cell dendritic length (Dean, 2012a), but that study involved a longer duration of treatment (7 days, PN7-13 daily) than the shorter duration used here (4 days, PN10-13 daily). This may explain why there was no significant effect of formestane on Purkinje cell dendrite length.

Representative pictures of Purkinje cells from each treatment group are shown in Figure 3.3B-E.

Figure 3.2: Treatment with PGE2 decreases Purkinje cell dendrite length but not spine density. Male and female pups were treated with PGE2 (2.5 μg through Foramen Magnum PN10 and PN12, collected on PN14). PGE2 decreased Purkinje cell dendritic length relative to vehicle-treated controls during the second postnatal week. **A)** Average Purkinje cell dendritic length, PGE2 treated vs vehicle treated animals, collapsed across sex (t-test, $t(12)=3.970$; $*p<0.05$). **B)** Average spine density, PGE2 treated vs vehicle treated animals, collapsed across sex (t-test, $t(12)=1.020$; $p=0.33$). **C)** Representative Purkinje cell from a vehicle-treated male. **D)** Representative Purkinje cell from a PGE2-treated male. Scale bar 25 μm .

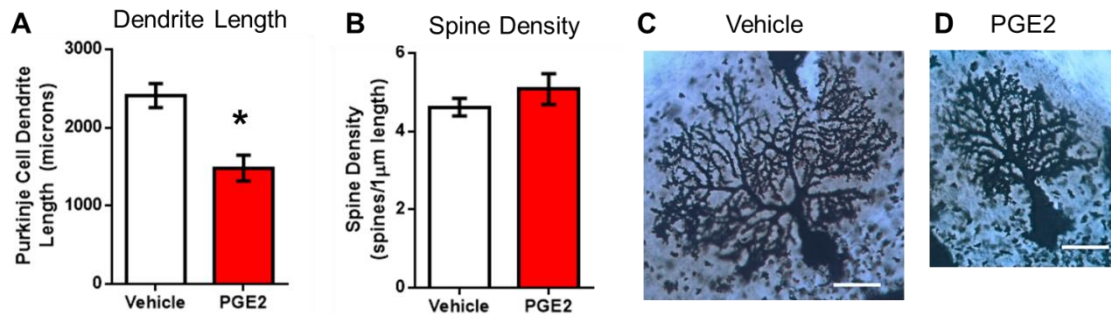
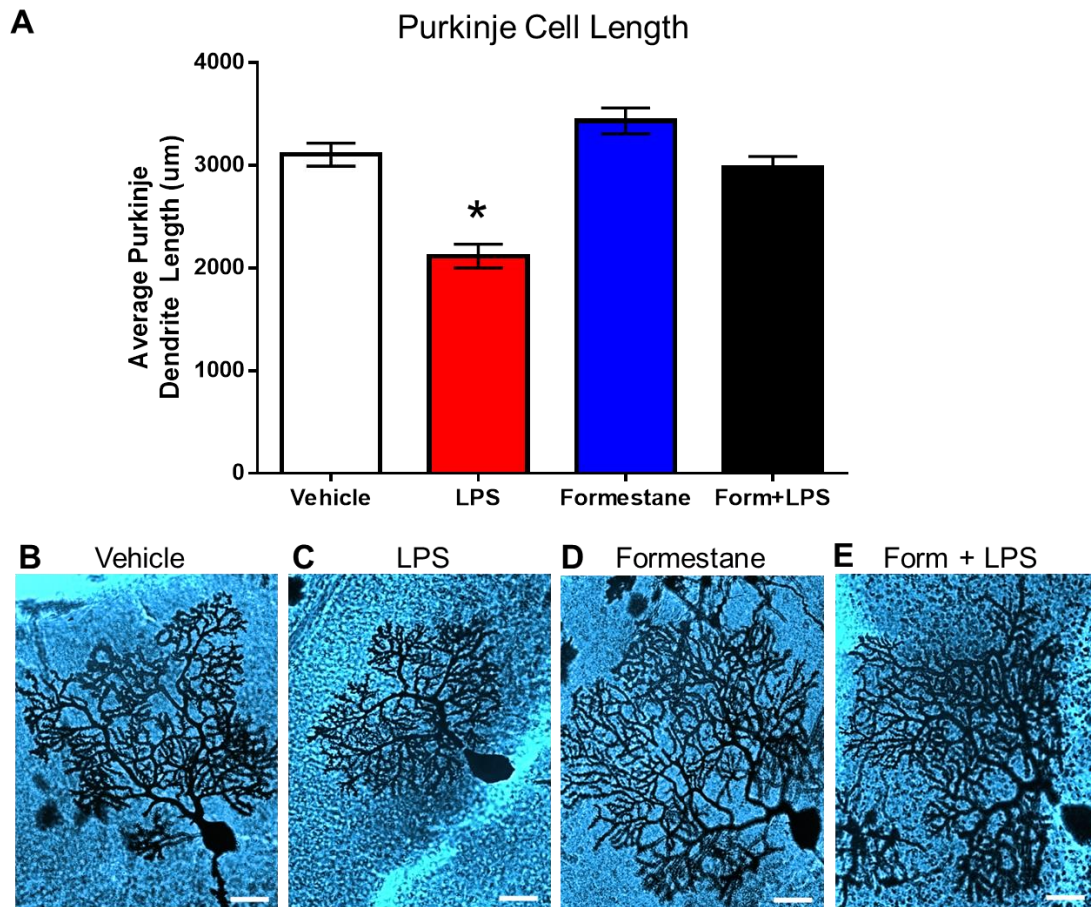
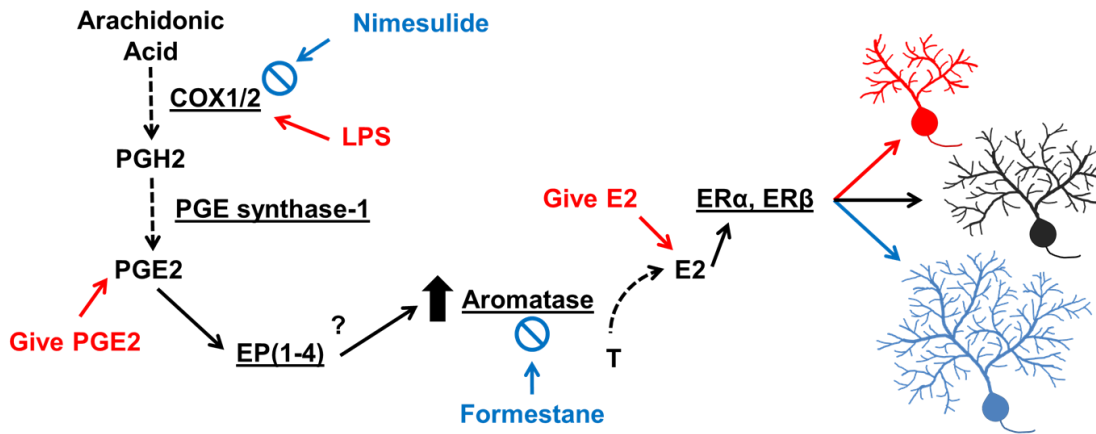


Figure 3.3: LPS treatment decreased Purkinje cell dendrite length, and this effect was blocked by pretreatment with the aromatase inhibitor formestane. Male and female pups treated with vehicle, LPS (200 $\mu\text{g}/\text{kg}$, ip PN10, 12), formestane (100 μg per injection, s.c. daily, PN10-14), or a combination (formestane + LPS, one hour in between injections) and collected on PN14 for golgi-cox impregnation and reconstruction of Purkinje cells. **A)** Average Purkinje cell dendritic length, vehicle treated vs PGE2 and LPS treated animals, collapsed across sex. LPS treatment decreased Purkinje cell dendritic length relative to vehicle-treated controls, which was prevented by pretreatment with formestane (ANOVA $F_{3, 42}=11.0$, $*p<0.001$; Bonferroni post-test, vehicle vs LPS: $t=4.323$, $p<0.001$; vehicle vs formestane: $t=0.3013$, $p=0.7659$; LPS vs Form+LPS: $t=2.789$, $p<0.05$). **B-E)** Representative images of Purkinje cells from pups treated with (B) vehicle, (C) LPS, (D) formestane, and (E) formestane + LPS.



We have confirmed and expanded upon the previously discovered PGE₂-E₂ signaling pathway in the rat cerebellum. In the second postnatal week, arachidonic acid is converted to PGH₂ by COX 1/2, and quickly converted to PGE₂ via PGE synthase-1. PGE₂ binds to its cognate receptors, EP(1-4), increasing aromatase activity and production of estradiol (E₂), which binds to its cognate receptors ER α and ER β , regulating PC dendritic growth. Induction of inflammation by LPS increases PGE₂ levels, and a similar mimicry of inflammation by directly increasing PGE₂ increases estradiol synthesis and decreases PC dendritic length, while decreased PGE₂ results in opposite effects. The current working model of this pathway and results of its manipulation is found in Figure 3.4.

Figure 3.4: Working model of the PGE2-E2 pathway in the cerebellum. Arachidonic acid is converted to PGH2 by the enzymes **COX 1/2**, which is then converted to PGE2 via **PGE synthase-1**. LPS increases COX2 expression, which increases PGE2 synthesis. PGE2 interacts with its receptors **EP(1-4)**, increasing **aromatase** activity to convert testosterone to estradiol, which binds to its receptors **ER α** and **ER β** and regulates Purkinje cell dendritic growth. Estradiol acts as a brake on growth – extrinsic factors that lead to an increase E2 stunt dendritic growth (red), and extrinsic factors that lead to a decrease E2 result in dendritic overgrowth (blue).



Discussion

We have shown here that in the rat cerebellum, during the second postnatal week the exogenous insult LPS results in an inflammatory response that increases PGE2 synthesis. An increased level of PGE2, either by LPS or direct injection of PGE2, drives an increase in aromatase activity and subsequent increase in estradiol synthesis. The resulting higher than normal levels of estradiol then stunt Purkinje cell dendritic growth, an effect which can be prevented by inhibiting aromatase activity via formestane prior to LPS exposure.

LPS acts as an endotoxin and elicits a strong immune response in animals, inducing the release of many prostanoids and cytokines. Prostaglandins are a critical component of the inflammatory response, yet can also impact normal brain development. It may be that prostaglandins prevent or prune off-target connections between climbing

fibers from the inferior olive or maturing granule cells in the cerebellum and their Purkinje cell targets, but it is not yet understood how PGE₂ might be acting. Our results suggest that PGE₂'s ability to increase estradiol levels through stimulation of aromatase activity is the mechanism by which LPS-induced inflammation can disrupt neuronal growth in the cerebellum.

Early life inflammation, either in utero or postnatally, is a major risk factor for development of disorders of mental health, including schizophrenia (Clarke, 2009; Li, 2009; reviewed in Fan, 2007) and autism spectrum disorder (Torres, 2003; Cohly, 2005; Persico, 2006). These disorders can also exhibit alterations in proteins controlling neuronal morphology: in some schizophrenic patients, synaptic but not cytoskeletal protein levels are reduced in the cerebellum relative to controls (Mukaetova-Ladinska, 2002) and the gene *NRG1*, whose product regulates dendritic spine maturation, is a candidate gene for schizophrenia (Barros, 2009). Further, the tumor-suppressor phosphatase with tensin homology (PTEN) protein is a negative regulator of dendritic branching, and mutations in PTEN have been associated with an increased risk of autism (O'Roak, 2012; Roberts, 2014; reviewed in Lv, 2013). Lack of PTEN in mice decreases spine density and dendritic organization of cerebellar Purkinje cells (Fraser, 2007), and haploinsufficiency of PTEN in mice results in autism-like social impairments in both males and females (Clipperton-Allen, 2014). In the human breast cancer cell line MCF-7, estradiol increases PTEN expression through Na(+)/H(+) exchanger regulatory factor 1 (NHERF1) (Longyan, 2011), and overexpression of PTEN in motoneurons appears to cause inactivation/dephosphorylation of Akt in neurons, resulting in increased cell death which can be prevented by estrogen exposure (Smith, 2009). This mechanism appears to

be in opposition to our finding that an increase in estradiol results in stunted dendritic trees, thus it is not likely that estradiol's mechanism of action is through PTEN in the cerebellum. Further, alterations to our pathway do not change spine density, but it is reasonable to believe that the difference in overall dendritic size would alter connections Purkinje cells normally make with the cerebellar network and result in downstream dysfunction similar to changes in spine density already demonstrated in neurodevelopmental diseases.

What has not been worked out yet is exactly how PGE₂ is capable of driving an increase in aromatase activity, or how estradiol can modulate Purkinje cell dendrite growth. In peripheral systems such as the breast, PGE₂'s ability to increase estradiol production is mediated by an increase in cAMP (Brueggemeier, 2001) and likely acts through EP₂ and/or EP₄ and PKA and PI3-kinase/Akt signaling pathways as both receptors increase cAMP (Wright, 2008; Han, 2010; Kim, 2010). Currently this mechanism is poorly understood and has not yet been explored in the brain. Evidence presented later in Chapter 4 will indeed suggest EP₄ as the most likely mechanism by which PGE₂ increases aromatase activity.

Estrogen receptors ER β and ER α are nuclear receptor transcription factors, activating gene transcription by binding to estrogen responsive elements in DNA. Another mechanism of estrogen/receptor signaling is through membrane estrogen receptors and their activation of mitogen-activated protein kinase (MAP kinase) pathways to activate other cellular processes that rely on the kinase/phosphatase balance (Mannella, 2006; Moriarty, 2006). The exact mechanism of estradiol's regulation of dendrite growth, particularly for Purkinje cells, is currently unknown. In the developing arcuate

nucleus, estradiol-induced reduction in synaptogenesis and dendritic branching involves an increased synthesis of gamma-aminobutyric acid (GABA) (Mong, 2002). GABA could be the mechanism for estradiol-induced stunting of Purkinje cells as well: preliminary data from our laboratory indicates estradiol increases glutamic acid decarboxylase (GAD) (Dean, 2009), the enzyme responsible for production of GABA. GABA is one of the factors involved in activity-dependent dendritic pruning of Purkinje cells during the second postnatal week (Sotelo, 2009). Estradiol might also affect cerebellar development is through retinoic acid-related (RAR) orphan receptor-alpha (RORA), a hormone dependent transcription factor involved in processes such as Purkinje cell differentiation (Hadj-Sahraoui, 2001), cerebellar development (Harding, 1997; Gold, 2007), and suppression of inflammation (Delerive, 2001). RORA is a direct transcriptional target of both androgen receptor and ER α , which can in turn regulate aromatase expression (Sarachana, 2001). Furthermore, RORA and aromatase proteins were significantly reduced in frontal cortex neurons from autistic subjects compared with matched controls, and the expression of aromatase protein in the neurons was strongly correlated with RORA expression (Sarachana, 2001). Levels of RORA in the cerebellum of autistic patients is unknown.

Yet another potential mechanism of estradiol action on cerebellar development is through brain-derived neurotrophic factor (BDNF), a secreted protein that acts to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses (Acheson, 1995; reviewed in Huang, 2001). It has been shown that knocking out the aromatase gene (ArKO) in mice results in decreased Purkinje cell dendritic growth, while administration of estradiol to neonatal wild-type or ArKO mice

increased dendritic growth, spinogenesis, and synaptogenesis in the Purkinje cell, and this occurs through an estradiol-induced increase in BDNF (Haraguchi, 2012). These data are in apparent direct contrast to our findings that elevating estradiol in the cerebellum results in *reduced* Purkinje cell dendritic growth and no change in synaptic density. Most of our work involved administration LPS or PGE2, which result in an increase in endogenous estradiol and subsequent stunting of Purkinje cell growth. LPS and PGE2 do not act in isolation during inflammatory responses; the role of other cytokines and even microglia and astrocytes must also be considered in determining the mechanism of estradiol regulation of Purkinje cell development. For example, cortical astrocytes of male or androgenized female rats exposed to LPS exhibited higher levels of mRNA for cytokines IL6, TNFa and IL1b, while IP10 mRNA was higher in control females, even though there were no sex differences in basal levels of these cytokines or in the LPS receptor TLR4 (Santos-Galindo, 2011). It is unknown whether this is also true in the cerebellum.

In summary, we have shown that in the rat cerebellum, the exogenous insult LPS results in an inflammatory response that increases PGE2 synthesis. An increased level of PGE2, either by LPS or direct injection of PGE2, drives an increase in aromatase activity and subsequent increase in estradiol synthesis. The resulting higher than normal levels of estradiol then stunt Purkinje cell dendritic growth. All of these treatments were performed and measured within the second postnatal week. Since that time is also highly dynamic for Purkinje cell development, in the next chapter we will determine whether the second postnatal week is a sensitive period for exogenous influence on the PGE2-E2 signaling pathway.

Chapter 4:

Identifying the sensitive period for exogenous influence on the PGE2-E2 pathway in the cerebellum

Introduction

A critical period is, in the simplest terms, a period of time during an organism's life in which the organism is more highly influenced by exogenous factors than other periods in its life. This concept has been around for a very long time, but was defined with a set of criteria by John Nash in the late 1970s. According to him, a critical period must have: 1) an identifiable beginning point, 2) an identifiable end point, 3) an intrinsic component (triggered by maturational events), and 4) an extrinsic component (a noninternal stimulus to which the organism is sensitive) (Nash, 1978). In 1982, in his review of the concept and study of critical periods, John Colombo added a fifth criterion: 5) a specified critical system affected by these components. The critical period concept has been applied to and examined in many systems, including visual development in the cat (Blakemore, 1973; Blakemore, 1974; Daw, 1976; Hubel, 1970), imprinting in birds (Hinde, 1962), and canine socialization (Scott, 1974). In the rat, barrel cortex formation and organization requires sensory input from the whiskers during a restricted developmental time period (Durham 1984), and sexual differentiation of hypothalamic brain regions controlling reproductive physiology and behavior is organized by hormone exposure during a critical period as well (Rhees, 1990). In humans, critical periods for infantile stimulation have been proposed to affect the development of adult reaction to stress (Bell, 1961; Levine, 1959), learning (Bell, 1963; Denenberg, 1960), emotionality (Denenberg, 1963; Lindholm, 1962), and activity/exploratory behavior (Bronstein, 1972;

Forgays, 1962). Some have argued that a critical period must have an immediate onset and offset, while others argue that it may be more accurate to include a gradual rise in sensitivity to the critical stimulus with a peak in sensitivity during which exposure is most effective. A critical period can last for hours, days, weeks, or years (Colombo, 1982).

It is important to note the subtle difference between a “critical period” and a “sensitive period.” Although the distinction is not made in every case, and many use the terms interchangeably, *critical period* refers to periods during which specific stimuli are necessary for the continuation of normal development, and *sensitive period* refers to periods during which the organism is especially vulnerable to harmful stimuli that usually disrupts normal development (Fox, 1970). There are several well defined examples of such developmental vulnerabilities to harmful stimuli in both rats and humans. Michael Meaney’s extensive work with maternal behavior shows us that maternal neglect results in pups with altered hypothalamic-pituitary-adrenal axis and stress responses (Meaney, 2001). Stress and traumatic events in early childhood have enduring consequences in the form of increased risk for later health problems, mental illness, and anxiety (Gunnar, 1998, 2007; Kuo, 2011; Norman, 2012). Another example of developmental vulnerability is inflammation during gestation, early childhood and even in the adult. These have been linked with an increased risk for developing schizophrenia, autism, and depression (Cyranowski, 2006). However, not every child that experiences trauma, stress, or disease develops mental disorders. One possible explanation for the variation is genetic predisposition, and another is the timing of an environmental insult. If there is a particular period when a developing system is most vulnerable to exogenous factors,

especially if a child has a genetic predisposition to a mental disorder, then defining that period more accurately could help us discover the best time for preventative interventions to reduce the risk for that disorder.

We have already discussed connections between early life inflammation and neurodevelopmental disorders such as schizophrenia and autism, and how the cerebellum has been implicated in that process (Chapter 1, Chapter 3). We know the second postnatal week in the rat is a dynamic period of Purkinje cell development, and a prostaglandin E2 – estradiol signaling pathway appears to play an important role in that process. While the expression patterns of some components of our proposed pathway have been examined previously, the data are a patchwork from multiple laboratories (Lavaque, 2006; Perez, 2003; Ikeda, 2006; Price, 2000; Sakamoto, 2003). A comprehensive and detailed analysis of all these components in the same animals or across a detailed timeline around our proposed sensitive period has not been examined previously. Given the dynamic timing of Purkinje cell development and our previous findings on the PGE2-E2 signaling pathway in the cerebellum and the effects of its disruption using COX and aromatase inhibitors (Dean 2012a, 2012b) or LPS and PGE2 (Chapter 3), we hypothesize that the second postnatal week (PN7-14) in the rat cerebellum is a sensitive period for exogenous influence on the PGE2-E2 pathway. From this we predict specifically that 1) LPS will increase PGE2 and nimesulide will decrease PGE2 when treatment occurs during week 2, but not when treatment is during weeks 1 or 3, 2) LPS and PGE2 will increase E2 levels when administered in week 2, but not in weeks 1 or 3, and 3) LPS and PGE2 will decrease Purkinje cell dendrite length when administered in week 2, but not in weeks 1 or 3. Further, we wished to understand which

components define this sensitive period, and hypothesized that one or more components of the PGE2-E2 signaling pathway in the rat cerebellum exhibit significant changes in expression and/or activity over the course of the first three postnatal weeks of life.

Methods

Rats and treatment paradigms

Sprague Dawley rats mated in our facility were allowed to deliver normally under standard laboratory conditions. All manipulations were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore. Treatment timing and type varies by experiment, so each figure's paradigm is summarized in Tables 4.1-4.5.

Table 4.1: Treatment paradigm for Figure 4.1

	Age of Injection	Treatment Groups	Age of Tissue Collection	Assay
Week 1	PN3	1) Vehicle (saline), i.p. 2) LPS (200 µg/kg), i.p.	1 hour post injection	COX1 and COX2 mRNA expression
Week 2	PN10			
Week 3	PN17			

Table 4.2: Treatment paradigm for Figure 4.2. Red text for injection ages corresponds to red arrows in the figure; blue text for injection ages corresponds to blue arrows in the figure.

	Age of Injection(s)	Treatment Groups	Age of Tissue Collection	Assay
Week 1	PN4	1) Vehicle (saline), i.p. 2) LPS (200 µg/kg), i.p.	8 hours post last injection	PGE2 content
	PN1-4 daily	1) Vehicle (oil), s.c. 2) Nimesulide (0.5 mg/injection), s.c		
Week 2	PN10	1) Vehicle (saline), i.p. 2) LPS (200 µg/kg), i.p.		
	PN7-10 daily	1) Vehicle (oil), s.c. 2) Nimesulide (0.5 mg/injection), s.c		
Week 3	PN17	1) Vehicle (saline), i.p. 2) LPS (200 µg/kg), i.p.		
	PN14-17 daily	1) Vehicle (oil), s.c. 2) Nimesulide (0.5 mg/injection), s.c		

Table 4.3: Treatment paradigm for Figure 4.3. Injections occurred at the red arrows in the figure.

	Age of Injections	Treatment Groups	Age of Tissue Collection	Assay
Week 1	PN3, 5	1) Vehicle (saline), F.M. 2) PGE2 (2.5 µg/injection), F.M.	PN7	Estradiol Content
Week 2	PN10, 12		PN14	
Week 3	PN17, 19		PN21	

Table 4.4: Treatment paradigm for Figure 4.4. Injections occurred at the red arrows in the figure.

	Age of Injections	Treatment Groups	Age of Tissue Collection	Assay
Week 1	PN3, 5	1) Vehicle (saline), i.p. or F.M.	PN7	Purkinje cell dendrite length
Week 2	PN10, 12	2) LPS (200 µg/kg), i.p.	PN14	
Week 3	PN17, 19	3) PGE2 (2.5 µg/injection), F.M.	PN21	

Table 4.5: Treatment paradigm for Figure 4.6. Injections occurred on the days indicated in blue, tissue collection occurred on days indicated in italics.

Ages of Injections and Tissue Collection (PN)	Set 1	5
	Set 2	5 6 7 8 9 10
	Set 3	5 6 7 8 9 10 11 12
	Set 4	5 6 7 8 9 10 11 12 13 14
	Set 5	5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Treatment Groups	1) Vehicle (oil), s.c. 2) Formestane (100 µg/injection), s.c.	
Assay	mRNA	

Measuring mRNA expression (Figures 4.1, 4.5, and 4.6)

Male and female pups were treated according to the paradigm in Table 4.1; 4 males and 4 females per group (Figure 4.1). Additionally, a second cohort of untreated animals was used to determine changes in mRNA expression across our developmental timecourse. Timed-pregnant dams were allowed to deliver normally and their pups cross-fostered to ensure equal numbers of sex per dam. Pups were then assigned to a male-female pair from each dam at one of 9 ages (PN3, 5, 7, 10, 12, 14, 17, 19, or 21) until there were 4 males and 4 females at each age (Figure 4.5). Further, a third cohort of animals was treated according to the paradigm in Table 4.5; 4 males and 4 females per group/age combination (Figure 4.6). Treatments occurred on the days indicated in blue, tissue collection occurred on the days noted in italics. Specifically, for set 1, animals were only treated with vehicle, not formestane, and tissue was collected at PN5. For set 2, animals were treated once daily from PN5-9 with vehicle or formestane, then tissue was collected at PN10. For set 3, animals were treated once daily from PN5-11, then tissue was collected at PN12. For set 4, animals were treated once daily from PN5-13,

then tissue was collected at PN14. For set 5, animals were treated once daily from PN5-13, no treatments occurred from PN14-18, and tissue was collected at PN19.

For all experiments, the cerebellar posterior vermis was collected from each animal and assayed for mRNA content as described fully in Chapter 2. In brief, RNA was extracted from tissue using a Qiazol and chloroform solution and purified using an RNeasy Mini Kit (Qiagen #74106/74104), and purity verified with a Nanodrop spectrometer (Wilmington, DE). Single stranded complementary DNA (cDNA) was generated from extracted RNA using the ABI High Capacity cDNA Reverse Transcription Kit #4368814 (Foster City, CA). A 1:3 serial dilution standard curve was created from a pool of all samples in an experiment and also served as the threshold for detection of amplification product. Standards and samples were run in triplicate on a 384 well plate, using an ABI ViiA7 machine, and SYBR green as the method of quantification. Cycles consisted of the following steps: 1) 95°C start, 2) 94°C melt, 3) primer incubation (temp depends on amplicon), 4) 72°C extension step, 5) 79°C or 80°C fluorescence read, 6) cycle step 2-5 for 40 repeats, 7) 72°C product stabilization, and 8) melting curve. To normalize for variations in the amount of DNA loaded, each sample was also run with a reference gene of GAPDH and samples were normalized to this GE. All results are expressed as a ratio of the target gene GE over corresponding GAPDH GE.

Specific primer information can be found in Table 2.1 (Chapter 2).

Measuring PGE2 content (Figure 4.2)

Male and female pups were treated according to the paradigm in Table 4.2 (bottom row); 8-10 males and 8-10 females per group (week 1), 15-18 males and 15-18

females per group (weeks 2 and 3). The cerebellar posterior vermis was collected from each animal and assayed for PGE2 content as described fully in Chapter 2. In brief, tissue was homogenized in RIPA buffer and assayed for total protein concentration via Bradford assay as described previously. An aliquot was then acidified and PGE2 extracted through a series of washes using octadecyl C18 minicolumns, eluted with ethyl acetate, evaporated to powder, and reconstituted in assay buffer from a PGE2 enzyme immunoassay kit (Arbor Assays). PGE2 concentrations for each sample were then determined by a colorimetric reaction with the kit, and values were normalized to their total protein concentration from the Bradford Assay before further analysis.

Measuring estradiol content (Figure 4.3)

Male and female pups were treated according to the paradigm in Table 4.3; 8-10 males and 8-10 females per group. The cerebellar posterior vermis was collected from each animal and assayed for estradiol content as described fully in Chapter 2. In brief, tissue was homogenized in RIPA buffer and assayed for total protein concentration via Bradford assay as described previously. An aliquot was then mixed thoroughly with diethyl ether and steroids separated out by separating and collecting the aqueous phase from the organic phase. Estradiol was extracted through a series of methanol washes using octadecyl C18 minicolumn, eluted with 100% methanol, evaporated to powder, and reconstituted in PBS. Samples were sent to the Center for Research in Reproduction (University of Virginia, Charlottesville, VA) and analyzed by radioactive immunoassay in duplicate. Reported estradiol values for each sample were then normalized to their total protein concentration from the Bradford Assay before further analysis.

Measuring Purkinje cell dendritic length (Figure 4.4)

Male and female pups were treated according to the paradigm in Table 4.4; 5-6 males and 5-6 females per group. The whole brain was collected from each animal and assayed for Purkinje cell dendritic length as described in Chapter 2. In brief, brains were placed in a Golgi-Cox solution (week 1- PN7, 12 days in solution, week 2- PN14, 16 days in solution, week 3 – PN21, 21 days in solution) followed by a sucrose solution prior to cerebelli being cut into 100 μm sagittal sections. Tissue was developed in 33% ammonium hydroxide and counterstained with Methylene blue to distinguish anatomical landmarks. Variations in timing and concentration of Methylene blue resulted in a range of image colors from brown to green to blue, but the neurons were all stained black and background color had no impact on the ability to reconstruct dendritic trees.

Reconstruction of cellular morphology was performed under a Nikon 60X objective using the NeuroLucida system (Nikon Eclipse E600 microscope, MicroBrightField CX900 CCD camera, MicroBrightField, Inc, Colchester, VT). Purkinje cells were identified by their morphology and location. The soma was traced as a reference location and the entire dendritic tree was reconstructed from soma to distal ends, after which the NeuroLucida program calculated the sum total of dendrite length traced. Three - five Purkinje cells from each animal were traced and averaged together for that animal. Individual animals were treated as the subjects for statistical examination, with 5-6 animals per sex/treatment and combined across sex as there was no significant sex difference.

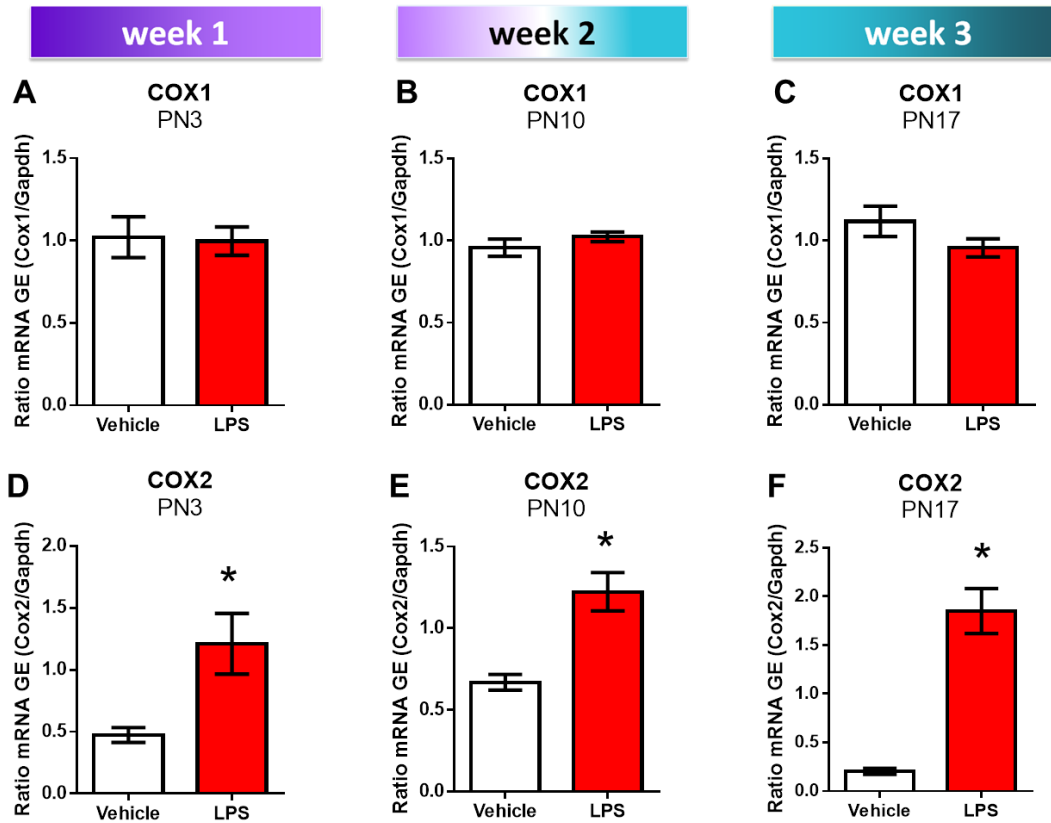
Statistical analysis

Group means for biochemical or morphological measures were compared via two-way ANOVA to determine if there was a significant effect of sex. No sex differences existed in any treatment or age group, and all groups were collapsed across sex and analyzed by one-way ANOVA (vehicle treatments across age, or treatments within age) with Tukey's post-hoc multiple comparison test to determine significance between groups. In cases where only two groups were compared, we used a student's t-test. For the changes in mRNA expression across time, for each individual gene, every age was compared back to PN3 with a Bonferroni correction with significance set at $p < 0.05$. All statistical tests used a value of $p < 0.05$ as the criterion for significance.

Results

It has been established that LPS increases COX2 expression in the periphery (Eliouopoulos, 2002), but it is necessary to show this effect also occurs in the cerebellum, and to determine if it occurs at all time points or only during our proposed sensitive period. Rat pups were treated with vehicle or LPS at three different time points (Table 4.1) and cerebellar vermis tissue collected 1 hour post injection. We found that as predicted, COX2 mRNA expression is induced by LPS in the second week ($t=4.04$, $*p < 0.01$) while COX1 expression is not ($t=1.17$, $p=0.15$). Further, we found that LPS is also capable of inducing COX2 mRNA expression within weeks 1 ($t=3.10$, $*p < 0.01$) and 3 ($t=3.10$, $*p < 0.01$), while COX1 mRNA expression remains unchanged by LPS at any time point (week 1 $t=0.15$, $p=0.31$, week 3 $t=0.78$, $p=0.08$).

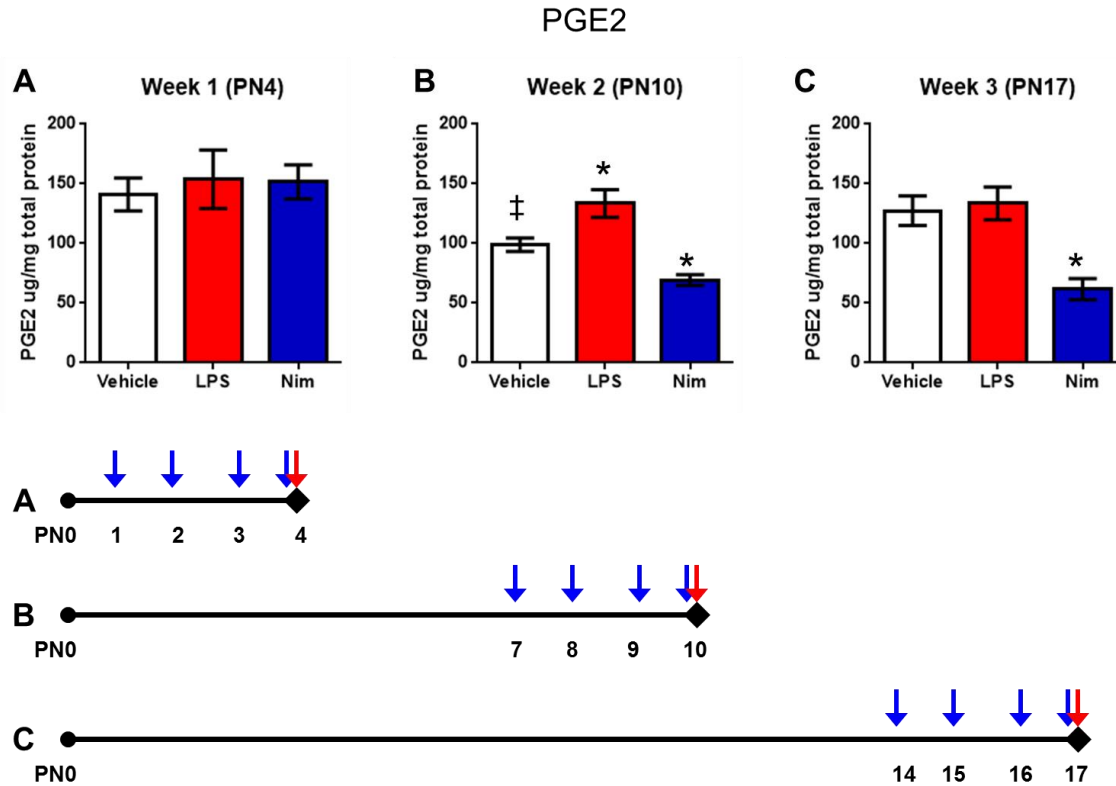
Figure 4.1: LPS induced COX2 expression across all three weeks. LPS (200 µg/kg in saline, i.p.) given on PN3 (week 1), PN10 (week 2), or PN17 (week 3); cerebellar vermis collected 1 hour post injection. **A-C)** COX1 expression is unchanged by LPS at any time point (PN3 $t=0.15$, $p=0.31$; PN10 $t=1.17$, $p=0.15$; PN17 $t=0.78$, $p=0.08$). **D-F)** COX2 expression is elevated by LPS over control at every time point (PN3 $t=3.10$ $*p<0.01$; PN10 $t=4.04$, $*p<0.01$; PN17 $t=3.10$, $*p<0.01$).



Since we've shown that COX2 mRNA is increased by LPS in all three weeks, the next step was to evaluate changes in PGE2 following LPS treatment. Pups were treated with vehicle, LPS, or nimesulide across the first three weeks as outlined in Table 4.2 and tissue was collected 8 hours after the last injection. Cerebellar posterior vermis was dissected and assayed for PGE2 content (Figure 4.2). Vehicle-treated groups were compared to reveal patterns across normal development. Basal PGE2 synthesis significantly decreased at PN10 (week 2) compared to weeks 1 or 3 (Figure 4.2 A, B, and C, white bars, ANOVA $F_{2,72}=4.20$, $*p<0.05$; PN4 vs PN10 $t=2.33$, $p<0.05$; PN10 vs

PN17 $t=2.47$, $p<0.05$). Inflammation induced by LPS had no effect on PGE2 synthesis within weeks 1 (ANOVA $F_{2,31}=0.14$, $p=0.87$) or 3 (ANOVA $F_{2,103}=10.68$, $p<0.01$; $t=0.36$, $p=0.99$), but significantly increased PGE2 levels over baseline in the second week (ANOVA $F_{2,89}=12.33$, $p<0.01$; $t=2.93$, $*p<0.01$). COX-2 inhibition by nimesulide had no effect on PGE2 synthesis in week 1 but significantly decreased PGE2 synthesis in week 2 as well as in week 3 ($t=2.30$, $*p<0.05$; $t=3.74$, $*p<0.01$ respectively), suggesting the second postnatal week is a sensitive period during which inflammation induces PGE2 synthesis against a backdrop of normally reduced basal PGE2 production.

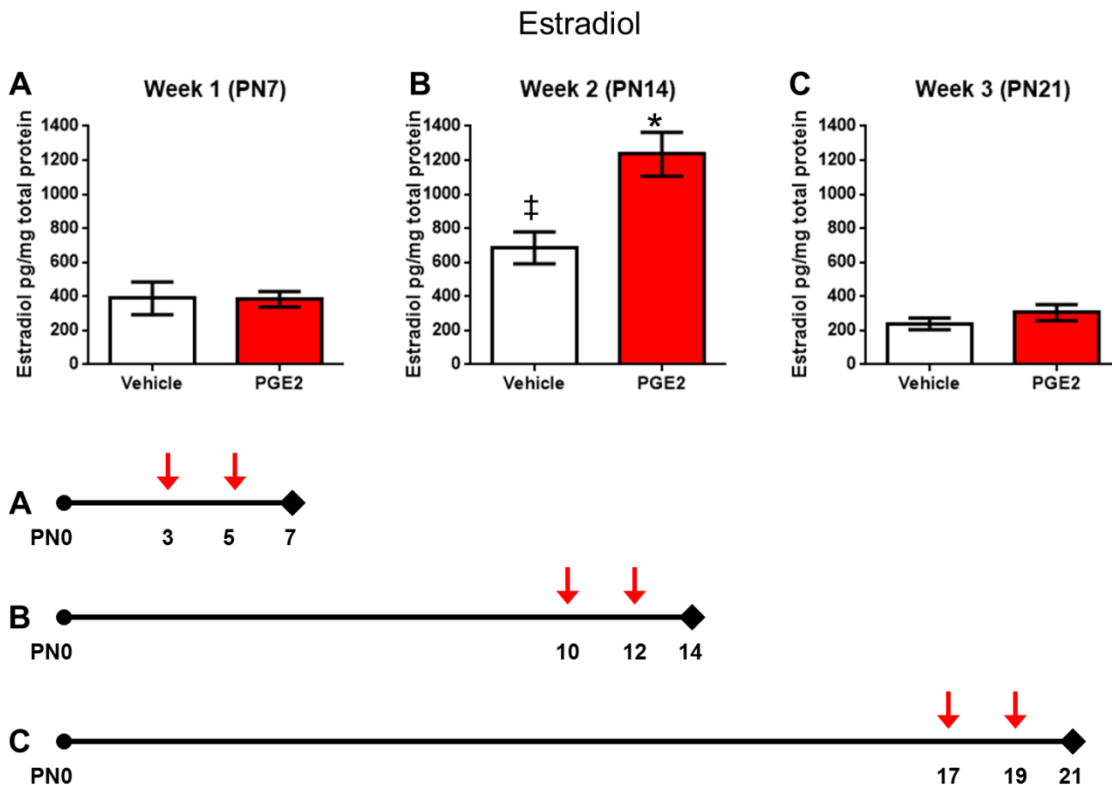
Figure 4.2: Effects of inflammation or anti-inflammatory treatment on PGE2 within each of the first three postnatal weeks of life. Male and female pups were treated with vehicle, LPS (200 μ g/kg), or nimesulide (0.5mg/injection). Blue arrows indicate days of nimesulide treatments, red arrows indicate days of LPS treatments. Tissue collection occurred on PN4, 10, or 17 8 hours following the last injection and was analyzed for PGE2 content. Timelines of injections and tissue collections are seen in the horizontal bars below the graphs. **A)** Within postnatal week 1, PGE2 content did not differ significantly from vehicle following treatment with either LPS or nimesulide (ANOVA $F_{2,31}=0.14$, $p=0.87$). **B)** Within postnatal week 2, PGE2 content was significantly higher than vehicle following LPS (ANOVA $F_{2,89}=12.33$, $p<0.01$; $t=2.93$, $*p<0.01$), and significantly lower than baseline following nimesulide ($t=2.30$, $*p<0.05$). **C)** Within postnatal week 3, PGE2 content did not differ significantly from vehicle following LPS (ANOVA $F_{2,103}=10.68$, $p<0.01$; $t=0.36$, $p=0.99$), but was significantly decreased following nimesulide ($t=3.74$, $*p<0.01$). **A-C)** Baseline PGE2 (vehicle treated animals, white bars) is significantly lower during the second postnatal week compared to weeks 1 or 3 (ANOVA $F_{2,72}=4.20$, $^{\ddagger}p<0.05$; PN4 vs PN10 $t=2.33$, $^{\ddagger}p<0.05$; PN10 vs PN17 $t=2.47$, $^{\ddagger}p<0.05$).



Previously we reported that within the second postnatal week, decreasing PGE2 synthesis with the COX-2 inhibitor nimesulide decreases aromatase activity and estradiol synthesis, but elevated PGE2 during the second postnatal week increases aromatase activity and estradiol (Dean, 2012a; Dean, 2012b). In order to determine if there was a sensitive period of extrinsic influences on the signaling pathway, pups were treated with vehicle or PGE2 across the first three weeks as outlined in Table 4.3. Cerebelli were collected at the end of the treatment week (PN7, PN14, or PN21 respectively) and the posterior vermis assayed for E2 content (Figure 4.3). In normally developing animals (vehicle treated) there was a significant increase in E2 levels at PN14 (week 2) compared to weeks 1 or 3 (PN7 vs PN14 trending, $t=2.31$, $p=0.06$; PN14 vs PN21 $t=3.70$, $^{\#}p<0.01$). Cerebellar infusion of PGE2 significantly increased E2 levels only within week 2

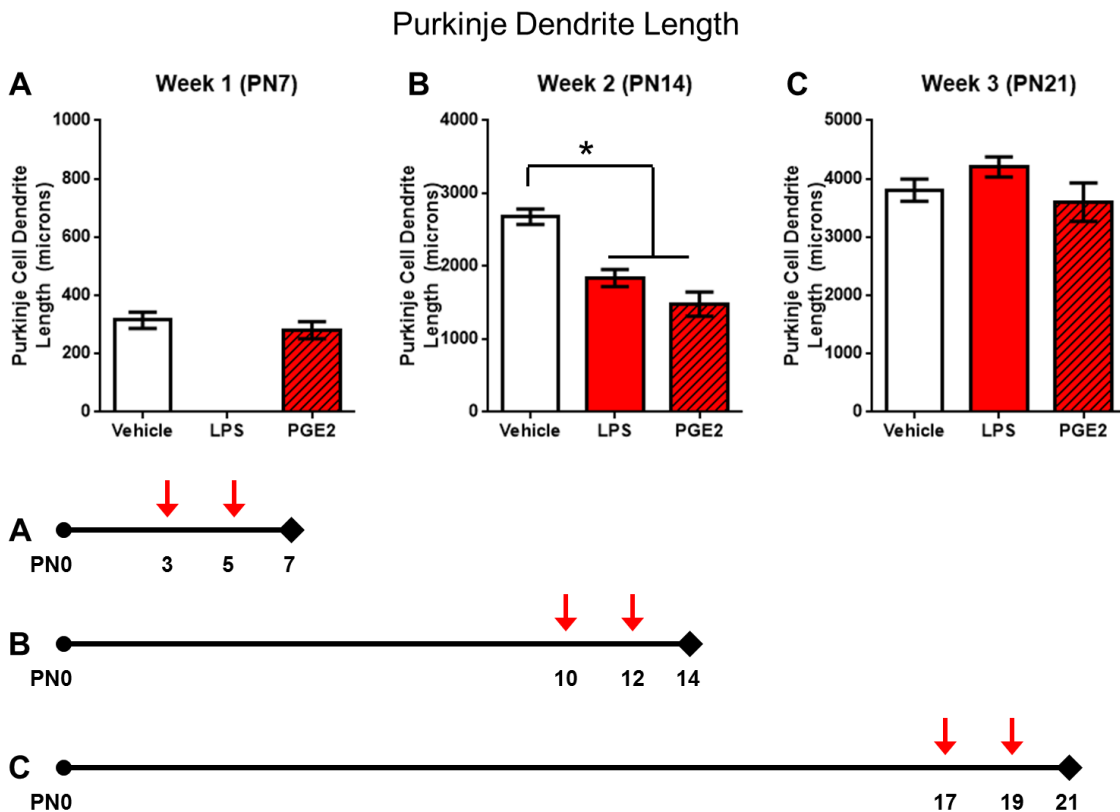
($t=4.706$, $*p<0.01$), with no effect within weeks 1 or 3 ($t=0.04$, $p=0.99$; $t=0.66$, $p=0.99$, respectively), suggesting a sensitive period for PGE2 actions on E2 synthesis. The slight basal elevation of E2 during the second week, a time when basal PGE2 is lower, further emphasizes the heightened sensitivity during this period.

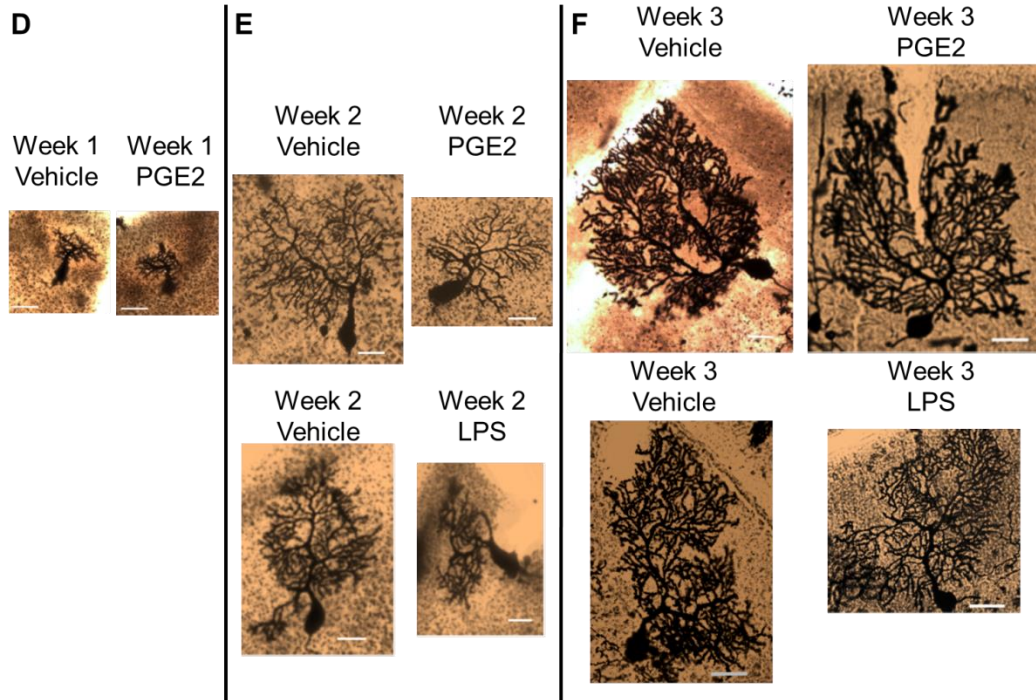
Figure 4.3: Effects of inflammation on estradiol within each of the first three postnatal weeks of life. Male and female pups were treated with vehicle or PGE2 (2.5 $\mu\text{g}/\text{injection}$) within one of three time periods: week 1 (PN3, 5), week 2 (PN10, 12), or week 3 (PN17, 19), indicated by the red arrows. Brains were collected at the end of the treatment week (PN7, PN14, or PN21 respectively) and tissue analyzed for estradiol content. Timelines of injections and tissue collections are seen in the horizontal bars below the graphs. A MANOVA revealed a significant effect of treatment ($F_{1,58}=10.67$, $p<0.01$), age ($F_{2,58}=43.97$, $p<0.01$), and interaction ($F_{2,58}=7.15$, $p<0.01$). Post-hoc analysis: vehicle-treated groups were compared to reveal patterns across normal development. Basal estradiol synthesis significantly increased at PN14 (week 2) compared to weeks 1 or 3 (PN7 vs PN14 trending, $t=2.31$, $p=0.06$; PN14 vs PN21 $t=3.70$, $^{\ddagger}p<0.01$). Within postnatal week 2, E2 content was significantly higher than vehicle following PGE2 treatment ($t=4.706$, $*p<0.01$), but not significantly different following treatment either in week 1 ($t=0.04$, $p=0.99$) or week 3 ($t=0.66$, $p=0.99$).



Previously we have shown that within the second postnatal week, decreasing PGE2 synthesis with the COX-2 inhibitor nimesulide increases Purkinje cell dendrite length, but elevated PGE2 during the second postnatal week decreases spinophilin in the cerebellar vermis (proxy for Purkinje cell dendrite length) and estradiol reduces Purkinje cell tree size (Dean, 2012a; Dean, 2012b). In order to determine if there was a sensitive period of extrinsic influences on Purkinje cell development we induced or mimicked inflammation within each of the first three weeks of postnatal life. Pups were treated with vehicle or PGE2 across the first three weeks as outlined in Table 4.4 and brains collected at the end of the treatment week (PN7, PN14, or PN21 respectively). Whole brains were immersed in Golgi-Cox solution, and Purkinje cells from sagittal slices of cerebellar posterior vermis traced. As would be expected from normal growth of the cerebellum, Purkinje cell dendritic length increased across the entire three week period (Figure 4.4A-C ANOVA $F_{2,42}=90.06$, $p<0.01$; PN7 vs PN14 $t=9.01$, $p<0.01$; PN14 vs PN21 $t=5.54$, $p<0.01$). As predicted, inflammation induced or mimicked by LPS or PGE2 significantly decreased Purkinje cell length but only when treatment occurred within the second postnatal week (ANOVA $F_{2,35}=25.19$; veh vs LPS $t=5.12$, $*p<0.01$; veh vs PGE2 $t=6.40$, $*p<0.01$), not within weeks 1 or 3 (students t-test $t=0.84$, $p=0.41$; ANOVA $F_{2,37}=1.55$, $p=0.23$, respectively). LPS at week 1 was not assessed. Representative images of Purkinje cells of all treatment groups and time points can be found in Figure 4.4D-F. These results further implicate a sensitive period for disruption of Purkinje cell morphology by inflammation.

Figure 4.4: Effects of PGE2 on Purkinje cell dendritic length within each of the first three postnatal weeks of life. Male and female pups were treated with vehicle, LPS (200 $\mu\text{g}/\text{kg}$), or PGE2 (2.5 $\mu\text{g}/\text{injection}$) within one of three time periods: week 1 (PN3, 5), week 2 (PN10, 12), or week 3 (PN17, 19), indicated by the red arrows, with the exception of week 1 LPS treatment. Brains were collected at the end of the treatment week (PN7, PN14, or PN21 respectively) and tissue analyzed for Purkinje cell morphology. Timelines of injections and tissue collections are seen in the horizontal bars below the graphs. Since LPS week 1 was not examined, an ANOVA across vehicles for all three ages was performed, as well as a t-test or ANOVA within each week. **A-C)** Purkinje cell dendritic length increased across the entire three week period of vehicle treated animals (ANOVA $F_{2,42}=90.06$, $p<0.01$; PN7 vs PN14 $t=9.01$, $p<0.01$; PN14 vs PN21 $t=5.54$, $p<0.01$). Both LPS and PGE2 significantly lowered Purkinje cell dendritic length when treated in week 2 (ANOVA $F_{2,35}=25.19$; veh vs LPS $t=5.12$, $*p<0.01$; veh vs PGE2 $t=6.40$, $*p<0.01$), but had no effect when treated in weeks 1 (students t-test veh vs. PGE2 $t=0.84$, $p=0.41$) or 3 (ANOVA $F_{2,37}=1.55$, $p=0.23$). **D)** Representative images of Purkinje cells from animals treated with either vehicle or PGE2 in week 1. **E)** Representative images of Purkinje cells from animals treated with vehicle, LPS, or PGE2 in week 2. **F)** Representative images of Purkinje cells from animals treated with vehicle, LPS, or PGE2 in week 3. Scale bar in all images equals 25 microns.

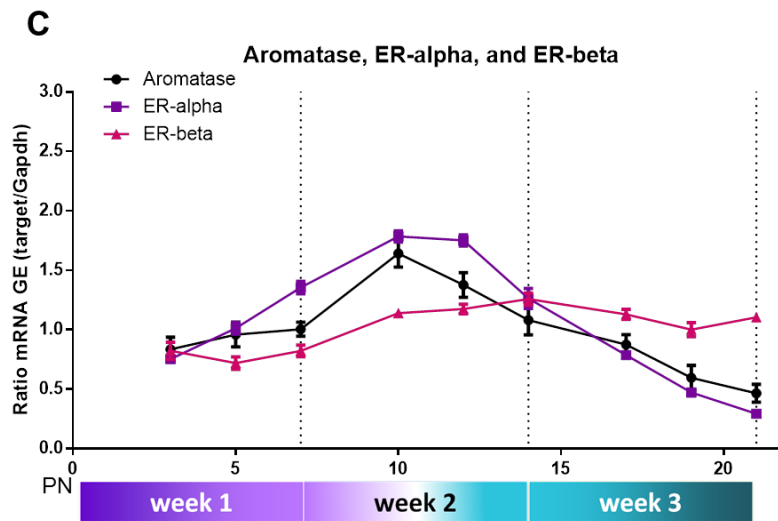
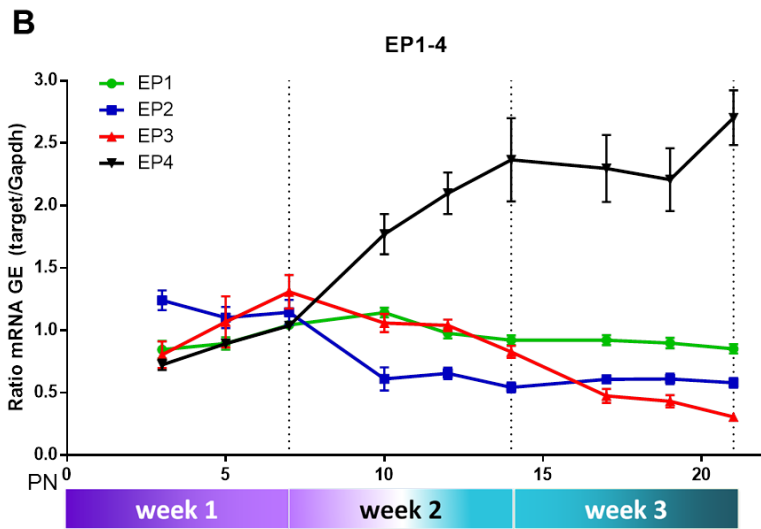
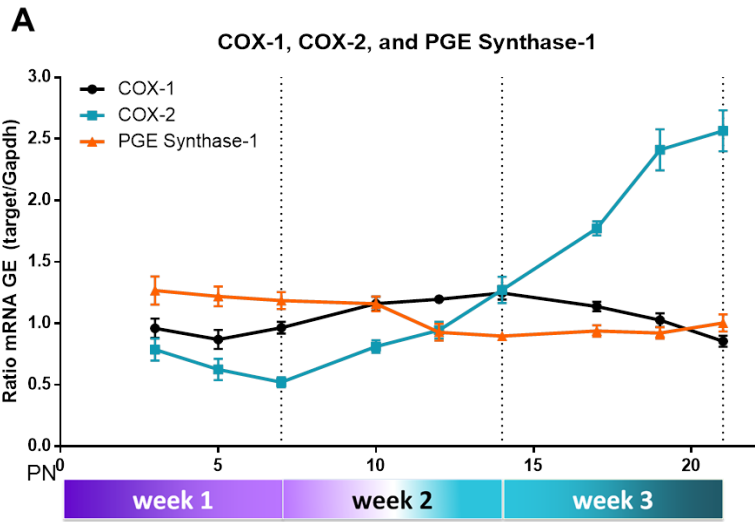




In order to get a better understanding of how different components of the PGE2-E2 signaling pathway could be determining the sensitive period we've seen thus far, we mapped the expression patterns of the following pathway components: COX1 (*PTGS1*) and COX2 (*PTGS2*), PGE synthase-1 (*PGES-1*), PGE2 receptors EP(1-4) (*PTGER1-4*), aromatase (*CYP19A1*), and estrogen receptors ER α (*ESR1*) and ER β (*ESR2*) (Figure 4.5). The cerebellar posterior vermis from male and female pups (4/sex/timepoint) were collected on PN3, PN5, PN7 (week 1), PN10, PN12, PN14 (week 2), and PN17, PN19, PN21 (week 3). These animals were used to examine all 10 of our target genes. Real-time RT-PCR was used to quantify mRNA for each target gene. There were no sex differences in any target genes at any age (1-way ANOVA) and samples were collapsed across sex for a total of 8 animals per group. For each gene, every age was compared back to PN3 with a Bonferroni correction with significance set at $p < 0.05$. COX2 mRNA

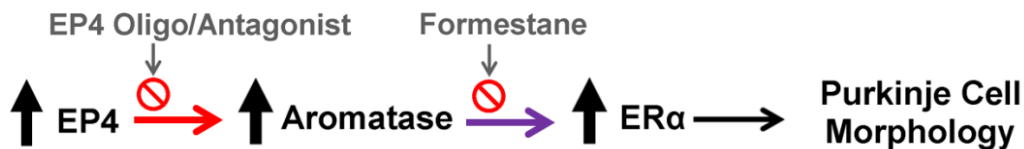
exhibited an insignificant drop across the first postnatal week, then began to rise and was significantly higher than PN3 from PN14 onward. COX1 and PGE Synthase-1 mRNA levels did not significantly change from PN3. EP4 was the most dynamic of the EP receptors, increasing significantly from PN7 onward, while EP1 was significantly higher than PN3 at PN10 only, EP2 was significantly lower at PN10 on but with no further change, and EP3 was significantly higher only at PN7 but significantly lower in the third postnatal week (PN17, 19, and 21). Both ER α and aromatase exhibited a peak in mRNA expression during the second postnatal week, being significantly higher than PN3 at PN7, 10, 12, and 14 (ER α) or PN10, and 12 (aromatase), and both were significantly lower at PN19 and 20. ER β was significantly higher than PN3 from PN10 onward, but with no further change. From these data a pattern emerges in which there are four likely candidates for defining our sensitive period: COX2 and EP4 mRNA are significantly elevated during the second postnatal week compared to the first, but they continue to rise during the third postnatal week. Their continued rise suggests that they are not the cause of the termination of the sensitive period, although it is possible a rise above some threshold triggers the initiation of the sensitive period and a further rise ends it. Conversely, both aromatase and ER α peak during the second postnatal week and then plummet to levels below the first week by the third postnatal week.

Figure 4.5: Developmental time-course of relevant signaling pathway component mRNAs in cerebellar vermis. Expression of each target gene is reported as a ratio of the target gene's genomic equivalent (GE) normalized to the Gapdh genomic equivalent as a loading control. Genomic equivalents are calculated by the SYBR green reporter fluorescence and based on a standard curve for each primer pair, in essence reporting the relative amount of that gene loaded.



Based on mRNA expression patterns, COX2, EP4, aromatase, and ER α are the four logical components most likely involved in defining the sensitive period in the second postnatal week of cerebellar development. However, since COX2 mRNA expression was induced by LPS at time points within all three weeks, it is likely not involved in initiating the sensitive period. Therefore we focused on the relationship between EP4, aromatase, and ER α in an attempt to further understand what was defining the sensitive period. Based on the model we suggested in Chapter 3, we hypothesized that in the cerebellar vermis, the sensitive period is defined by a serial mechanism wherein the initial increase in EP4 expression drives the increase in aromatase expression, which in turn drives the peak in ER α expression (Figure 4.6).

Figure 4.6: Predicted relationship between EP4, aromatase, and ER α mRNA during the sensitive period. Our proposed model within the PGE2-E2 signaling pathway where the initial increase in EP4 expression drives the increase in aromatase expression, which in turn drives the peak in ER α expression during the second postnatal week.



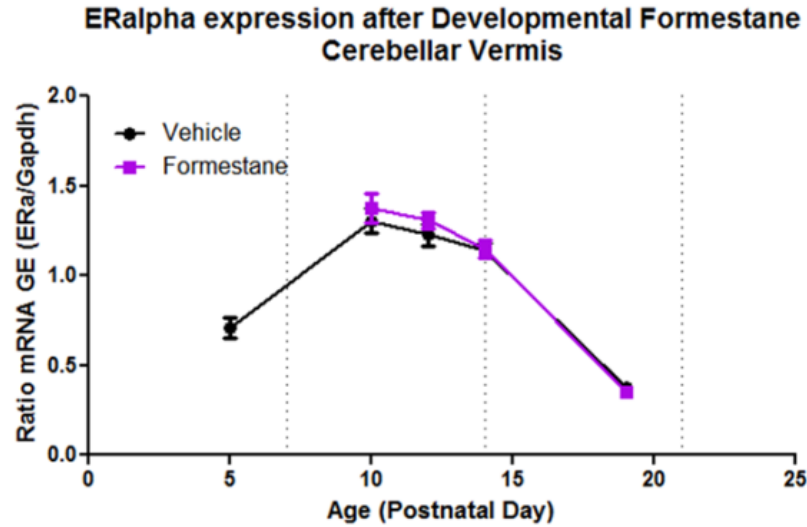
From this we made several predictions:

- 1) if the increase in EP4 expression was driving the increase in aromatase mRNA, then blocking EP4 translation with an antisense oligonucleotide would prevent the increase in aromatase mRNA
- 2) blocking EP4 protein function with an antagonist would also prevent the increase in aromatase mRNA

- 3) if the increase in aromatase expression was driving the increase in ER α mRNA, then blocking aromatase activity with formestane would prevent the increase in ER α mRNA
- 4) if this pathway was truly *serial*, then blocking EP4 protein production or function would *also* prevent the increase in ER α mRNA, as well as preventing the increase in aromatase mRNA.

To test these predictions, we planned to treat animals between PN5-13 with either an EP4 antisense oligonucleotide or an antagonist and measure aromatase or ER α mRNA at different time points throughout the three postnatal weeks, and treat another cohort of animals with formestane in the same paradigm and measure ER α mRNA. Unfortunately, although we were able to use an EP4 antisense oligonucleotide previously established in our laboratory, we were unable to determine whether it was effective in decreasing EP4 protein production due to complications with EP4 antibody reliability. Similarly, we were unsuccessful in obtaining an EP4 inhibitor that has been referenced in the literature but is no longer available. We were able to treat animals with formestane and examine changes in ER α mRNA however, so animals were treated according to the paradigm in Table 4.5 and results are seen in Figure 4.6C. Formestane had no effect on ER α expression throughout the sensitive period ($F_{1,70\text{-treat}}=0.70$, $p=0.40$).

Figure 4.7: Relationship between aromatase and ER α mRNA during the sensitive period. Formestane was not capable of preventing the peak in ER α mRNA although there was a significant peak in expression over time (ANOVA, treatment $F_{1,70}=0.70$, $p=0.40$; age $F_{4,70}=121$, $p<0.01$; interaction $F_{4,70}=0.35$, $p=0.84$).



Discussion

We have shown that an increase in PGE2 induced either through the systemic administration of the inflammation inducer LPS or through direct infusion increases estradiol production in the cerebellum, as well as reduces Purkinje cell dendrite size, but only when exposure to these extrinsic factors occurred within the second postnatal week, not the first or third. All of these findings strongly indicate a sensitive period in which normal cerebellar development is at risk of disruption by extrinsic factors. Further, upon examining the mRNA expression pattern of components of the PGE2-E2 signaling pathway, four of them initially appear to be potential candidates for defining the sensitive period: COX2 and EP4 both increase steadily beginning at the second postnatal week, potentially driving the opening of the sensitive period. Both aromatase and ER α peak

during the second postnatal week and then decrease to levels below the first week by the third postnatal week. Further, although levels of expression between different genes cannot be directly compared in the method they were run here, the efficiencies of the ER α and ER β primers were similar, with an average cycle threshold (CT) value of 26.012 in week 2 and 27.495 in week 3 for ER α and 27.267 in week 2 and 27.140 in week 3 for ER β . A lower cycle threshold indicates a higher level of cDNA present in the initial sample. This means that in week 2, there is a greater amount of ER α present than ER β , but in week 3 this switches and there is a greater amount of ER β present than ER α . ER β opposes actions of ER α in some tissues, and ER β inhibits ER α when the proteins dimerize. The switch in relative expression from week 2 to week 3 could cause the close of the sensitive period. Thus it is potentially the steroid components of the pathway that constrain the sensitive period.

Despite the natural increase in COX2 mRNA from the second postnatal week onward, it is actually an unlikely driver of the sensitive period because LPS was able to induce COX2 at the middle time point of all three postnatal weeks. Why, then, was LPS unable to increase PGE2 at all three weeks? We only showed that COX2 *mRNA* expression goes up; we didn't examine a change in the actual protein level or even activity. It could be that the sensitive period in LPS inducing PGE2 comes from a change in translation, rather than transcription, of COX2, something that should be explored further. Another possible explanation is that there may be a shift in synthesis of the other prostanoids across the three weeks. Increasing COX2 expression is not a guarantee that only PGE2 will go up: as shown in Figure 1.5, COX1 and COX2 are responsible for converting arachidonic acid to PGG2 which is quickly converted to PGH2, which is in

turn converted to one of five prostanoids by other PG-synthases. PGE-synthase exhibited practically no change across the first three weeks, but it is possible that the comparative levels of the five synthases are changing relative to one another, thus driving synthesis of other prostanoids, rather than PGE₂, at this time. Additionally, other researchers have reported that rats under a week old do not exhibit a febrile response or increase in PGE₂ following exposure to endotoxins such as LPS (Kasting, 1987; Kentner, 2010), although the exact mechanism is not currently understood. These questions are worth following up in future experiments to understand the full picture of the seeming disconnect between COX2 and PGE₂.

In Chapter 1 we discussed expression of some of the pathway components in the neonatal rat cerebellum. Tsutsui (2008) reported that mRNA for aromatase peaked between PN5 and 10 and fell thereafter, with a smaller, later peak in females. Our data indicate no significant sex difference in the peak expression times of aromatase, although the peak in aromatase around PN10 is consistent with their findings. Sakamoto (2003) reported that estradiol levels were higher in the cerebellum than the plasma during the first week of life, but that no differences were found between cerebellum and plasma later in life. They actually looked at PN3 versus adults, and did not compare cerebellar estradiol levels across multiple time points within the first three postnatal weeks that we did. Ikeda (2006) reported that ER α mRNA expression was higher in the first three postnatal weeks than adulthood, and showed a peak at week 2 (PN14). This is consistent with our finding that ER α expression peaked within the second postnatal week; due to our expanded time course of 9 time points instead of 3, we showed that the peak is actually around PN10. A comparison of our values around PN7, 14, and 21 and their

reported values shows a similar expression pattern. Further, we show that ER β is relatively unchanged across the first three postnatal weeks, also consistent with reports by Ikeda (2006). However, these results are not consistent with those by Jakab (2001), which show ER β mRNA expression in the cerebellum is low through most of the first postnatal week, peaks on PN10, and then falls to adult levels.

We have shown for the first time expression patterns of 10 different genes in the cerebellum across 9 days within the first three postnatal weeks of cerebellar rat development, several of which exhibit patterns consistent with a sensitive period in the second postnatal week. We propose that the second postnatal week, PN7-14, in rats is in fact a sensitive period in cerebellar development wherein normal Purkinje cell maturation is especially vulnerable to inflammatory events. Consider again each of Nash's criteria for a critical period:

- 1) *an identifiable beginning point* – around PN7
- 2) *an identifiable end point* – around PN14
- 3) *an intrinsic component* – the PGE2-E2 signaling pathway, especially a natural decrease in PGE2 during this period
- 4) *an extrinsic component* – LPS specifically, but any exogenous source of infection, pushing PGE2 and E2 beyond their normal endogenous levels
- 5) *a specified critical system affected* – Purkinje cell morphology (in the next chapter, we discuss how this also effects socially-relevant behaviors).

It is appropriate to refer to this period as a sensitive period rather than a critical period because it is a time when neural development is most vulnerable to *disruption* by external

stimuli, rather than a point when exogenous stimuli are *necessary* for normal development.

This particular sensitive period in cerebellar development is important because PN7-14 in the rat corresponds to between birth and a year or two old in the human, a time when children are especially prone to infection. Given that early life inflammation is associated with an increased risk of autism (Torres, 2003; Cohly, 2005; Persico, 2006) and schizophrenia (Clarke, 2009; Li, 2009; reviewed in Fan, 2007), this is an appropriate model for the study of the etiology of neurodevelopmental diseases in humans.

To further investigate the potential correlation between expression in the PGE2-E2 signaling pathway during this time frame in the rat and human, our lab is currently examining human cerebellar tissue from the NICHD Brain Bank. We have more than 50 samples from birth to 5 years, male and female, across a variety of ethnic backgrounds, and are examining changes in mRNA expression for the 10 genes examined in Figure 4.5 in the rat. If patterns in human mRNA expression match up with expression patterns we have seen here in the rat, it would confirm a strong correlation between developmental ages of the rat and human, and help us identify a similar sensitive period in human development. Identification of a period of potential increased risk for autism from inflammation could lead to more directed preventative practices in healthcare that could reduce the incidence of future neurodevelopmental diseases.

Chapter 5:

Characterization and prevention of behavioral deficits following perturbation of the PGE2-E2 pathway mediating the sensitive period in cerebellar development

Introduction

Mental disorders have always been a part of human history, but it's only recently that we've begun to understand them on a biochemical and neurophysiological level. The term schizophrenia, whose Greek roots mean "split mind," coined by Swiss psychiatrist Eugene Bleuler in 1911, was previously understood as dementia precox (Kraepelin, 1992). The work begun by Kraepelin and Bleuler divided schizophrenia into five subtypes when it first appeared in the DSM-III: disorganized, catatonic, paranoid, residual, or undifferentiated. These categories are loosely still used today, but have not provided much insight into causes of this disorder. Diagnosis and research currently focuses more on "positive" vs "negative" symptoms, the progression of the disorder in terms of type and severity of symptoms over time, and the co-occurrence of other mental disorders and syndromes.

The term Autism, whose Greek root means "self," was also coined by Bleuler around the same time, referring more to the social withdrawal aspect of negative symptoms in schizophrenia (Gay, 1989). In the early 1940s Hans Asperger, a scientist in Germany, identified a similar condition that's now called Asperger's syndrome (Asperger, 1968), and Leo Kanner, a doctor from Johns Hopkins University, used Autism to refer to children with a particular form of early onset schizophrenia (Eisenberg, 1956). It wasn't until the late 1960s that autism began to be seen as a disease distinct from

schizophrenia, and was finally separated from childhood schizophrenia when it was added to the DSM-III in 1980 (for review Johnson, 2008). In the late 1990s the DSM-IV expanded the diagnosis of Autism to include Asperger's syndrome, but in 2013 the DSM-V removed several subcategories defined in the DSM-IV and collapsed it into one umbrella diagnosis of autism spectrum disorder (ASD), defined by two categories: 1) impaired social communication and/or interaction and 2) restricted and/or repetitive behaviors. All of these changes in schizophrenia and autism diagnostics relied heavily on observational behaviors, with little to no insight into specific causes, or better treatments, until very recently.

Schizophrenia generally manifests in the late teens or early twenties, with an average age of onset in men at 18 and in women at 25, and frequently is less severe in women. Onset during childhood is rare and only 0.01% of children are diagnosed with schizophrenia (Kalapatapu, 2008). The disorder is characterized by both positive symptoms (sensory hallucinations, delusions, disorganized speech, disorganized behavior, or catatonia) and negative symptoms (flattening of affect, poverty of speech, lack of social interest, general apathy, and general inattentiveness), and most current medications are only capable of addressing the positive symptoms. Schizophrenia affects 1% of the general population, but it occurs in 10 percent of people who have a first-degree relative with the disorder, such as a parent, brother, or sister. People who have second-degree relatives (aunts, uncles, grandparents, or cousins) with the disease also develop schizophrenia more often than the general population. The risk is highest for an identical twin of a person with schizophrenia, with a 40-60 percent chance of developing the disorder, indicating a strong but not exclusive genetic component of Schizophrenia.

Due to the complex diagnoses of Autism Spectrum Disorder (ASD), the exact prevalence of ASD is unknown. Current estimates place it at approximately 1 in 1,000 children in the US, with a similar risk across racial, ethnic, and social groups (NICHD, 2005; Whiteley, 2010). Regardless of prevalence, there is a three- to four-fold higher incidence of ASD in males versus females (Berg, 2009). Symptoms generally appear between 18 months and 3 years – evidence that it is an early developmental disorder. ASD is characterized by inappropriate social interactions, impairments in social communication, repetitive and stereotyped behaviors, resistance to change, and abnormal responses to sensory stimuli, among others. Currently there is no cure, and the best therapies are psycho-social therapy sessions with varying degrees of success. ASD is one of the most common serious childhood disorders, with both direct and indirect costs of caring for an autistic person across their lifetime. Discovery of risk factors for development of ASD could lead to preventative methods to reduce the incidence, and possibly provide therapy options for those with ASD.

It is nearly impossible to study positive symptoms of schizophrenia in the rat, as we have no way of knowing if a rat is hallucinating or experiencing disorganized speech. Negative symptoms of both Schizophrenia and ASD overlap, but likely are a similar phenotype due to disparate origins, and so are only capable of providing face validity in a study. That said, several of these behaviors have well-defined analogous tests in mice and rats such as social play, social choice chambers, resident-intruder tasks, ultrasonic vocalization recordings, hole-poke boards, object exploration and responses to von Frey filaments (Crawley, 2007). Knowing this we can begin to examine potential risk factors for development of early life mental disorders.

Early life inflammation, either in utero or postnatally, is a major risk factor for development of disorders of mental health, including schizophrenia (Clarke, 2009; Li, 2009; reviewed in Fan, 2007) and ASD (Torres, 2003; Cohly, 2005; Persico, 2006). However, not every child that suffers fever and inflammation develops these disorders – this suggests there may be a specific sensitive period in which intrinsic and extrinsic variables converge and change the course of normal neuronal development in genetically susceptible individuals. The concept of a “sensitive period” in brain development has been established in several neural systems such as visual cortex, barrel cortex, and sexual differentiation of hypothalamic brain regions (Blakemore, 1973; Blakemore, 1974; Daw, 1976; Hubel, 1970; Rhee, 1990). With this in mind, we seek to define the postnatal sensitive period within which inflammation may increase the risk for development of ASD.

We have already observed behavioral deficits in males after treatment with the COX2 inhibitor, nimesulide during postnatal week 2 - only males show a decrease in social play behavior, though both males and females showed an increase in total object exploration (Dean, 2012a). Social play is a sexually differentiated behavior controlled by multiple brain regions including the amygdala (van Kerkhof, 2013) and prefrontal cortex (Bell, 2009). The cerebellum has indirect connections with these regions, which could explain the prevalence of cerebellar pathologies in ASD diagnoses (see references in Chapter 1).

The cerebellum is highly involved in motor activity, and if an animal has abnormal motor ability or activity levels it would confound any results in other tasks. To ensure that our rats had normal motor behavior, three different tasks were utilized. The

negative geotaxis task (performed at PN13) tests a natural righting reflex, the wire hang task (performed at PN19) tests grip strength, and the open field task (here performed at PN25 and PN60) measures general activity (Petrosini, 1990).

Another potential confounding variable for social interaction tasks is whether an animal is capable of detecting socially relevant odors and pays them more attention than non-socially relevant odors. This was assessed with the Q-tip olfactory test.

To examine the autistic-like behavior of increased object interest, also known as perseverance, we utilized the Novel Object task. Normally used to test changes in memory, animals are given two identical objects to interact with one day (becoming the “familiar object”), and 24 hours later are given one of the familiar objects and a different (“novel”) object to interact with. Generally animals will spend more time with the novel object than the familiar object. Previously when we used this task in animals treated with nimesulide in the second postnatal week, we found that although there were no differences in object memory by treatment, there was an increase in total object interest (Dean, 2012a). Thus the same behavioral task was used here.

To examine the autistic-like feature of abnormal social interaction, two different tasks were used. The first was a social play task run from PN28-38, assessing juvenile aggressive play behaviors (chasing, pouncing, wrestling, pinning, and boxing). Males are naturally more aggressive than females and typically have higher play scores in this task (Oleson, 2005; Casto, 2003; Ward, 1991; Meany and McEwen, 1986). Since previously we saw a decrease in male play behavior with no change in female play behavior, which was already very low (Dean, 2012a), we considered the potential of a floor effect in play behavior in females below which they could not be further reduced. To examine a

different aspect of social interaction that does not have a sex bias, we used a second social task: the social choice chamber. Performed at PN70, animals are given a choice of investigating a box containing a stranger rat (age and sex matched) or an empty box (Crawley, 2007).

After establishing the existence of a sensitive period for Purkinje cell development in the second postnatal week we next sought to determine if there is also a sensitive period for induction of behavioral abnormalities. We hypothesized that abnormalities in Purkinje cell development from exogenous insults only during the sensitive window in postnatal week 2 would result in behavioral impairments. From this hypothesis we predicted that 1) induction or mimicking of inflammation will result in behavioral deficits, 2) these deficits can be prevented in the same way changes to Purkinje cell morphology were prevented, and 3) these deficits will be limited to animals experiencing inflammation only in the second postnatal week.

Methods

Behavioral tasks

Specifics of each behavioral task are outlined in the general methods section (Chapter 2). A summary of the timing of each task and its purpose is outlined in Table 5.1. For each treatment paradigm, animals went through multiple behavioral tasks; there was not a separate treated cohort for each individual task. While we recognize that order effects of exposure to different tasks could influence behavior in later tasks, this was unavoidable as the paradigm we examined was based on developmental changes and the tasks had to occur at age-specific times due to the nature of the task.

Table 5.1: Behavioral Task Timeline

Age	Task	Outputs / Measurements
PN 13	<i>Negative Geotaxis</i>	<i>Time to turn 180°</i>
PN 19	<i>Wire Hang</i>	<i>Time to fall (grip strength)</i>
PN 25	<i>Open Field – Young (Novel Object Day 1)</i>	<i>Line crossings (general movement) / center crossings (anxiety)</i>
PN 26	Familiar Object Presentation (Novel Object Day 2)	No Data
PN 27	Novel Object Presentation (Novel Object Day 3)	Object interest / object memory
PN 28 – 38	Social Play	Play groups of 6-8 animals, tally of play behaviors
PN 60	<i>Open Field – Adult (Novel Object Day 1)</i>	<i>Line crossings (general movement) / center crossings (anxiety)</i>
PN 61	Familiar Object Presentation (Novel Object Day 2)	No data
PN 62	Novel Object Presentation (Novel Object Day 3)	Object interest / object memory
PN 67	<i>Q-tip olfactory test</i>	<i>Deficits in social odor cues</i>
PN 70	Social Choice Chamber	Adult social deficits
PN 76	Brain collection	Golgi staining for cerebellar morphological changes

Rats and treatment paradigms

Sprague Dawley timed pregnant dams were allowed to deliver normally under standard laboratory conditions. Following treatments within the second or third postnatal week, animals were weaned on PN21 and housed in groups of 2 or 3 with like sex, mixed treatment. Specified behavioral tasks were performed, and brains collected for later evaluation of cerebellar morphology.

2nd postnatal week – PGE2 Treatment Paradigm

Pups were treated on PN10 and PN12 with PGE2 (2.5 µg/1µl) or saline vehicle via the Foramen Magnum, injection volume of 1 µl. N= 7-9 males, 7-9 females per group. This cohort performed the negative geotaxis, wire hang, open field / novel object

(at the young age), social play, open field / novel object (as adults), Q-tip olfactory test, and social choice chamber tasks.

2nd postnatal week – LPS and formestane Treatment Paradigm

Pups were treated with 1) vehicle; 2) LPS (200 µg/kg, i.p. PN10, PN12); 3) formestane (100 µg per injection, s.c. daily, PN10-14); or 4) a combination of formestane and LPS, one hour in between injections. N= 7-9 males, 7-9 females per group. This cohort performed the negative geotaxis, wire hang, open field / novel object (at the young age), social play, and open field / novel object (as adults) tasks.

2nd postnatal week – LPS and nimesulide Treatment Paradigm

Pups were treated with 1) vehicle; 2) LPS (200 µg/kg, i.p. PN10, PN12); 3) Chronic nimesulide (0.5mg per injection, s.c. daily, PN7-14); 4) Acute nimesulide (0.5 mg per injection, s.c. daily, PN10-14); or 5) a combination of Acute nimesulide and LPS, one hour in between injections. N= 7-9 males, 7-9 females per group. This cohort performed the open field / novel object (at the young age) and social play tasks.

3rd postnatal week – PGE2 Treatment Paradigm

Pups were treated on PN17 and PN19 with PGE2 (2.5 µg/1 µl) or saline vehicle via the Foramen Magnum, injection volume of 1 µl. N= 7-9 males, 7-9 females per group. This cohort performed the open field / novel object (at the young age), social play, and adult open field / novel object (as adults) tasks.

Statistical Analysis

Group means for behavioral measures were compared via two-way ANOVA, with Tukey's post-hoc tests performed in the event of a significant main or interaction effect of treatment and sex. All statistical tests used a value of $p < 0.05$ as the criterion for significance.

Results

In order to confirm there were no potential confounding variables in object interest or social tasks we utilized a variety of motor and olfactory discrimination tasks. As expected, there were no significant differences between sex or treatment groups in any of the negative geotaxis or wire hang tasks (Figure 5.1). For the open field task, there were no significant differences induced by any treatment, but several cohorts showed a trending or significant difference according to sex where females were more active than males overall: 1) second week PGE2 cohort, PN25 ($^{\#}F_{1,26\text{-sex}}=3.94$, $p=0.06$) and PN60 ($^*F_{1,26\text{-sex}}=9.40$, $p < 0.01$); 2) second week LPS/formestane cohort, PN60 ($^*F_{1,45\text{-sex}}=48.96$, $p < 0.01$), 3) third week PGE2 cohort, PN25 ($^*F_{1,25\text{-sex}}=14.44$, $p < 0.01$) and PN60 ($^*F_{1,25\text{-sex}}=22.52$, $p < 0.01$) (Figure 5.2).

In the novel object task, when examining the preference for examining a novel object over a familiar object, there were no significant differences induced by any treatment, but one cohort showed a significant difference according to sex where females showed a greater preference for the novel object than the males: second week LPS/Formestane cohort at both PN27 ($^*F_{1,44\text{-sex}}=4.91$, $p < 0.05$) and PN62 ($^*F_{1,46\text{-sex}}=12.48$, $p < 0.01$) (Figure 5.3). Most animals showed a preference percentage of about

50%, however, indicating that there was actually no preference for the novel object over the familiar one. This indicates that the memory aspect of this task is not a valid test for changes in memory, and could be due to a few factors. First, animals were tested 24 hours after a short (5 minute) exposure to the familiar objects, which is a long retention time, especially for the younger (PN7) animals. Secondly, the initial objects used were rather large – a mug and a large box; these were used in the PGE2 week 2 and week 3 treated cohorts. These could have been intimidating to the rats, especially the younger ones, and upon switching to a smaller box and smaller cylinder the rats did show an increase in the percent time spent with the novel object (see LPS/formestane cohort). To more accurately test potential changes in memory retention, a different task protocol would be necessary. However, this does not affect overall interest in objects, a potential perseverance behavior, which we previously found to be significantly increased in males treated with nimesulide in the second postnatal week (Dean, 2012a).

Rats in the second week PGE2-treatment paradigm were tested for their ability to detect and habituate to two different odorants, one non-socially relevant (citral), one socially-relevant (cage swab), and water. Rats were able to detect both odorants and habituate to them, with a greater interest in the socially relevant odor, with no significant differences in sex or treatment (Figure 5.4).

Figure 5.1: Neonatally treated rats exhibit no deficits in general motor ability. **A)** Rats treated with vehicle or PGE2 in the second postnatal week. Negative geotaxis task performed at PN13. No significant differences in treatment ($F_{1,26}=0.64$, $p=0.43$), sex ($F_{1,26}=0.22$, $p=0.64$), or interaction ($F_{1,26}=0.07$, $p=0.79$). **B)** Rats treated with vehicle or PGE2 in the second postnatal week. Wire hang task performed at PN19. No significant differences in treatment ($F_{1,26}=0.18$, $p=0.67$), sex ($F_{1,26}=0.49$, $p=0.49$), or interaction ($F_{1,26}=0.01$, $p=0.91$). **C)** Rats treated with vehicle, formestane, LPS, or formestane + LPS in the second postnatal week. Negative geotaxis task performed at PN13. No significant differences in treatment ($F_{3,45}=0.55$, $p=0.65$), sex ($F_{1,45}=0.10$, $p=0.76$), or interaction ($F_{3,45}=0.53$, $p=0.66$). **D)** Rats treated with vehicle, formestane, LPS, or formestane + LPS in the second postnatal week. Wire hang task performed at PN19. No significant differences in treatment ($F_{3,45}=1.10$, $p=0.36$), sex ($F_{1,45}=1.83$, $p=0.18$), or interaction ($F_{3,45}=0.14$, $p=0.94$).

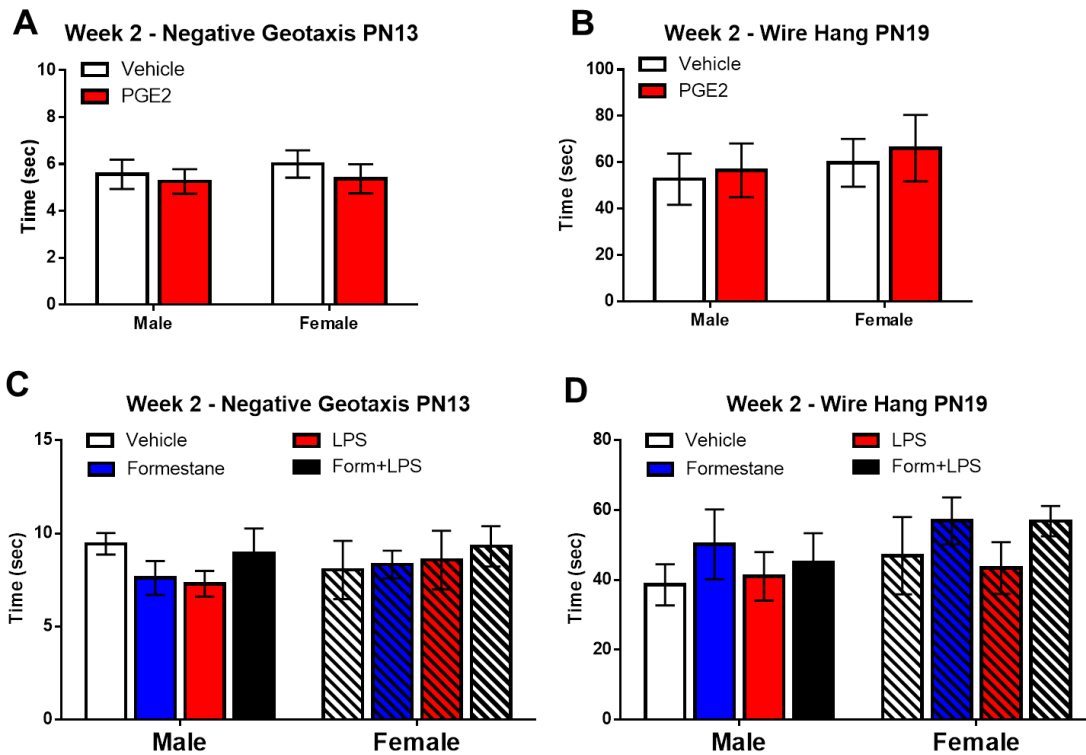


Figure 5.2: Neonatally treated rats exhibit no deficits in general motor activity. Open field assessment performed either at PN25 (“Young”) or PN60 (“Adult”). **A)** Rats treated with vehicle or PGE2 in the second postnatal week, assessed in open field at young age. No significant differences in treatment ($F_{1,26}=0.04$, $p=0.84$) or interaction ($F_{1,26}=0.07$, $p=0.79$), with a trending difference in sex ($^{\#}F_{1,26}=3.94$, $p=0.06$). **B)** Rats treated with vehicle or PGE2 in the second postnatal week, assessed in open field in adults. No significant differences in treatment ($F_{1,26}=0.01$, $p=0.94$) or interaction ($F_{1,26}=0.46$, $p=0.50$), with a significant difference in sex ($^*F_{1,26}=9.40$, $p<0.01$). **C)** Rats treated with vehicle, formestane, LPS, or formestane + LPS in the second postnatal week, assessed in open field at young age. No significant differences in treatment ($F_{3,45}=0.75$, $p=0.53$), sex ($F_{1,45}=1.00$, $p=0.32$), or interaction ($F_{3,45}=0.41$, $p=0.75$). **D)** Rats treated with vehicle, formestane, LPS, or formestane + LPS in the second postnatal week, assessed in open field in adults. No significant differences in treatment ($F_{3,45}=1.23$, $p=0.31$) or interaction ($F_{3,45}=0.13$, $p=0.94$), with a significant difference in sex ($^*F_{1,45}=48.96$, $p<0.01$). **E)** Rats treated with vehicle, Acute nimesulide, Chronic nimesulide, LPS, or Acute nimesulide + LPS in the second postnatal week, assessed in open field at young age. No significant differences in treatment ($F_{4,73}=0.60$, $p=0.66$), sex ($F_{1,73}=2.50$, $p=0.12$), or interaction ($F_{4,73}=0.84$, $p=0.51$). **F)** Rats treated with vehicle or PGE2 in the third postnatal week, assessed in open field at young age. No significant differences in treatment ($F_{1,25}=0.15$, $p=0.70$) or interaction ($F_{1,25}=0.01$, $p=0.94$), with a significant difference in sex ($^*F_{1,25}=14.44$, $p<0.01$). **G)** Rats treated with vehicle or PGE2 in the third postnatal week, assessed in open field in adults. No significant differences in treatment ($F_{1,25}=0.73$, $p=0.40$) or interaction ($F_{1,25}=0.01$, $p=0.91$), with a significant difference in sex ($^*F_{1,25}=22.52$, $p<0.01$).

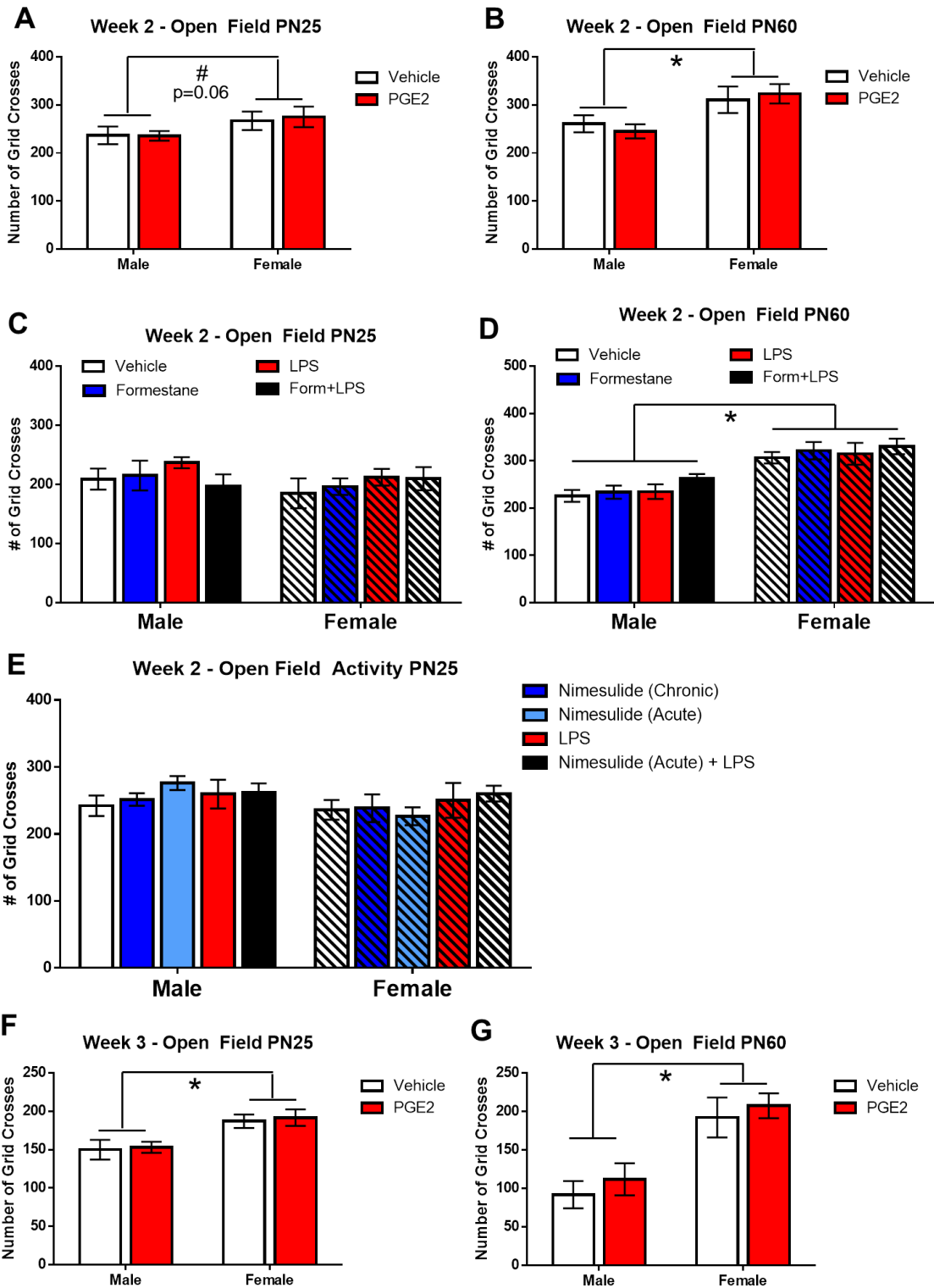


Figure 5.3: Neonatally treated rats exhibit no differences in preference for a novel object. Pups were treated with vehicle or PGE2 (for A and B) or vehicle, formestane, LPS, or formestane+LPS (for C and D) during the second postnatal week. In E and F pups were treated with vehicle or PGE2 during the third postnatal week. Novel object preference, the percent time spent investigating the novel object out of the entire time spent investigating both objects, was assessed at PN27 (“young”) or PN62 (“adult”). **A)** Novel object preference at young age: Two-way ANOVA revealed there no significant effects of treatment ($F_{1,25}=0.01$, $p=0.94$), sex ($F_{1,25}=1.16$, $p=0.29$), or interaction ($F_{1,25}=2.01$, $p=0.17$). **B)** Novel object preference in adults: Two-way ANOVA revealed there were no significant effects of treatment ($F_{1,26}=0.19$, $p=0.66$), sex ($F_{1,26}=0.07$, $p=0.80$), or interaction ($F_{1,26}=0.10$, $p=0.75$). **C)** Novel object preference in young age: Multi-way ANOVA revealed there was a main effect of sex ($F_{1,44}=4.91$, $p<0.05$) where females preferred the novel object more than males, but there was no effect of treatment ($F_{3,44}=1.19$, $p=0.33$) or interaction ($F_{3,33}=0.12$, $p=0.95$). **D)** Novel object preference in adults: Multi-way ANOVA revealed there was a main effect of sex ($F_{1,46}=12.48$, $p<0.01$) where females preferred the novel object more than males, but there was no effect of treatment ($F_{3,46}=0.57$, $p=0.64$) or interaction ($F_{3,46}=0.35$, $p=0.79$). **E)** Novel object preference at young age: Two-way ANOVA revealed there no significant effects of treatment ($F_{1,16}=0.01$, $p=0.98$), sex ($F_{1,16}=0.01$, $p=0.94$), or interaction ($F_{1,16}=1.23$, $p=0.28$). **F)** Novel object preference in adults: Two-way ANOVA revealed there were no significant effects of treatment ($F_{1,19}=0.06$, $p=0.81$), sex ($F_{1,19}=0.01$, $p=0.96$), or interaction ($F_{1,19}=0.16$, $p=0.70$).

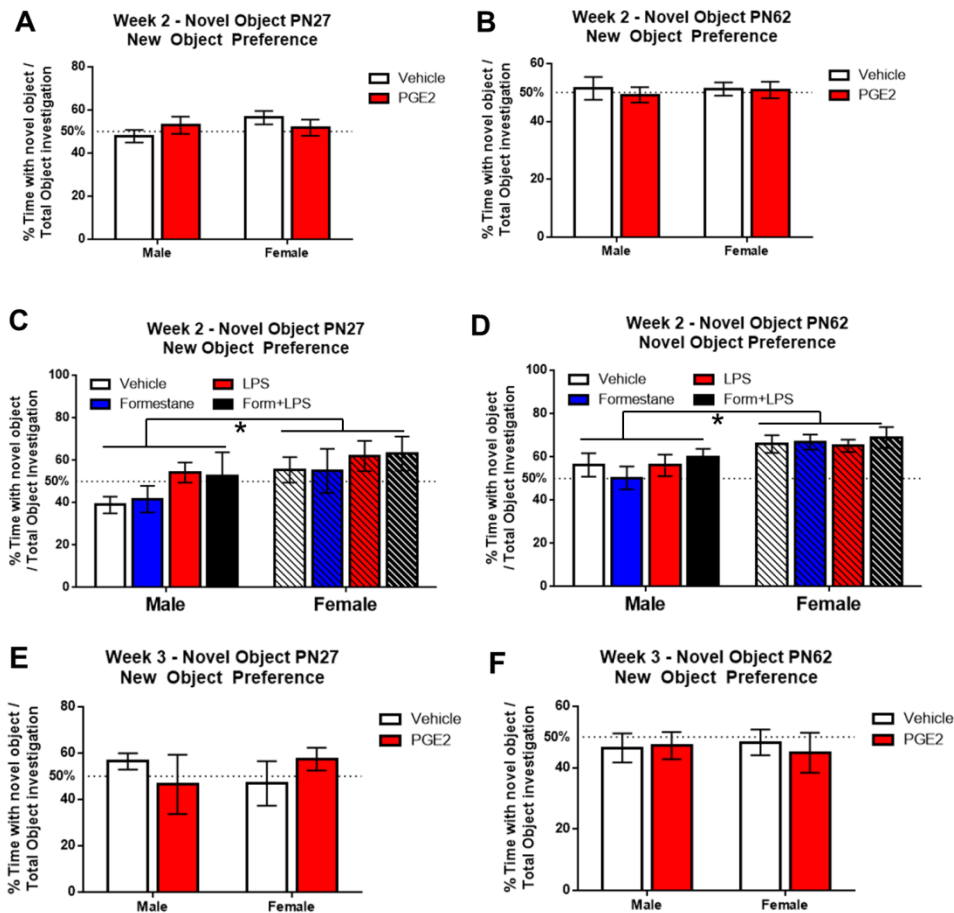
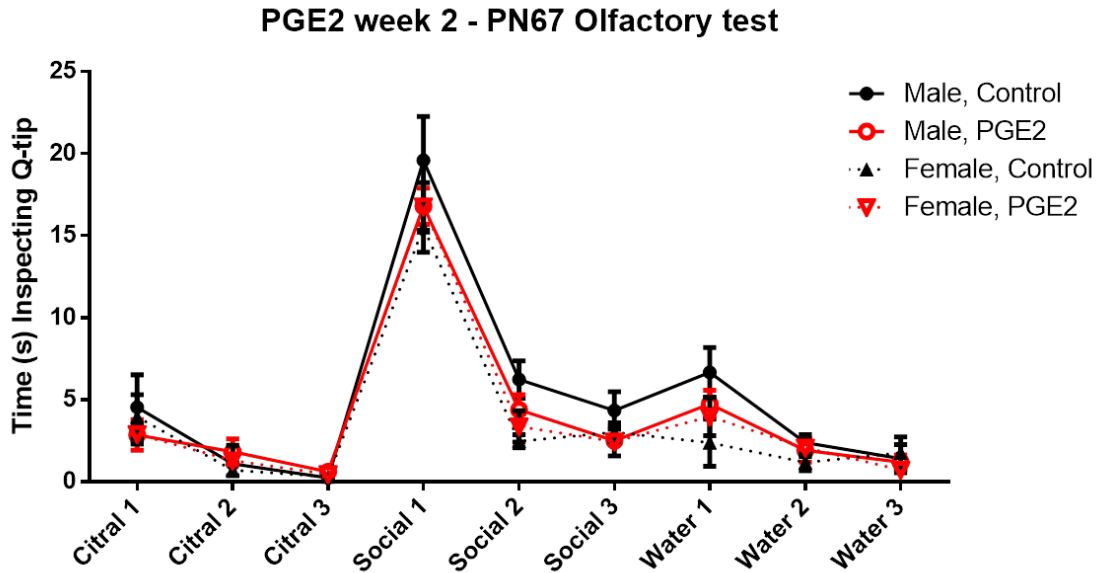


Figure 5.4: Neonatally treated rats exhibit no deficits in social odor detection. Rats treated with vehicle or PGE2 during postnatal week 2 were presented with a series of Q-tips scented with citral (3 trials), social odor (3 trials), and water (3 trials). Average total time investigating each Q-tip presentation per group was analyzed. Animals habituated to subsequent presentations of each scent trial scent, and spent more time investigating the first presentation of the socially relevant scent than the citral scent. Post hoc comparisons revealed no differences existed between sex or treatment group (MANOVA, scent $F_{8,234}=7.49$, $p<0.00$; treatment/sex $F_{3,234}=4.70$, $p<0.01$; interaction $F_{24, 234}=0.75$, $p=0.79$).



Induction of inflammation was achieved by treatment with LPS, and mimicking of inflammation was achieved by treatment with PGE2. Since we predicted that both would impact behavior, and that the impact could be prevented, we also co-treated animals with the aromatase inhibitor, formestane, or the COX2 inhibitor, nimesulide and examined changes in behavior. When tested on PN27, both males and females had increased total object exploration after either LPS (Figure 5.5B, males $t=3.06$, $*p<0.05$; females $t=3.12$, $*p<0.05$) or PGE2 (Fig 5.5A, $F_{1,24-treat}=5.90$, $*p<0.05$). There was no effect of either LPS or PGE2 treatment on object interest at PN60, although there was a general sex difference in the LPS/formestane cohort, with females showing more total

interest than males ($F_{1,46\text{-sex}}=15.94$, $*p<0.01$). Further, the increased object exploration in animals exposed to LPS during the second postnatal week was prevented by pretreatment with formestane in both males ($t=3.65$, $*p<0.01$) and females ($t=3.10$, $*p<0.01$), with no effect of formestane alone (Fig 5.5C).

As expected, males were more socially playful than females in every play cohort (Figure 5.6A $t=2.22$, $*p<0.05$; B $t=4.170$, $*p<0.01$; C $t=5.61$, $^ap<0.01$), but only the males exhibited a decrease in social play after treatment with LPS (Figure 5.6B, $t=3.42$, $**p<0.05$) or PGE2 (Figure 5.6A, $t=3.50$, $**p<0.01$). The deficit in male social play induced by exposure to LPS was partially prevented by pretreatment with formestane: Form+LPS is neither significantly different from vehicle ($t=1.98$, $p=0.24$) nor LPS alone ($t=1.44$, $p=0.66$). However, this is confounded by the observation that treatment with formestane alone also reduced male social play (Figure 5.6B, $t=3.42$, $p<0.05$). To examine this confounding variable from a different angle, rats were given nimesulide in a “chronic” dose (the same dose given in Dean, 2012a which resulted in a decrease in social play in males) or an “acute” dose (a shorter exposure we expected to have no effect on play). As expected, LPS exposure still decreased play behavior only in males (Figure 5.6C, $t=4.21$, $^bp<0.05$), as did chronic nimesulide treatment ($t=3.91$, $^bp<0.05$), but the acute treatment had no effect ($t=1.09$, $p=0.99$). Thus we can be confident the acute nimesulide treatment given 1 hour prior to LPS did in fact prevent the decrease in play behavior in males as opposed to an independent effect of nimesulide when treated in the second postnatal week (Figure 5.6C, $t=4.65$, $^cp<0.05$).

To examine another potential social deficit, animals in the second postnatal week PGE2 paradigm were given the choice to investigate a box with a stranger rat or an empty

box. There was no significant effect of sex ($F_{1,26}=0.14$, $p=0.71$), treatment ($F_{1,26}=1.47$, $p=0.24$), or interaction ($F_{1,26}=0.77$, $p=0.39$) (Figure 5.7) on preference of investigating the stranger rat over the empty box.

Figure 5.5: Inflammation within the second postnatal week increased total object interest, which was blocked by inhibition of aromatase activity. Pups were treated with vehicle or PGE2 (for A and B) or vehicle, formestane, LPS, or formestane+LPS (for C and D) during the second postnatal week. Object investigation was assessed at PN27 (“young”) or PN62 (“adult”). **A)** Object investigation at young age: Two-way ANOVA revealed there was a main effect of treatment ($F_{1,24}=5.90$, $*p<0.05$ - PGE2 increased object interest in both males and females), but not sex ($F_{1,24}=1.93$, $p=0.17$) or interaction ($F_{1,24}=0.56$, $p=0.46$). **B)** Object investigation in adults: Two-way ANOVA revealed there were no significant effects of treatment ($F_{1,26}=0.83$, $p=0.37$), sex ($F_{1,26}=0.92$, $p=0.35$), or interaction ($F_{1,26}=0.22$, $p=0.64$). **C)** Object investigation in young age: Multi-way ANOVA revealed there was a main effect of treatment ($F_{3,39}=8.92$, $p<0.01$), but not sex ($F_{1,39}=3.34$, $p=0.08$) or interaction ($F_{3,39}=0.52$, $p=0.67$). For males, post-hoc tests revealed there was no difference between vehicle and formestane alone ($t=0.39$, $p=0.99$) or formestane +LPS ($t=0.62$, $p=0.99$). LPS treatment greatly increased investigation time over vehicle ($t=3.06$, $*p<0.05$) and this was prevented by pretreatment with formestane ($t=3.65$, $*p<0.01$). For females, post-hoc tests revealed there was no difference between vehicle and formestane treatment alone ($t=1.63$, $p=0.23$) or combined treatment with formestane+LPS ($t=0.40$, $p=0.70$). LPS treatment greatly increased object investigation time compared to vehicle treatment ($t=3.12$, $*p<0.05$) and this was blocked by pretreatment with formestane ($t=3.10$, $*p<0.01$). **D)** Object investigation in adults: Multi-way ANOVA revealed there was a main effect of sex ($F_{1,46}=15.94$, $*p<0.01$ – females overall investigated more than males), but not treatment ($F_{3,46}=1.25$, $p=0.30$) and there was no interaction between sex and treatment ($F_{3,46}=0.13$, $p=0.94$).

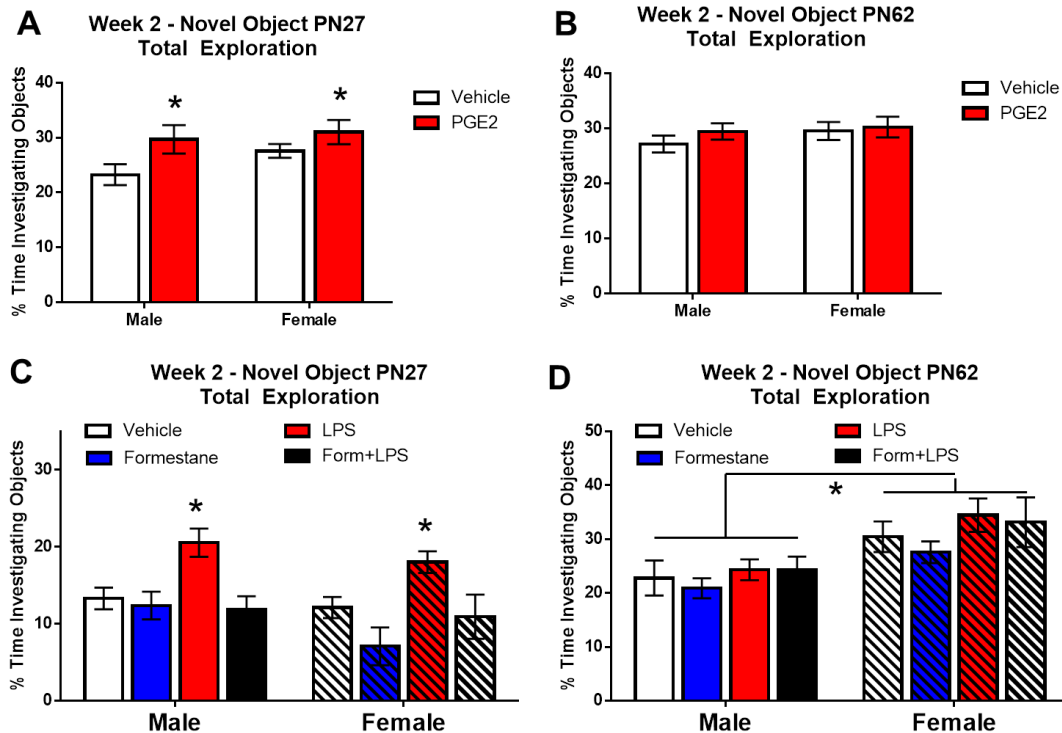


Figure 5.6: Inflammation within the second postnatal week decreased play behavior by males, which was blocked by inhibition of COX or aromatase activity. All treatments occurred within the second postnatal week; social play behavior was assessed from PN28-38. **A)** Pups treated with vehicle or PGE2. Two-way ANOVA revealed there was a main effect of treatment ($F_{1,26}=6.78$, $p<0.05$) but not sex ($F_{1,26}=0.273$, $p=0.61$), and a significant interaction ($F_{1,26}=5.49$, $p<0.05$). Post-hoc tests revealed vehicle-treated males had significantly more play events than vehicle-treated females ($t=2.22$, $*p<0.05$), and only play behavior in males was significantly decreased by PGE2 treatment ($t=3.50$, $**P<0.01$). **B)** Pups treated with vehicle, formestane, LPS, or formestane prior to LPS. Multi-way ANOVA revealed a main effect of treatment ($F_{3,45}=3.40$, $p<0.05$), sex ($F_{1,45}=10.94$, $p<0.01$), and interaction ($F_{3,45}=2.98$, $p<0.05$). Post-hoc tests revealed vehicle-treated males had significantly more play events than vehicle-treated females ($t=4.17$, $*p<0.01$), and only play behavior in males was significantly decreased by formestane treatment ($t=3.42$, $**p<0.05$) or LPS treatment ($t=3.42$, $**p<0.05$). Form+LPS-treated male play behavior was not significantly different from either vehicle-treated males ($t=1.98$, $p=0.24$) nor LPS alone in males ($t=1.44$, $p=0.66$). **C)** Pups treated with vehicle, “chronic” nimesulide, “acute” nimesulide, LPS, or “acute” nimesulide prior to LPS. Multi-way ANOVA revealed a main effect of treatment ($F_{4,71}=3.51$, $p<0.05$), sex ($F_{1,71}=43.18$, $p<0.01$), and interaction ($F_{4,71}=6.58$, $p<0.01$). Post-hoc tests revealed vehicle-treated males had significantly more play events than vehicle-treated females ($t=5.65$, $^ap<0.01$), and only play behavior in males was significantly decreased by “chronic” nimesulide treatment ($t=3.91$, $^bp<0.05$) and LPS treatment. “Acute” nimesulide-treated males were no different than vehicle-treated males ($t=1.09$, $p=0.99$) in play behavior, and “acute” nimesulide prior to LPS in males blocked the decrease in social play by LPS ($t=4.65$, $^cp<0.05$) without being significantly different from vehicle-treated males ($t=0.76$, $p=0.99$).

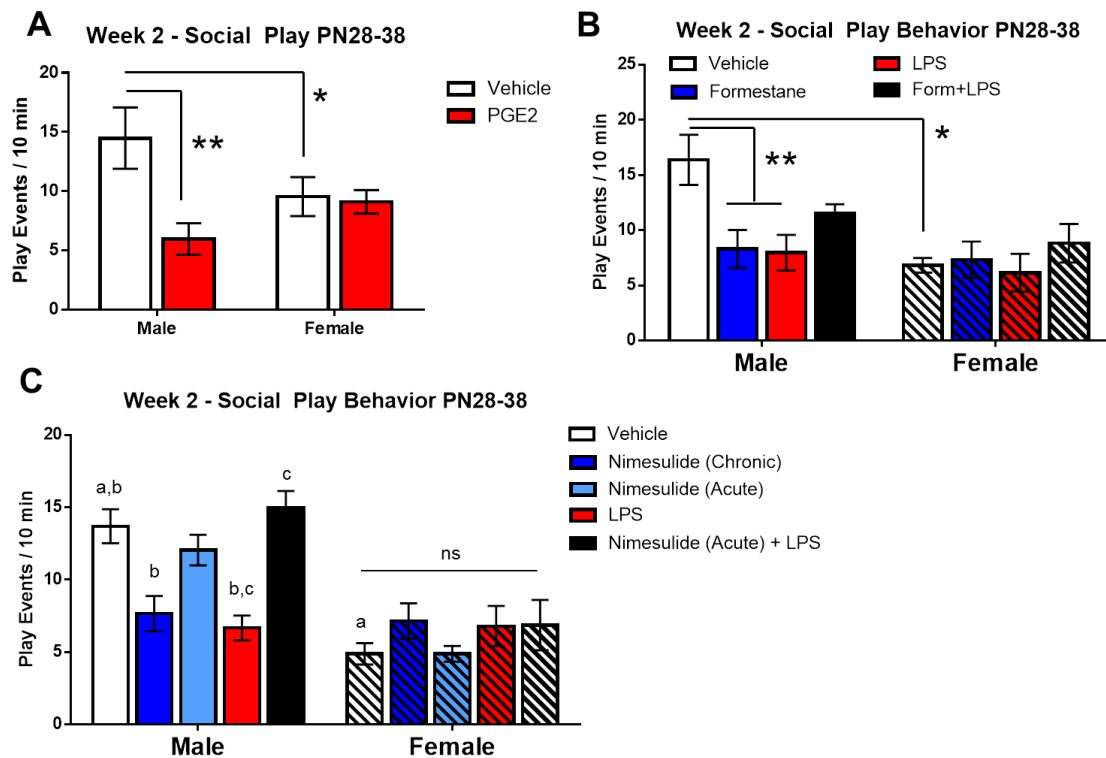
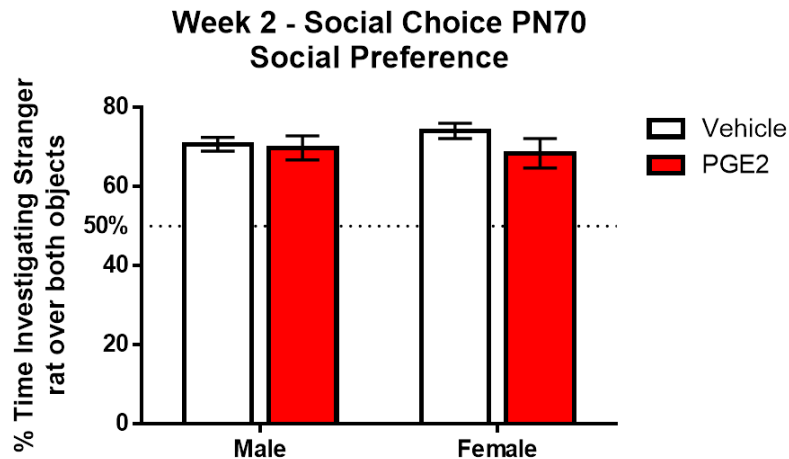
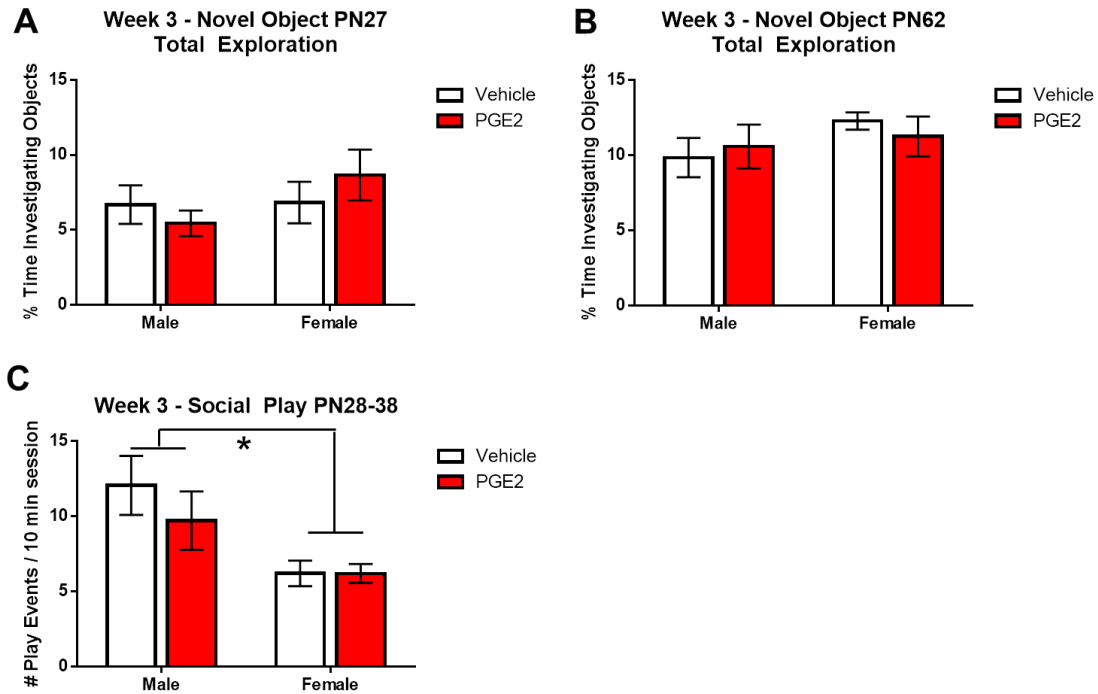


Figure 5.7: Inflammation within the second postnatal week has no effect on social investigation in adults. Animals were treated with PGE2 during the second postnatal week. Social choice task was assessed at PN70; 50% time investigating stranger indicates no preference, <50% indicates preference for the empty box, >50% indicates preference for the stranger rat. There were no significant differences in treatment ($F_{1,26}=1.47$, $p=0.24$), sex ($F_{1,26}=0.14$, $p=0.71$), or interaction ($F_{1,26}=0.77$, $p=0.39$) in percent time investigating the stranger rat over the empty box.



Our third prediction was that the observed behavioral deficits would be limited to animals experiencing inflammation only in the second postnatal week. To test this we examined animals treated in the third postnatal week PGE2 paradigm for the same behavioral tasks utilized in the second postnatal week PGE2 paradigm. As predicted, there were no longer any significant differences due to inflammation in total object exploration (young, $F_{1,20-treat}=0.05$, $p=0.82$) or social play behavior ($F_{1,23-treat}=0.584$, $p=0.42$), although animals did exhibit the expected sex difference in play, with play events being more frequent in males ($F_{1,23-sex}=9.34$, $*p<0.01$) (Figure 5.8).

Figure 5.8: Inflammation during the third postnatal week has no effect on later object interest or social play. Animals treated with PGE2 during the third postnatal week. **A)** “Young” Novel Object exploration assessed at PN27; no significant differences in treatment ($F_{1,20}=0.05$, $p=0.82$), sex ($F_{1,20}=1.58$, $p=0.22$), or interaction ($F_{1,20}=1.32$, $p=0.26$). **B)** “Adult” Novel Object exploration assessed at PN62; no significant differences in treatment ($F_{1,21}=0.01$, $p=0.90$), sex ($F_{1,21}=1.67$, $p=0.21$), or interaction ($F_{1,21}=0.54$, $p=0.47$). **C)** Social play assessed from PN28-38; males had significantly more play events than females (sex $F_{1,23}=9.34$, $p<0.01$), but there were no significant differences in treatment ($F_{1,23}=0.58$, $p=0.42$) or interaction ($F_{1,23}=0.58$, $p=0.42$).



Discussion

Previous work has shown that during the second postnatal week, treatment with nimesulide, an anti-inflammatory drug, results in decreased social play behavior and increased object interest only in males. We expanded on these findings by examining effects of inducing (LPS) or mimicking (PGE2) inflammation rather than reducing it. Animals in all treatment groups tested normal in assessments of locomotor activity and olfactory perception, therefore deficits in play behavior cannot be attributed to

confounding variables of general cerebellar disruption. By contrast, treatment with either LPS or PGE2 increased object fixation behavior in males and females, though only when tested at a young age, and systemic pretreatment with the aromatase inhibitor formestane prevented the LPS effect. Further, both LPS and PGE2 treatment resulted in decreased social play behavior in males only, and this is prevented by pretreatment with formestane and nimesulide. An interesting question, then, is why formestane reduced social play in males but did not affect object interest? At this point in time we do not know, but there are a few potential mechanisms. The systemic dose of formestane very likely reduced estradiol synthesis throughout the brain, not just the cerebellum. Other brain regions involved in social play and object interest, like the amygdala, hippocampus, and frontal cortex, may have different sensitivities or thresholds for changes in local estradiol, resulting in differential behavioral impairment. In order to tease out the role of cerebellar estradiol on behavior, experiments are currently underway in the same formestane/LPS paradigm but a low dose of formestane is injected directly into the cerebellum and behavior will be assessed as before.

We found no sex differences in any endpoint measured in the first three postnatal weeks following disrupted or increased prostaglandin synthesis, but, as animals matured, a selective vulnerability of males emerged in the case of social play behavior. The cerebellum is not generally considered a sexually dimorphic brain region; some have found sex differences in cerebellar size in early development (Geidd, 1996) and adulthood (Raz, 2001), while others have not (Henery, 1989; Nopoulous, 2000). Sex differences in cell number (Mayhew, 2001) or traditional motor performance (Nguon, 2005) have not been reported, either. While histological and functional differences in the

cerebellum between males and females are either nonexistent or are so small as to be lost in statistical noise, a sex difference does begin to appear when we look at vulnerabilities to insults and injuries affecting the cerebellum. For example, normal male and female rats do not differ in performance on the rotarod task, traditionally used to assess cerebellar-related motor function. However, following perinatal exposure to polychlorinated biphenyls, a common industrial toxin that stunts the development of the Purkinje cell dendritic tree (Kimura-Kuroda et al., 2007), males show a slightly greater reduction in cerebellar mass and fare considerably worse on rotarod performance (Nguon et al., 2005). This indicates male cerebellar development may have a greater vulnerability to environmental insults than females.

Subtle differences in protein expression or function may lay the basis for the sex difference in vulnerability of damage to cerebellar development. Retinoic acid-related (RAR) orphan receptor-alpha (RORA) is a nuclear receptor unique to Purkinje cells. It is a hormone dependent transcription factor involved in processes such as Purkinje cell differentiation (Hadj-Sahraoui, 2001) and cerebellar development (Harding, 1997; Gold, 2007). RORA is also a direct transcriptional target of both androgen receptor and ER α , and they work in opposition to regulate RORA expression (Sarachana, 2001), which may be at least one basis for sex differences in compensation mechanisms against insult. In the *staggerer* mutant, where the RORA gene is nonfunctional, homozygotes have severely reduced numbers of Purkinje cells, and those that remain have abnormal cytoarchitecture. Homozygotes also undergo granule cell death during the first month of life. Heterozygotes exhibit a sex difference, with male mice losing their Purkinje cells at one month of age, but females do not begin to lose cells until 9 months of age, although

by 13 months male and female Purkinje cell numbers are the same (Doulazmi, 1999). This delayed cell loss in females may provide a period within which cells have the opportunity to mature more, or for other systems to compensate for cell loss, potentially allowing them to become more resistant to insult than males. Another cerebellar protein where sex differences are not apparent in normal expression, but are evident when its mutated form is expressed, is reelin. Reelin is an extracellular matrix glycoprotein that modulates synaptic plasticity by enhancing the induction and maintenance of long-term potentiation (Weeber, 2002; D'Arcangelo, 2005), and stimulates dendrite and dendritic spine formation (Niu, 2008). In *reeler* mice heterozygous for a mutation in reelin, males lose Purkinje cells as they age, but females remain unaffected (Hadj-Sahraoui, 1996), again providing a source of increased vulnerability in males. Although our model does not use rats with mutated reelin genes, it is possible that the lack of a sex difference in Purkinje cell morphology following LPS and appearance of a male-specific deficit in play behavior could be due to a mechanism in which females are capable of compensating for the insult but males are not. In addition to differences in vulnerability due to possible changes in proteins following injury or inflammation, sex differences in behavioral deficits following inflammation may be attributed to changes in other relevant brain areas and disruptions to the entire circuit.

While not directly involved in social behavior, the cerebellum does have a role in multi-sensory integration (Kern, 2002), has been implicated in emotional and cognitive processing, has indirect connections to the medial prefrontal cortex (Hayhow, 2013; Rogers, 2013), and has reciprocal connections with the amygdala (Konarski, 2005; Heath, 1978). Both the medial prefrontal cortex and the amygdala are strongly

implicated in social play behavior (Kirkhof, 2013), and the amygdala has been implicated in autism (Auger, 2009; Rutishauser, 2013). An important question then becomes the functional impact of altered Purkinje cell dendritic development. Purkinje cell dendrites make direct synaptic connections to many cell types: climbing fibers (1:1), parallel fibers (200,000:1), stellate cells, and other Purkinje cells. Their function is to collect and interpret many connections to produce an appropriate output. If the dendritic tree is larger than normal, the potential exists for more synaptic inputs than necessary that would produce confusing inputs. Alternately, a smaller than normal dendritic tree would potentially provide fewer than normal possible synaptic inputs, losing vital information Purkinje cells would normally process for a correct response. Additionally, changes in cell size could result in changes in membrane capacitance or resistance, which would change the cell's ability to send or receive electrical signals. These changes to normal signaling capability of Purkinje cells would then result in altered downstream signaling to brain regions important to behaviors such as social play or object interest. Electrical stimulation of Purkinje cells increases extracellular levels of dopamine in the medial prefrontal cortex (MPFC), a brain area implicated in social play behavior (Rogers, 2011; Bell, 2010). The targets of dopaminergic activity in the MPFC, including synapsin II, regulate social play behavior (Bell, 2010; Dyck, 2012). Moreover, decreases in Purkinje cells, and therefore activity, mimic the effects of excitotoxic lesions to the MPFC and result in increased repetitive behavior, a hallmark of autism (Martin, 2010).

Although it is known that prostaglandins alter development in the preoptic area, we previously demonstrated that the same changes in spinophilin found in the cerebellum are not found in the amygdala, hippocampus and cortex when COX inhibitors are

administered systemically, showing that altering prostaglandin production during this period does not lead to alteration in spinophilin expression in all brain regions (Dean, 2012a). More importantly, we showed that COX inhibitors injected directly into the cerebellum are sufficient to induce the same behavioral effects seen following systemic treatment (Dean, 2012a). This suggests cerebellar pathology plays a causal role in the observed behavioral changes, but still falls short of anything more than a correlation between morphology and behavior. To more directly test the relationship between Purkinje cell morphology and behavior, it is necessary to determine how estradiol modulates Purkinje dendrite growth. Once the mechanism through which estradiol regulates dendrite growth is better understood, it would be possible to activate or inhibit those secondary events induced by estradiol to experimentally control Purkinje dendrite growth more directly. Since both stunting and excessively growing Purkinje cell dendritic trees result in similar behavioral patterns, future research should focus on altering dendritic tree size in both directions and examining the correlation between degree of change in morphology and severity of behavioral deficits. Even so, our findings of altered social behavior and object fixation following manipulation of cerebellar development are consistent with the increasingly well-understood role of the cerebellum in non-motor behavior (for further review, see Moulton, 2010; Strick, 2009; Stoodley & Schmahmann, 2010, 2009).

Autism and schizophrenia are complex neurodevelopmental diseases with both high heritability and strong sensitivity to environmental factors, particularly during certain periods of development, and exhibit a strong gender bias (for review see Dean and McCarthy, 2008). Our observation that induction or mimicry of inflammation can alter

behavior in a way that aligns with autistic-like symptoms and a male-bias in social dysfunction, but only if the inflammation occurs within the second postnatal week, suggests that during this narrow developmental period, fever and inflammation may increase the risk for neurodevelopmental disorders by altering cerebellar growth. It is likely that this particular sensitive period is more relevant to autism than schizophrenia for several reasons. First, much of the literature on early life inflammation and schizophrenia indicates that *in utero* exposure is the important time period, and we exposed postnatal animals to LPS. This is consistent with the literature on early life inflammation and autism. Second, symptoms of schizophrenia appear later in life, in late adolescence or early adulthood in humans, and the behavioral deficits we have seen here in the rat appear in preadolescence and are not present in later ages. Autism, however, has an early symptomology that does correspond with the behavioral deficits we see. Third, the behavioral tasks examined are not all-inclusive of potential symptoms of autism, but are even less-so for schizophrenia. Rats showed no deficit in social investigation, which would be expected for schizophrenia, and we did not examine anhedonia or any kind of communication deficits, both of which are important to schizophrenia. It would be well worth examining changes in ultrasonic calls in rat pups across different developmental ages; deficits in animals exposed to LPS would strengthen the case that early life inflammation is a risk factor for neurodevelopmental disorders. Further, while our observation of an increase in object interest suggests a perseverance behavior typical in autistic patients, it is not the only method of examining stereotypy. A further expansion of the battery of behavioral tasks should include assessment of hole-pokes, marble burying, open field corner preference, circling behavior, and repetitive

grooming to more accurately assess such behavior. Also, it is important to note that we are not proposing that our model is of an autistic or schizophrenic rat. The specific behavioral deficits identified here do share characteristics of neurodevelopmental disorders, however, and so suggest a potential mechanism by which children may be at an increased risk for developing these disorders if they experience fever or inflammation during this sensitive period.

Chapter 6:

General Discussion

Knowledge and appreciation for the cerebellum's involvement in sensory perception and higher cognitive functions are expanding rapidly. Additionally, pathologies in cerebellar development have begun to be associated with neurodevelopmental diseases such as autism spectrum disorder and schizophrenia. While their exact causes are unknown, these types of diseases have been strongly associated with environmental insults during early life, a time when cerebellar development is the most dynamic. In the rat, the second postnatal week is a time of active growth, selective pruning, and synapse formation for Purkinje cell dendritic trees (Sotelo, 2009; Cesa, 2009) and this process is mediated by a variety of intrinsic and extrinsic factors. Previous work from our lab discovered a common pathway in the cerebellum between the intrinsic factor, estradiol, and upstream PGE₂, an important intrinsic signaling molecule that is also subject to extrinsic regulation by infectious agents, injury, or trauma. Here we show that an infectious agent, LPS, increases PGE₂ and drives synthesis of estradiol by increasing aromatase activity. This leads to stunted Purkinje cell dendritic growth as well as an increase in object perseverence in males and females, and a decrease in social play behavior in males. Further, we establish that the second postnatal week is actually a narrow sensitive period where normal development is most at risk from external factors such as bacterial infection. Exploring the relationship between timing, early life inflammation, and neurodevelopmental disease could provide insights into preventative measures to reduce the risk of developing such diseases.

Lipopolysaccharide and PGE2 disrupt normal cerebellar development through estradiol synthesis

Previous work has shown that inhibiting PGE2 synthesis with cyclooxygenase inhibitors decreases aromatase activity and hence estradiol production, and this allows excessive growth of Purkinje cell dendritic trees (Dean, 2012a, 2012b). Here we show that LPS increases aromatase activity and estradiol synthesis, and this stunts Purkinje cell dendritic growth. During normal cerebellar development, estradiol levels in the cerebellum are mediated, at least in part, by PGE2 levels, and estradiol acts as a brake on Purkinje cell dendrite growth. In the periphery, LPS increases PGE2 production by increasing COX2 expression; we show this to be true in the cerebellum as well, as LPS increases COX2, but not COX1, mRNA within an hour of exposure. Nimesulide, a COX2 inhibitor, administered prior to LPS exposure prevents the increase in PGE2, as well as that in aromatase activity and estradiol. Direct intracerebellar injection of PGE2 similarly stunts Purkinje cell dendrite growth. Further, when the aromatase inhibitor formestane is administered prior to LPS exposure, we prevent Purkinje cell stunting and the dendritic trees are able to grow normally. An exogenous insult such as LPS creates a spike in PGE2 synthesis through an induction of COX2 expression, and excess PGE2 in turn drives a greater than normal increase in aromatase activity and elevated estradiol production. In this way, external environmental insults that initiate fever and inflammatory responses can alter normal development and result in stunted cerebellar growth.

What has not been worked out yet, however, is exactly how PGE2 is capable of driving an increase in aromatase activity, or how estradiol can modulate Purkinje cell

dendrite growth. In peripheral systems such as the breast, PGE₂'s ability to increase estradiol production is mediated by an increase in cAMP (Brueggemeier, 2001) and likely acts through EP₂ and/or EP₄ and PKA and PI3-kinase/Akt signaling pathways (Han, 2010; Kim, 2010) as both receptors increase cAMP (Wright, 2008). Currently this mechanism is poorly understood and has not yet been explored in the brain. We hypothesized that EP₄ is the PGE₂ receptor most likely involved in increasing aromatase activity in the cerebellum due to its signaling mechanism and increase in expression across the second postnatal week, but unfortunately were unable to test this due to technical limitations.

The exact mechanism of estradiol's regulation of dendrite growth, particularly for Purkinje cells, is currently unknown. Our studies suggest estradiol is acting as a brake on dendritic growth. There is an endogenous peak of estradiol levels in the cerebellum during the second postnatal week, but an increase over that level stunts dendritic growth, potentially providing too much "stop signal" for dendrite branching. Conversely, preventing the increase in estradiol during the second postnatal week allows Purkinje cell dendrites to grow excessively, potentially not providing enough of a "stop signal." This mechanism could conceivably be either a prevention of growth or a retraction in growth, and one way to examine this would be using live cell imaging techniques. Seeing how Purkinje cells in cerebellar slice cultures exposed to estradiol change in real time could provide some insight to the mechanism.

We hypothesize that ER α is the primary estradiol receptor mediating the changes in Purkinje cell morphology and the timing of the sensitive period due to its peak in mRNA expression during the second postnatal week. If ER α is actually the primary

mediator of Purkinje cell growth, a strong candidate for its mechanism of action is retinoic acid-related orphan receptor-alpha (RORA). RORA is under transcriptional control of ER α and is involved in Purkinje cell differentiation, cerebellar development, and suppression of inflammation (Hadj-Sahraoui, 2001; Harding, 1997; Gold, 2007; Delerive, 2001). Levels of aromatase and RORA proteins in neurons were strongly correlated and reduced in frontal cortex tissue from autistic subjects compared with matched controls, although levels in the cerebellum weren't examined (Sarachana, 2001). This potential link between changes in estradiol, Purkinje cell growth, and neurodevelopmental disorders is worth further investigation in our model.

Another potential and unexplored mechanism of estradiol in cerebellar development is through an estradiol-induced increased synthesis of gamma-aminobutyric acid (GABA). Preliminary data from our laboratory indicates estradiol increases glutamic acid decarboxylase (GAD) (Dean, 2009), the enzyme responsible for production of GABA, and GABA is one of the factors involved in activity-dependent dendritic pruning of Purkinje cells during the second postnatal week (Sotelo, 2009).

Yet another potential mechanism of estradiol action on cerebellar development is through brain-derived neurotrophic factor (BDNF), a secreted protein that acts to support the survival of existing neurons, and encourage the growth and differentiation of new neurons and synapses (Acheson, 1995; reviewed in Huang, 2001). It has been shown that knocking out the aromatase gene (ArKO) in mice results in decreased Purkinje cell dendritic growth, while administration of estradiol to neonatal wild-type or ArKO mice increased dendritic growth, spinogenesis, and synaptogenesis in the Purkinje cell, and this occurs through an estradiol-induced increase in BDNF (Haraguchi, 2012). These data are

in apparent direct contrast to our findings that elevating estradiol in the cerebellum results in *reduced* Purkinje cell dendritic growth and no change in synaptic density. Although we did show that systemic administration of estradiol decreased Purkinje cell dendrite length (Dean, 2012b), most of our work focus on administering LPS or PGE2, which result in an increase in estradiol and subsequent stunting of Purkinje cell growth. LPS and PGE2 do not act in isolation during inflammatory responses; the role of other cytokines and even microglia and astrocytes must also be considered in determining the mechanism of estradiol regulation of Purkinje cell development. For example, cortical astrocytes of male or androgenized female rats exposed to LPS exhibited higher levels of mRNA for cytokines IL6, TNFa and IL1b, while IP10 mRNA was higher in control females, even though there were no sex differences in basal levels of these cytokines or in the LPS receptor TLR4 (Santos-Galindo, 2011). It is unknown whether this is also true in the cerebellum, but if it is, this difference in response of male and female astrocytes to LPS provides a tantalizing explanation for our observed sex difference in social play behavior following LPS but lack of any observed sex differences in Purkinje cell morphology. Currently our laboratory is examining differences in basal microglia between the sexes and their responses to LPS across the first three postnatal weeks to address this question.

LPS and PGE2 disrupt social play and object perseverance

Previous work has shown that during the second postnatal week, treatment with nimesulide, an anti-inflammatory drug, results in decreased social play behavior and increased object interest only in males. We expanded on these findings by examining

effects of inducing (LPS) or mimicking (PGE2) inflammation rather than reducing it. Animals in all treatment groups tested normal in assessments of locomotor activity and olfactory perception, therefore deficits in play behavior cannot be attributed to confounding variables of general cerebellar disruption. Treatment with either LPS or PGE2 increased object fixation behavior in males and females, though only when tested at a young age, and pretreatment with the aromatase inhibitor formestane prevented the LPS-induced increase in object fixation. Further, both LPS and PGE2 treatment resulted in decreased social play behavior in males only. Formestane treatment prevented the LPS-induced deficit in male social play, but this result is confounded because formestane treatment alone also induced a deficit in male social play, and so we are unable to determine whether formestane is acting directly in the cerebellum or elsewhere within the brain. We are currently completing a new experiment where we assess play behavior under the same paradigm, but with formestane treatment administered via intracerebellar injection to better address this question. A shorter period of nimesulide treatment prevented the LPS induced deficit in male social play without affecting play itself, while a longer nimesulide treatment replicated the reduction in male social play. These results, along with associated changes in Purkinje cell size, are summarized in Table 6.1.

Table 6.1: Summary of morphological and behavioral changes following PGE2-E2 pathway disruption. Columns under the heading “@week 2” indicate treatments were administered during the second postnatal week, and “@week3” indicates treatments were administered during the third postnatal week. Blue indicates treatments that decrease the inflammatory response and ultimately result in decreased estradiol, while red indicates treatments that induce the inflammatory response and ultimately result in increased estradiol. Arrow direction indicates an increase (up) or decrease (down) in outcome over vehicle, a dash indicates no change. M = male, F = female.

	@Week 2						@Week 3
	Chronic Nimesulide	"Acute" Nimesulide	PGE2	LPS	Formestane + LPS	Ac. Nim + LPS	PGE2
Motor Control (confounds)	normal	normal	normal	normal	normal	normal	normal
Object Interest Young – PN27			M ↑ F ↑	M ↑ F ↑	M – F –		M – F –
Social Play PN28-38	M ↓ F –	M – F –	M ↓ F –	M ↓ F –	M +/- F –	M – F –	M – F –
Object Interest Adult – PN62	M ↑ F –		M – F –	M – F –	M – F –		M – F –
Social Choice Adult – PN70			M – F –				
Purkinje Cell Size (early)	M ↑ F ↑		M ↓ F ↓	M ↓ F ↓	M – F –		M – F –

In dealing with complex behaviors and a network of brain regions, it is important to address the distinction between brain pathology that is a simple marker of behavioral dysfunction, or a true cause. Although it is known that prostaglandins alter development in the preoptic area, we previously demonstrated that the same changes in spinophilin found in the cerebellum are not found in regions such as the amygdala, hippocampus and cortex when COX inhibitors are administered systemically, showing that altering prostaglandin production during this period does not lead to alteration in spinophilin expression in all brain regions (Dean, 2012a). More importantly, we showed that COX inhibitors injected directly into the cerebellum are sufficient to induce the same behavioral effects seen following systemic treatment (Dean, 2012a). This suggests cerebellar pathology plays a causal role in the observed behavioral changes, but still falls short of anything more than a correlation between morphology and behavior. To more directly test the relationship between Purkinje cell morphology and behavior, it is first necessary to determine how estradiol modulates Purkinje dendrite growth, potentially via RORA or GAD mentioned previously. Use of that mechanism to gain experimental

control over the degree of stunting or extension of the Purkinje cell dendritic arbor and examining the severity of behavioral deficits would get us one step closer to understanding the role of Purkinje cell morphology on behaviors relevant to neurodevelopmental disorders. Even so, our findings of altered social behavior and object fixation following manipulation of cerebellar development is consistent with the increasingly well-understood role of the cerebellum in non-motor behavior (for further review, see Moulton, 2010; Strick, 2009; Stoodley, 2010, 2009).

Even though it is increasingly apparent that the cerebellum plays a role in non-motor behavior, it is not yet well understood how this occurs. Anecdotally, humans with cerebellar lesions exhibit disruption in their emotional, cognitive, and executive functioning (Schmahmann, 1998; Timmann, 2007). Activation of some areas in the cerebellum during fMRI measures previously dismissed as only involved in motor requirements of the task are now appreciated as activity separate from motor movements (Petersen, 1989). Structurally, the closed circuit of the cerebro-cerebellar circuit suggests a constant communication loop between the cerebellum and cerebral cortex used in higher functions. In addition to the many inputs the cerebellum receives from throughout the brain, the cerebellar nuclei project out to multiple subdivisions of the ventrolateral thalamus, which then projects to many cortical areas including frontal, prefrontal, and posterior parietal cortex, making it physically possible for the cerebellum to influence more than motor pathways (Strick, 2009). Functionally, there are three current theories as to how cerebellar signaling is capable of influencing cognition and affect. First, in motor control the cerebellum plays a role in timing of movements and predictions of movements (Holmes, 1939; Ivry, 1989). If the cerebellum loses its ability to track or

control precise timing, it could lead to problems with task-shifting and executive control. Second, the cerebellum is responsible for processing a lot of sensory-motor inputs to create sensory-motor imagery: imagined speech or mental representations of a movement that will occur (Hanakawa, 2008). If the cerebellum uses similar mental representations for cognitive tasks, especially an internal voice used to represent, maintain, and organize task-relevant information, disruption of this ability would result in disorganized cognitive function. Third, it is known that the cerebellum plays a crucial role in adaptive plasticity needed for learning fine motor skills. The process uses internal models that predict sensory inputs that should occur following a motor output or motor movements necessary to achieve a goal. The cerebellum then calculates errors between these predicted and actual outcomes, and uses this error to adapt new models for greater accuracy (Wolpert, 1998). This error adaptation method could also be applied to internal cognitive representations and outcomes of their goals. Disruption would not allow the organism to adapt to social learning, for example. These three mechanisms of cerebellar involvement in non-motor activities are not necessarily mutually exclusive, and are beginning to be examined in greater detail (Barsalou, 1999; Ito, 2008).

We found no sex differences apparent in any endpoint measured in the first three postnatal weeks following disrupted or increased prostaglandin synthesis, but, as animals matured, a selective vulnerability of males emerged in the case of social play behavior wherein treated males exhibited reduced play but treated females did not. The high level of rough-and-tumble play by adolescent males is one of the most robust and reliable of sex differences across species (Olesen, 2005). Since males are much more playful than females, our observed lack of a decrease in female play behavior may simply reflect a

floor effect below which females can go no further. However, this argument is weakened by the fact that play activity of treated males went below the level of control females. Further, there was no difference in the social choice chamber task, which does not involve the aggressive play bias towards males. The apparent disconnect between sex differences in morphology and behavior may be explained by differences in how male and female brains react to early extrinsic insults. The total cerebellar volume of mature, adult males was larger than age matched females, and selectively reduced in males treated with COX inhibitors during the preadolescent period (Dean, 2012a). The cerebellum is not commonly considered to be a sexually dimorphic brain region, although a larger cerebellar volume in human males has been detected by some (Giedd, 1996), but not others (Henery, 1989), and recent reports have demonstrated gonadal steroid-independent sex differences in the expression of cerebellar proteins such as calbindin (Abel, 2011), Foxp2 (Hamson, 2009) and JaridC (Xu, 2008). In addition to these relatively subtle sex differences, there is a sex-based difference in developmental vulnerability, with males frequently developing greater pathology than females in response to the same insult (reviewed in Dean & McCarthy, 2008). For example, normal male and female rats do not differ in performance on the rotorod task, traditionally used to assess cerebellar-related motor function, but following perinatal exposure to polychlorinated biphenyls, a common industrial toxin that stunts the development of the Purkinje cell dendritic tree (Kimura-Kuroda, 2007), males show a slightly greater reduction in cerebellar mass and fare considerably worse on rotorod performance (Nguon, 2005). Male rats prenatally exposed to cocaine also show greater motor impairments later in life than females (Markowski, 1998), which has been interpreted as a

potential sign of greater cerebellar impairment. Further, estradiol administration restores deficiencies in reelin mRNA levels caused by heterozygote expression of the null mutation and thereby increases the survival of Purkinje cells in males, but not females (Biamonte, 2009). Reelin is a secreted extracellular matrix glycoprotein associated with regulating the process of neuronal migration and positioning, and has been suggested to have a role in psychiatric disorders such as schizophrenia (Tueting, 2006), bipolar disorder (Goes, 2010), and autism (Pardo, 2007).

Given the male bias in autism and the sex difference in onset and severity in schizophrenia, it is interesting to see sex differences in affected behavior emerge from a lack of sex differences in immediate responses to an early life inflammatory insult. Further examination of morphology at later ages and compensatory mechanisms between the sexes is necessary to elucidate what role locally synthesized steroids in the brain are playing in the establishment of sex differences, especially differences in vulnerability to neurodevelopmental disorders.

The second postnatal week is a sensitive period for exogenous influence on cerebellar development and behavior

In the rat cerebellum, the second postnatal week is the most dynamic period for Purkinje cell dendritic growth, mediated by a variety of intrinsic and extrinsic factors. We have shown here that intrinsically, baseline PGE₂ in the cerebellum is lower during the second postnatal week than the first or third, while baseline E₂ is higher in the second postnatal week than the first or third. COX1 mRNA expression does not change significantly across the three postnatal weeks, but COX2 begins to steadily increase at the

start of the second postnatal week and continues through the third postnatal week. LPS is capable of inducing COX2 (but not COX1) mRNA within all three postnatal weeks, but we only see a spike in PGE2 production when LPS is administered in the second postnatal week. While PGE-synthase mRNA does not change significantly across the three postnatal weeks, this could be due to differences in the ratio of synthesis in other members of the prostanoid family, or in availability of the precursor arachidonic acid, and requires further investigation to understand how this disconnect is actually possible. PGE2 receptors EP1, 2, and 3 mRNA remain relatively unchanged throughout the three week period, but EP4 also exhibits a significant increase in expression beginning at the second postnatal week and continuing until it levels off for the third postnatal week. EP4 thus appears to be the most likely PGE2 receptor involved in this signaling pathway between PGE2 and aromatase and could be a constraint for the beginning of the sensitive period in cerebellar development. Further, both aromatase and estradiol receptor ER α mRNA expression rise and fall across the first three postnatal weeks, with a peak right in the middle of week 2. The observation that the ratio of expression between ER α and ER β reverses at the end of the second week, potentially inhibiting ER α activity, suggests it is the steroid components of the pathway that appear to mediate both the onset and close of the sensitive period. Additionally, only PGE2 administered in the second postnatal week, not in the first or third, is capable of increasing estradiol, which has previously been shown capable of stunting Purkinje cell growth (Dean, 2012b). Both LPS and PGE2 are also capable of stunting Purkinje cell growth, but again only when administered in the second postnatal week, not the first or third. Even more compelling evidence for the cerebellar sensitive period is that we only see behavioral deficits when

PGE2 is increased in the second postnatal week, but no deficits resulted after PGE2 induction within week 3.

Even though there are minor inconsistencies within the literature about expression of steroidogenic enzymes and ER levels in the neonatal rat cerebellum, thus far most studies have focused on a few days either concentrated in the first week of life, or a single time point for each of the first three postnatal weeks. No studies have examined an extended time course, or more than a couple of genes in the same animals across that time course. We show for the first time mRNA expression patterns of 10 different genes in the cerebellum across 9 days within the first three postnatal weeks of cerebellar rat development, several of which (EP4, aromatase, and ER α) exhibit changes consistent with a sensitive period in the second postnatal week. However, future research will require confirmation of similar changes in protein level expression of these genes to better understand their role in the sensitive period.

We have provided convincing evidence that the second postnatal week is a sensitive period in the cerebellum where normal development is most at risk from exogenous, environmental factors. Our findings met all 5 of John Nash and John Colombo's criterion for a critical period 1) *an identifiable beginning point* – around PN7; 2) *an identifiable end point* – around PN14; 3) *an intrinsic component* – the PGE2-E2 signaling pathway, including a natural decrease in PGE2 during this period; 4) *an extrinsic component* – LPS specifically, but potentially any exogenous source of infection, that pushes PGE2 and E2 beyond their normal endogenous levels; and 5) *a specified critical system affected* – the cerebellum beyond its traditional role in motor function, evidenced by changes in Purkinje cell morphology as well as behavioral deficits that parallel

symptoms of human neurodevelopmental disorders (ASD, schizophrenia). Further, we emphasize the idea that it is more appropriate to refer to this period as a sensitive period rather than a critical period because it is a time when neural development is most vulnerable to *disruption* by external stimuli, rather than a point when exogenous inflammation is *necessary* for normal development.

We have shown that changes to Purkinje cell morphology in either direction (stunted or overgrown) lead to similar behavioral deficits: if Purkinje cell morphology directly correlated with the direction of behavioral deficit, then we would expect to see directionality in the behavioral deficits. For example, stunted Purkinje cells would result in an increase in social play behavior and a decrease in object interest, while overgrown Purkinje cells would result in a decrease in social play behavior and an increase in object interest. But this is not what we see. Instead there appears to be an inverted U-shaped response where morphology disruption away from the norm in any direction results in decreased social play and increased object interest, and this only occurs during the sensitive period of the second postnatal week. More confusing is that we do not see a sex difference in biochemistry or morphology in the first three postnatal weeks, but at older ages we see a sex difference in behavior. At later ages (PN40) a sex difference in cerebellar volume appeared as well (Dean, 2012a), indicating there may be some form of recovery or compensatory mechanisms that differ between the sexes. The second postnatal week is a time when Purkinje cell cells are making their most important connections with climbing fibers and parallel fibers. Even if Purkinje cells are able to recover their morphology later in life, the fact that they were unable to make their normal synaptic connections (either too many with a larger dendritic tree, or too few with a

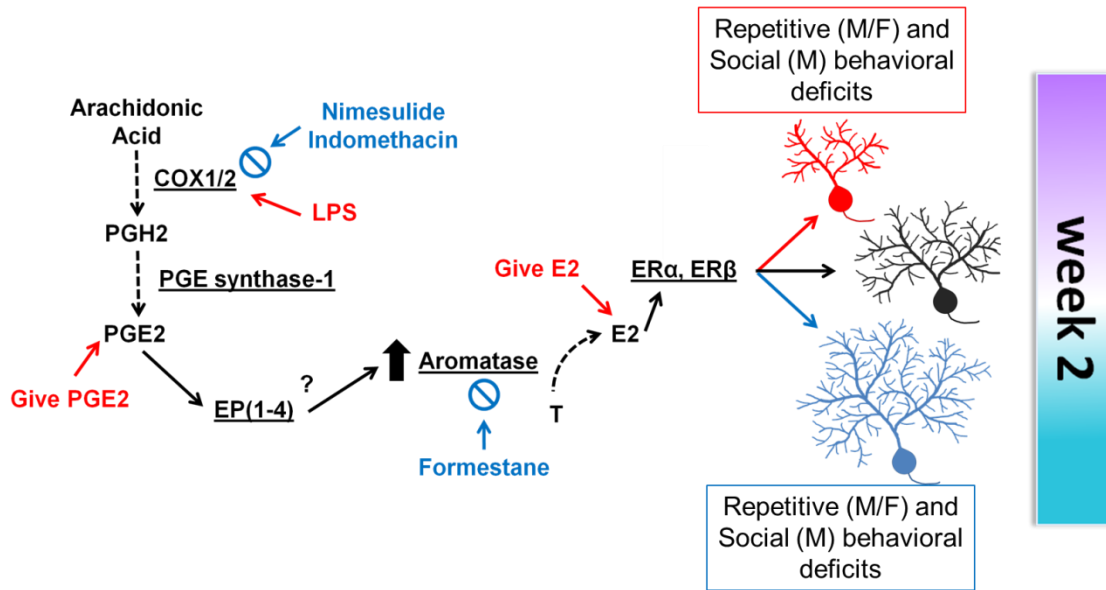
smaller dendritic tree) leaves them incapable of consolidating inputs to generate the necessary output for normal behavior. Timing of the disruption is very important.

Through comparisons of predominantly structural changes in brain development we can make the general statement that in rats the second and third postnatal week (PN7-21) correspond to human full term birth to toddler (gestational day 36 to 2-3 years).

However, there is a lack of a detailed correlation between more specific events of the rat developmental time line and the human developmental timeline for this period, especially for the cerebellum. Thus it is important to examine other aspects of development, such as changes in gene expression, in both rats and humans to further establish corresponding developmental events between the two species and the factors that influence them. We hope that our ongoing investigation into human brain tissue will provide such insight.

Considering this work collectively, we propose an updated model of the PGE2-E2 signaling pathway (Figure 6.1). The biochemical pathway described in Chapter 3 (Figure 3.4) can be updated to emphasize the effects of LPS, and that a disruption in the pathway in either direction results in multiple, similar behavioral deficits. Further, the second postnatal week is the only time in which these disruptions can occur.

Figure 6.1: An updated working model of the PGE2-E2 pathway in the cerebellum. Arachidonic acid is converted to PGH₂ by the enzymes **COX 1/2**, which is then converted to PGE₂ via **PGE synthase-1**. COX2 can also be stimulated to increase PGE₂ synthesis by LPS. PGE₂ interacts with its receptors **EP(1-4)**, increasing **aromatase** activity to convert testosterone to estradiol, which binds to its receptors **ER α** and **ER β** and regulates Purkinje cell dendritic growth. Estradiol acts as a brake on growth – extrinsic factors that ultimately increase E2 stunt dendritic growth (red), and extrinsic factors that ultimately decrease E2 allow for dendritic overgrowth (blue). Both disruptions in the pathway lead to behavioral deficits (boxes), and pathway disruptions are only possible when they occur within the second postnatal week of life.



Implications for human disease

The vast majority of children will, at some point and more than once in their lives, experience an illness that involves fever and/or inflammation. Millions of them are then exposed to prescription and over-the-counter medications that inhibit prostaglandins. However, relatively few of these children develop autism spectrum disorder or schizophrenia. Why? Both disorders have a strong but not exclusive genetic component (Fatemi, 2008; O'Donovan, 2003, 2009; Currenti, 2009; Tanaka, 2009), and one of the greatest challenges with these disorders is understanding how genes and environment interact to create greater risk for their development. Early life inflammation, either in utero or postnatally, is a major environmental risk factor for development of disorders of mental health, including schizophrenia (Clarke, 2009; Li, 2009; reviewed in Fan, 2007) and autism (Torres, 2003; Cohly, 2005; Persico, 2006). These observations together strongly suggest some individuals may have a genetic predisposition that makes them

susceptible to deleterious effects of either inflammation or specific anti-inflammatory medications, thus greatly increasing their vulnerability for development of neuropsychiatric disorders. Genetic variability in the cyclooxygenases contributes to relative risk of adverse cardiac events in adult populations by altering the base level of PGE₂ synthesis across the lifespan (Ali, 2005) and potential variation in the EP receptors is just beginning to be explored (Namba, 1993; Adam, 1994). Future work investigating effects of inflammation on the PGE₂-E₂ pathway overlying genetic variations such as these in COX1/2 and EP receptors, especially during the sensitive period, could provide insight into the subtle intricacies of gene and environmental interaction in neurodevelopmental disorders.

Prior to our previous work and the work presented here, there were no studies linking early life inflammation and cerebellar pathology, even though cerebellar pathology has been linked to both schizophrenia (Weinberger, 1979; Mukaetova-Ladinska, 2002; Riehmman, 2001) and autism (Courchesne, 1987, 1988, 1994; Allen, 2003; Wills, 2009; Levitt, 1999). Our work is a step forward in understanding how the timing of intrinsic and extrinsic factors can come together to alter normal development and affect mental health. It is clear that the second postnatal week is a carefully balanced period of development in the rat cerebellum. Extrinsic factors such as NSAIDS which block PGE₂ synthesis and thus decrease estradiol levels result in extensive Purkinje cell dendrites, but at the same token extrinsic factors such as LPS that result in increased PGE₂ synthesis and thus increased estradiol levels result in stunted Purkinje cell dendrites. However, if Purkinje cell dendritic growth is beyond some normal homeostatic level, regardless of whether it is larger or smaller, animals exhibit abnormal

behaviors reminiscent of neurodevelopmental disorders. This narrow sensitive period (PN7-14) in the rat corresponds to between birth and one or two years of age in the human. Children of this age are especially prone to infection, experiencing 8-12 colds a year, due in part to their immature immune system and underdeveloped sinuses (A.D.A.M, 2013; American Academy of Otolaryngology, 2014).

Given that early life inflammation is associated with an increased risk of autism and schizophrenia, our findings seem to be a strong model for the study of increased risk for neurodevelopmental diseases. Most importantly, if the sensitive period we find here holds true in human development, then we may be able to reduce the risk of developing these disorders by examining different pathways and mechanisms for treating fever and inflammation during this time. First and foremost methods of avoiding infection during this time period could reduce the incidence of future disorders. Since NSAIDS alone are also a potential risk factor, careful monitoring of their use and dose during this period could also reduce disorder incidence. Clearly the current method of knocking down all prostanoid synthesis systemically is too broad with unwanted side effects. Our new understanding of the cerebellum in the link between early life inflammation and neurodevelopmental diseases could lead to more targeted fever therapeutics. For example, given that increased PGE₂ results in increased estradiol during this period, and pre-treatment with formestane during the second postnatal week prevented Purkinje cell morphological and behavioral deficits, it is possible formestane could be used as a preventative therapeutic during fever and inflammation. However, this will not be practical without further careful study regarding timing and method of administration as aromatase is present throughout the body and brain and systemic application of its

inhibitor could result in multiple side effects. If administration could be directed to the cerebellum during the human equivalent of this sensitive period, though, it could prove to be a novel and effective method of neurodevelopmental disorder risk reduction.

Similarly, anti-fever medications might instead be targeted to febrile-response regions such as the preoptic area and avoid disrupting the cerebellum at all.

Autism spectrum disorder and schizophrenia are two neurodevelopmental disorders prevalent in our society, but their causes are not well understood. Prevention and treatment methods are currently limited. Understanding how a sensitive period in rat cerebellar development corresponds to human age will provide a time period in which we could potentially be more effective in reducing the risk for developing such disorders, and thus reduce their future incidence. Our work has highlighted an important period in development to focus future research and reevaluate the way we deal with fever and inflammation.

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