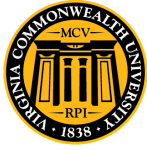

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Effects of Early Alcohol Exposure on Ocular Dominance Plasticity in Mice

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARND	Alcohol-Related Neurodevelopmental Disorder
BDNF	Brain Derived Neurotrophic Factor
BEC	Blood ethanol content
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
cpd	Cycles per Degree
CREB	cAMP response element-binding
DMSO	Dimethyl sulfoxide
FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
G	Gestational day
GABA	gamma-aminobutyric acid
LGN	Lateral Geneculate Nucleus
LTD	Long term depression
LTP	Long term potentiation
MD	monocular deprivation
MWM	Morris Water Maze
NMDA	*N*-Methyl-D-aspartate
OD	Ocular Dominance

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ODI	Ocular Dominance Index
ODP	Ocular Dominance Plasticity
OI	Optical Imaging
P	Postnatal day
PDEi	phosphodiesterase inhibitor
PDEi1	phosphodiesterase type 1 inhibitor
PDEi4	phosphodiesterase type 4 inhibitor
PDEi5	phosphodiesterase type 5 inhibitor
pFAS	partial Fetal Alcohol Syndrome
PKA	Protein Kinase A
PKG	Protein Kinase G
SRF	Serum Response Factor
V1	Primary Visual Cortex
VEP	Visually Evoked Potentials

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Abstract

EFFECTS OF EARLY ALCOHOL EXPOSURE ON OCULAR DOMINACNE

PLASTICITY IN MICE

By Crystal L. Lantz

A dissertation submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Major Director: Alexandre E. Medina, PhD
Assistant Professor, Department of Anatomy and Neurobiology

Fetal alcohol spectrum disorder is the leading cause of mental retardation in the western world and is associated with numerous sensory processing deficits. Some of these deficits are a result of faulty neuronal plasticity. Previously our lab has used ferrets to demonstrate that alcohol exposure during the third trimester of human gestation results in impaired ocular dominance plasticity (ODP). Here we have extended this model to mice. Animals treated with 5 mg/kg of ethanol on postnatal days 5, 7 and 9, exhibit a lack of ODP plasticity after 10 days of monocular deprivation (MD) during the critical period of visual cortex plasticity, as seen by optical imaging of intrinsic signals. This deficit in ODP was rescued by a treatment with a phosphodiesterase type 1 inhibitor (PDEi1), vinpocetine. This rescue did not occur after treatment with a PDEi4 (rolipram) or a PDEi5 (vardenafil) inhibitor alone. Interestingly when these drugs were given concurrently, ODP was rescued. To further explore the effect of early alcohol exposure on ODP, we used Visually Evoked Potentials to examine the potentiation and depression components of ODP. Here we saw that although saline animals exhibited

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the expected depression and potentiation of contralateral ipsilateral eye responses, respectively, ethanol animals exhibited only a depression of contralateral eye responses. In conclusion we translated our previous findings from ferrets to mice, demonstrating that early alcohol exposure disrupts ODP. Moreover, we also confirmed our recent studies showing that PDEi1 inhibitor restores this disruption.

Using this model we showed for the first time that early alcohol exposure disrupts the potentiation, but not the depression component of ODP. Our results also suggested they need to both cAMP and cGMP cascades in the restoration of ODP by PDEis. These finding should contribute to our understanding of the mechanisms underlying disruption of neuronal plasticity by alcohol.

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Chapter 1

Introduction

Consumption of alcohol during pregnancy can lead to Fetal Alcohol Spectrum Disorders (FASD) in the developing child. Despite the preventable nature of this condition, it remains the leading cause of neurodevelopmental disabilities in the western world. In fact, the number of FASD cases per year out number cases of spina bifida and Down's syndrome combined (May, 2009; Klug and Burd, 2003) and has an estimated annual cost of $3.6 billion annually in the United States (Sampson et al., 1997). FASD is characterized by growth deficiencies and a wide range of neurobehavioral problems (Hoyme et al., 2005). The type and severity of these deficits is dependent on the timing and amout of alcohol exposure. Therefore, children with FASD can exhibit pathologies that can vary from gross malformations such as microcephaly and agenesis of the corpus callosum, to subtle learning and memory deficits. Despite the severity and prevealence of FASD there is no effective treatment and the mechanisms behind the effects of early alcohol exposure are poorly understood.

Mounting studies over the past decade have indicated that problems in neuronal plasticity may underlie the cognitive deficits associated with FASD. In fact, animal models of FASD have consistently shown impairments in different paradigms of neuronal plasticity. For instance early alcohol exposure can affect various types of plasticity such as barrel cortex plasticity (Rema and Ebner, 1999), eye-blink conditioning

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(Johnson et al., 2008), and long-term potentiation (LTP) (Puglia and Valenzuela, 2010). Additionally alcohol can disrupt learning in numerious behavioral tests such as the Morris Water Maze (MWM), object recognition and passive avoidance (Filgueiras et al., 2010; Kim et al., 1997; Girard et al., 2000; Summers et al., 2008).

A classical measure of neocortical plasticity is called ocular dominance plasticity (ODP). ODP is a measure of how eye dominance in the visual cortex (V1) changes based on visual experience. In classical ODP, an animal has one eye sutured closed (monocular deprivation, MD) during the critical period of visual cortex development (Hubel and Wiesel, 1970). This unilateral change in visual input results in a shift in eye dominance in V1. This shift occurs through biphasic mechanisms: an initial decrease in responses from the deprived eye (depression component), followed by an increase in responses from the experienced (open) eye (potentiation component) (Frenkel and Bear, 2004). Using this ODP paradigm, our lab has previously demonstrated that exposure to ethanol during the 3rd trimester equivalent of human gestation in ferrets results in permanent deficits in ODP (Medina et al., 2003; Medina and Ramoa, 2005). This disruption can be reversed by treating ferrets with the phosphodiesterase type 1 inhibitor (PDEi1) vinpocetine (Medina, 2006). PDE1, converts cAMP to AMP and cGMP to GMP (Keravis and Lugnier, 2010), therefore inhibition of this enzyme can lead to increase intracellular levels of the cyclic form of these nucleotides. Both cAMP and cGMP can activate cascades which will ultimately lead to phosphorylation of transcription factors that are important for the expression of plasticity related genes (Frank and Greenberg, 1994; Atkins et al., 1998; Etkin et al., 2006). Among these transcription factors are the cAMP responsive binding protein (CREB) and the serum

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response factor (SRF). In fact, overexpression of a constitutively form of SRF by viral mediated gene transfer also resulted in restoration of ODP in the ferret model of FASD (Paul et al., 2010).

While ferrets can be very useful as animal models, their high cost and the lack of transgenic animals can be limiting. Therefore, extending our previous results to mice may be extremely useful. Our first goal would be to test whether ocular dominance plasticity in mice is disrupted after early alcohol exposure and whether this disruption can be reversed by the PDEi1, vinpocetine. Because PDEi1 acts on both cyclic nucleotides, it is not know whether the positive effects of vinpocetine treatment are due cAMP or cGMP cascades. Therefore, our second goal is to test the efficacy of a PDEi4 (specific for cAMP) and a PDEi5 (specific to cGMP) in restoring ODP in mice.

As mentioned previously, ODP relies on the depression and potentiation of responses of the deprived and experienced eye respectively. However, it is unknown whether alcohol exposure equally affects both of these components. Because most studies suggest that early alcohol exposure affects LTP more severely than long term depression (LTD) (Puglia and Valenzuela, 2010; Izumi et al., 2005; Richardson et al., 2002; Sutherland et al., 1997; Servais et al., 2007; Titterness and Christie, 2008); and since ODP is closely related to these two forms of synaptic plasticity, our third goal is to test the hypothesis that early alcohol exposure will affect mainly the potentiation component of ODP. The accomplishment of these goals should contribute to our better understanding of how early alcohol exposure disrupts cortical plasticity and what mechanisms underlie the PDE mediated restoration of neuronal plasticity in models of FASD.

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Chapter 2

Background and Significance

Early Alcohol Exposure in Humans

Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term for the variety of effects that occur in a child whose mother imbibes alcohol during pregnancy. FASD includes several conditions of varying severity, including Fetal Alcohol Syndrome (FAS), Partial Fetal Alcohol Syndrome (pFAS) and Alcohol Related Neurodevelopment Disorders (ARND) (Reviewed in (Riley et al., 2011)). Estimates put the prevalence of FASD at approximately 1 in 100 live births, making it the leading cause of mental retardation in the western world (Sampson et al., 1997). Moreover, the cost of treating affected children is reported to be $3.6 billion annually in the U.S. However these numbers are for diagnosed FAS alone; therefore, the actual cost of treatment for people affected by the more general FASD is certainly much higher.

The most obvious effect of early alcohol exposure is the stereotypical facial phenotype displayed by children with FAS. This phenotype is described as including small palpebral fissures, flattened philtrum, thin upper lip, small mid-face and low nasal bridge (Figure 1). Some children also show a 'hockey-stick' palmar crease and 'railroad track' ears (Hoyme et al., 2005). The severity of these deficits is thought to vary based

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Figure 1

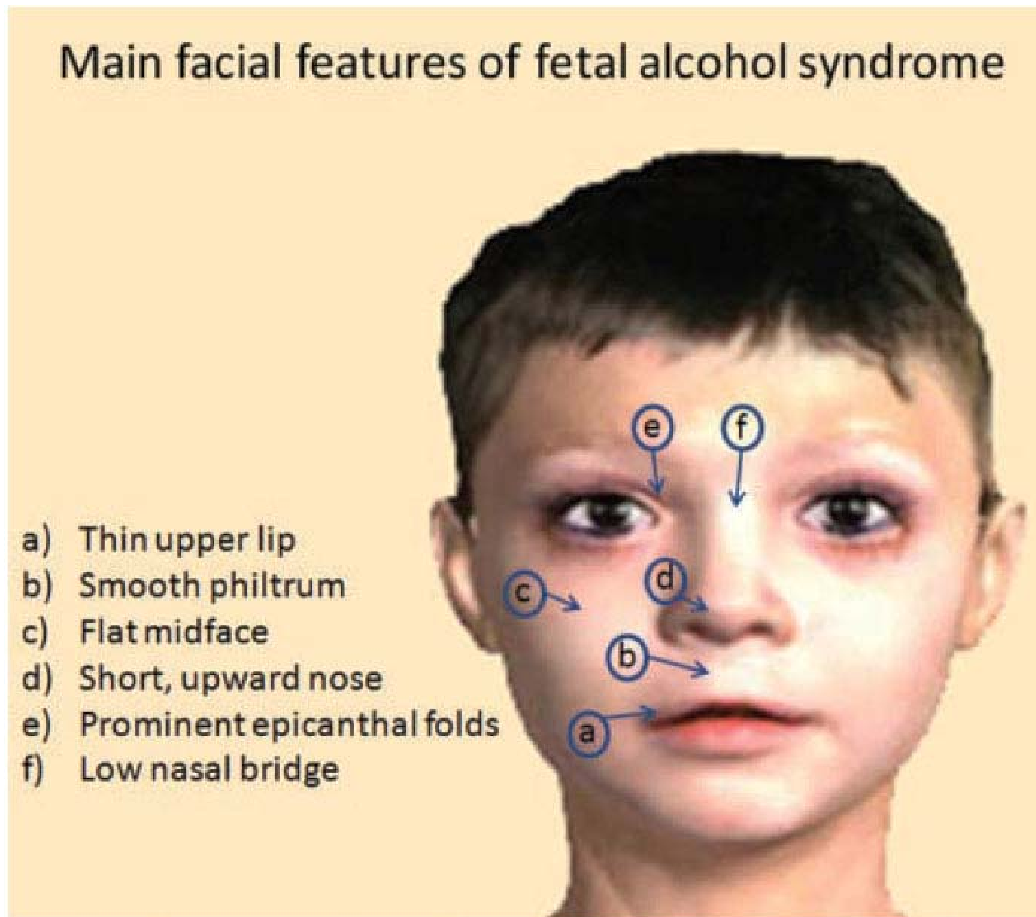

Figure 1. Facial Features of Fetal Alcohol Syndrome. Children with FAS exhibit a series of stereotypical facial features after exposure to alcohol *in utero.* These features include a) a thin upper lip and b) a smooth philtrum. Additionally there are many mid face malformations including c) flat midface, d) short upward turned nose, e) prominent epicanthal folds and f) a low nasal bridge. (Medina, 2001)

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on amount, timing of maternal alcohol intake and genetic factors. To be diagnosed as having FAS a child must exhibit at least two of the traditional facial features, growth retardation and brain growth deficiencies. While in pFAS, in addition of two facial features, the patient exhibits either growth retardation or brain growth deficiencies but not both (Hoyme et al., 2005). A recent study on FAS facial features observed a marked facial asymmetry, such that midline components are slightly shifted towards the right side of the face (Klingenberg et al., 2010). The alterations in the face observed in FAS can be replicated in animal models. Studies using rodents determined that the two weeks after conception is the period when alcohol exposure can induce this type of dysmorphology (Sulik et al., 1981).

Despite the lack of the typical FAS facial features, an exposure to alcohol during other periods of the gestation can still produce severe brain growth deficits and cognitive abnormalities. Such is the case in children diagnosed as having ARND. However, because of the lack of an evident feature (the FAS facial features) and the difficulties to prove consumption of alcohol during pregnancy, many children with this condition remain undiagnosed.

Brain alterations in FASD have also been extensively studied. While, microcephaly can be observed in severe cases, some children with FASD exhibit an increase in cortical thickness (Yang et al., 2011; Sowell et al., 2008). This paradoxical increase is hypothesized to be a result of a decrease in cortical cell pruning, a process of neuronal plasticity associated with brain maturation (Katz and Shatz, 1996; White and Fitzpatrick, 2007). Fiber tracts seem to be particularly affected in FASD. For instance, a reduction or a complete agenesis of corpus callosum is commonly observed (see (Riley

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and McGee, 2005) for review). Not only are these midline structures disrupted but they are also less functional as shown by fMRI functional connectivity measures (Wozniak et al., 2011).

Children affected by FASD show a wide spectrum of neurobehavioral problems. It is estimated that as many as 95% of children with FASD have hyperactivity or Attention Deficit Hyperactivity Disorder (ADHD) (Bhatara et al., 2006; Fryer et al., 2007). Another common behavioral problem seen in children affected by FASD is a deficit in executive function and problem solving. Since executive function is crucial for goal oriented behavior (Zelazo et al., 2004). It is not surprising then, that children with FASD show repeated deficits in learning and reduced IQ throughout their lifespan (Steinhausen and Spohr, 1998).

Similarities between Human and Animal Models of FASD

Although much can be learned from studying children with FASD, there are often many confounding factors, such as the use of other drugs of abuse during pregnancy and malnourishment. Furthermore, the great variability of the quantity and timing of alcohol consumption, together with the wide range of the effects observed, make the study of the mechanisms of alcohol exposure on brain development particularly challenging. In this regard, the use of numerous types of animal models has been pivotal in advancing our knowledge of alcohol teratology. During the equivalent of the 1st trimester of human gestation, (gestational day (G) 5 to G11 in rodents) organogenesis and neural tube formation is occurring. Animals exposed to ethanol during this period of development display a facial dysmorphology similar to that seen in

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FASD, with shortened palpebral fissures, as well as lack of a philtrum and a shortened mid-face (Sulik et al., 1981). When the brain of these mice is examined, the corpus callosum is found to be significantly smaller or missing completely (Sulik and Johnston, 1982). Finally, MRI studies demonstrated that mice treated with ethanol at G8 show overall decrease in brain volume as well as a total decrease in whole body volume. The association between these abnormalities and alcohol exposure during early development was confirmed in human studies (Ernhart et al., 1987). Animals exposed to ethanol during this time period also display deficits in object recognition, a type of hippocampal learning (Summers et al., 2008).

Exposure to alcohol during the 2nd trimester equivalent to human gestation (G12 to 21 in rodents) can depress proliferation and migration of neurons throughout the brain (see Review (Guerri, 1998)). More specifically, ethanol accelerates the transformation of radial glia to astrocytes. This premature loss of radial glia causes disrupted migration of late forming neurons (Miller and Robertson, 1993). Behaviorally, ethanol exposure between G7 to G18 in rodents causes increased locomotion in open field tests, resembling the hyperactive phenotype seen in children with FASD (Lehotzky et al., 1988). In general, alcohol exposure during the second trimester is less characterized, as most studies tend to look at exposure throughout gestation or third trimester alone.

During the third trimester equivalent of human gestation (roughly between P4-P10 in rodents) the brain goes through a phase of intense synaptogenesis and circuit refinement called the 'brain growth spurt' (Dobbing and Sands, 1979). A single dose of ethanol during this time period causes widespread neuronal death, through a process

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mediated by the pro-apoptotic protein BAX (Ikonomidou, 2000)(Young et al., 2003), aberrant myelination and alters synapse formation (Lancaster et al., 1984; Guerri, 1987). Behaviorally, animals exposed to ethanol during the 'brain growth spurt' exhibit hyperactivity, similar to what is seen in 2nd trimester equivalent exposure (Nunes et al., 2011). Additionally, these animals show marked deficits in tasks of learning and memory. Mice exposed to ethanol during this developmental window displayed deficits in task acquisition and recall in the Morris Water Maze (MWM) (Filgueiras et al., 2010; Thomas et al., 2007), impairment of trace-fear conditioning and altered exploration of the 8-arm radial maze (Pick et al., 1993).

Plasticity in FASD

Neuronal plasticity is the brain's ability to change based on experience through strengthening and weakening of neuronal connections. This process is essential to normal development, when new synapses are made and circuits are refined, as well as throughout life in learning and memory processes (Katz and Shatz, 1996; White and Fitzpatrick, 2007). Impairments in these processes have been demonstrated in FASD models through the use of several plasticity paradigms. The most common behavioral paradigm to test hippocampal plasticity is the MWM. The MWM tests spatial learning and the ability of an animal to use visual clues to find a hidden platform. Ethanol exposed animals consistently show impairments in this task, taking more trials to reliably find the platform, as well as decreased swimming in the goal quadrant during the probe trial (Kim et al., 1997; Girard et al., 2000; Richardson et al., 2002). Another common learning task is object recognition. Here an animal is allowed to explore two

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identical objects. Following exploratory phase, one of the original objects is replaced with a novel object. The animal should then spend more time exploring the novel object than the original one. When given this object-recognition task, ethanol exposed animals explore the novel object and old object equally (Summers et al., 2008). Both of these measures indicate deficits in learning and memory, based in hippocampal plasticity (Broadbent et al., 2009; Morris et al., 1982).

It is quite established that long-term potentiation (LTP) and long-term depression (LTD) are the cellular basis of learning and memory (Etkin et al., 2006; Teyler, 1987; De Roo et al., 2008; Malinow and Malenka, 2002; Bear and Malenka, 1994; Malenka and Bear, 2004). In general, these processes work through changes in AMPA receptor trafficking, gene regulation and cell responsiveness after specific types of stimulation (Malenka and Bear, 2004). Typically, LTP is involved in a strengthening of neuronal synapses and responses based on synchronous or regular patterned activity. This patterned activity causes cell depolarization through AMPA receptors, removing the Mg^{+} blockade of NMDA receptors, allowing them to be activated (Brown et al., 1988). This activation causes additional incorporation of AMPA receptors at the synapse and responses are greatly enhanced (Bredt and Nicoll, 2003). In contrast, LTD is, typically, the weakening of synapses strength due to asynchronous or irregularly patterned activity, and removal of AMPA receptor from the synapse through endocytosis (Kessels and Malinow, 2009). Although AMPA receptor endocytosis is the proto-typical model for LTD, mechanisms can vary throughout the brain layers and structures (see review, (Massey and Bashir, 2007)). For instance, in visual cortex, LTD is mediated by AMPA

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receptor trafficking and canabinoid transmission in layers 4 and 2/3 respectively (Liu et al., 2008).

Early alcohol exposure can affect many molecules that are related to synaptic plasticity. For example, animals with early alcohol exposure show an increase in the NR2A, but not the NR2B, subunit of the NMDA receptor (Samudio-Ruiz et al., 2010; Honse et al., 2003; Nixon et al., 2002). Additionally, early ethanol exposure can reduce the overall number of NMDA and AMPA receptors (Rema and Ebner, 1999; Bellinger et al., 2002) and alter the glutamate binding (Savage et al., 1991). In addition to its effects on excitation, ethanol is also an acute GABA agonist (Alfonso-Loeches and Guerri, 2011) and, chronically, can disrupt GABAergic transmission. For instance it has been shown that early alcohol exposure can decrease the amplitude of inhibition potentials for several days post-treatment (Durand and Carlen, 1984; Durand and Carlen, 1984; Hsiao et al., 1999). A disruption in LTP was seen in animals exposed to ethanol in the 1st, 2nd, and 3rd trimester equivalents (Puglia and Valenzuela, 2010; Sutherland et al., 1997). Despite the great number of studies demonstrating disruption of LTP after alcohol exposure, it is not clear whether it affects LTD (Titterness and Christie, 2008). For instance Izumi and collaborators demonstrated that exposure to alcohol at P0 or P7 (2 injections of 2.5 mg/kg s.c.; BEC = ~500mg/dL) can disrupt LTD in the hippocampus of rats at P30 (Izumi et al., 2005). In contrast exposure to moderate levels of alcohol (BEC = ~200mg/Kg) during the whole of rat gestation did not affect LTD *in vivo* (Titterness and Christie, 2008).

The effect of alcohol may be different according to brain region. In the cerebellum, LTD has been shown to be disrupted in the parallel fibers of ethanol

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exposed animals. In this paradigm parallel fiber stimulation is paired with climbing fiber stimulation, normally resulting in LTD. Yet, when this stimulus paradigm is applied in ethanol exposed animal parallel fibers, responses are potentiated, leading to LTP instead of LTD (Servais et al., 2007).

Ocular dominance plasticity (ODP) is a classical model of cortical neuronal plasticity. It is characterized by anatomical and/or functional changes in the visual cortex after one eye is occluded (monocular deprivation, MD) for days or weeks (Hubel and Wiesel, 1970; Hubel et al., 1977). In higher mammals the primary visual cortex contains alternating columns which respond to inputs from the contralateral and ipsilateral eye, in most animals the representation of the former is greater than the latter. After the contralateral eye is deprived of vision, by eyelid suture for a period of days, this dominance shifts to favor the ipsilateral eye. OD plasticity is a type of activity-dependent plasticity based on mechanisms that work to strengthen synapses whose activity coincides with target depolarization beyond some threshold level (Hebb, 1949), and to eliminate synapses whose activity is not correlated with postsynaptic activity (Stent, 1973). The NMDA receptor may function as the correlation detector that signals when pre- and post-synaptic activities are synchronous (Hebb, 1949). Influx of calcium ions through NMDA receptors, activates protein kinases that, in turn, phosphorylate transcriptional regulators. One protein kinase that plays a critical role is PKA. In fact, OD plasticity can be blocked by Rp-8-Cl-cAMPS, a classic PKA inhibitor (Beaver et al., 2001). PKA is activated by increased levels of cAMP and has a major role in phosphorylating the transcription factors CREB (Sands and Palmer, 2008), Elk-1 (Kovalovsky et al., 2002) and serum response factor (SRF) (Blaker et al., 2009) .

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Visual Cortex Plasticity in Mice

Some of the most important developmental benchmarks of the visual system in rodents occur during the 3rd trimester equivalent of human gestation. It is during this time that the retina fires Stage I, II and III retinal waves which play a major role in establishing the organization, binocularity and connectivity of the visual system (Galli and Maffei, 1988; Maffei and Galli-Resta, 1990; Bansal et al., 2000). While these retinal waves are occurring, the photoreceptor outer segments are maturing and begin to form synapses with horizontal and bipolar cells at P6. After these synapses form between P8 and eye opening (P12) the retina goes through intense synaptogenesis (Olney, 1968). While these events are occurring, the optic tract finishes innervating the LGN at P2 (Guido, 2008). This innervation is characterized by significant overlapping of ipsilateral and contralateral eye projections (Clancy et al., 2001; Jaubert-Miazza et al., 2005). The refinement of eye projections to the LGN is evident after P7, with adult like patterning of ipsilateral and contralateral projections appearing by P12. Axons from the LGN innervate cortical layer 4 of V1 by P2. After this innervation, pyramidal cells in the V1 begin maturing, and forming synapses, with mature synapses and pyramidal cells recognizable by P7 (Li et al., 2010).

An important aspect of the visual system is the binocular representation present in the primary visual cortex (V1). Although all mammals have binocular cells in V1, its organization varies between species. In most gyrencephalic mammals (humans, primates, ferrets), V1 contains alternating columns of cells which respond to input from each eye separately called ocular dominance columns (Rockland, 1985; LeVay et al.,

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1975; Horton et al., 1990). Although both hemispheres contain columns that respond to each eye, there is usually a bias towards inputs from the contralateral eye. This bias is also seen in rodents, although they do not have ocular dominance columns (Drager, 1975). Instead, mice and rats have a V1 that is composed of two different areas: a large monocular zone, which comprises 2/3rds of its total size, and a small binocular region, which occupies the remaining third. While the former receives only input from the contralateral eye, the latter receives input of both, but a contralateral bias is still seen (Figure 2) (Coleman et al., 2009). This bias can be shifted towards the ipsilateral eye, if the contralateral eye is deprived of vision for a period of days. This procedure is done by suturing the eyelids (MD) and leads to an increase and a decrease of the deprived and experienced (open) eye responses respectively (Gordon and Stryker, 1996). These two components of ocular dominance plasticity (increase and decrease of responses) occur at two different times and use different mechanisms (See Figure 3). The first of these processes to occur is the decrease in responsiveness to the deprived eye. In mice this occurs as soon as 24hrs after MD, and can be reliably recorded after 3 days (Frenkel and Bear, 2004). This depression component of OD plasticity shares many mechanisms with LTD, such as weak activation of the NMDA receptor, followed by internalization of AMAP receptors by a clathrin mediated process (Smith et al., 2009). The potentiation of ipsilateral eye response occurs after the first 5 days of MD, increasing until 7 days of MD (Frenkel and Bear, 2004). This potentiation component of OD plasticity shares mechanisms with LTP, with NMDA receptor activation leading to increased insertion of AMPA receptors into the synapse. Together these modifications make up a complete ocular dominance shift (Smith et al., 2009).

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Figure 2

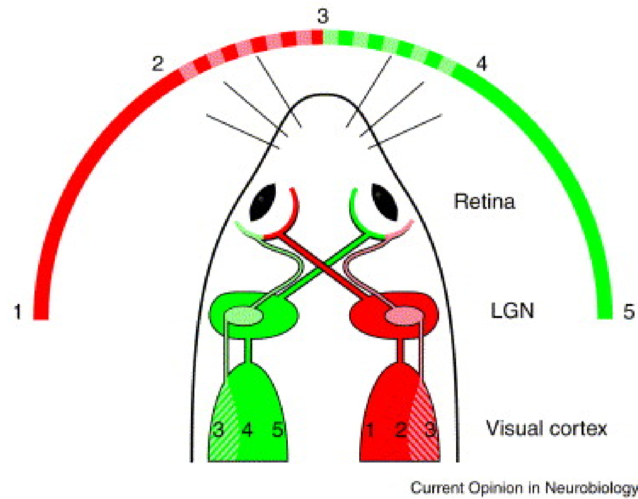

Figure 2. Mouse Visual System. The left and right sides of the mouse visual system and their respective visual fields are represented here, green for left hemisphere and red for right hemisphere. The primary visual cortex is divided into two areas based on their response pattern. These two areas are the binocular and monocular zones. The monocular zones represent input from only the contralateral eye, and its nasal visual field, seen here numbered 1, 2, 4 & 5. In contrast the binocular zone responds to inputs from both eyes and their temporal visual fields, representing inputs from both the contralateral and ipsilateral eyes, labeled here as 3. It is in this binocular zone that visual cortex plasticity is present. (Hubner, 2003)

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Figure 3

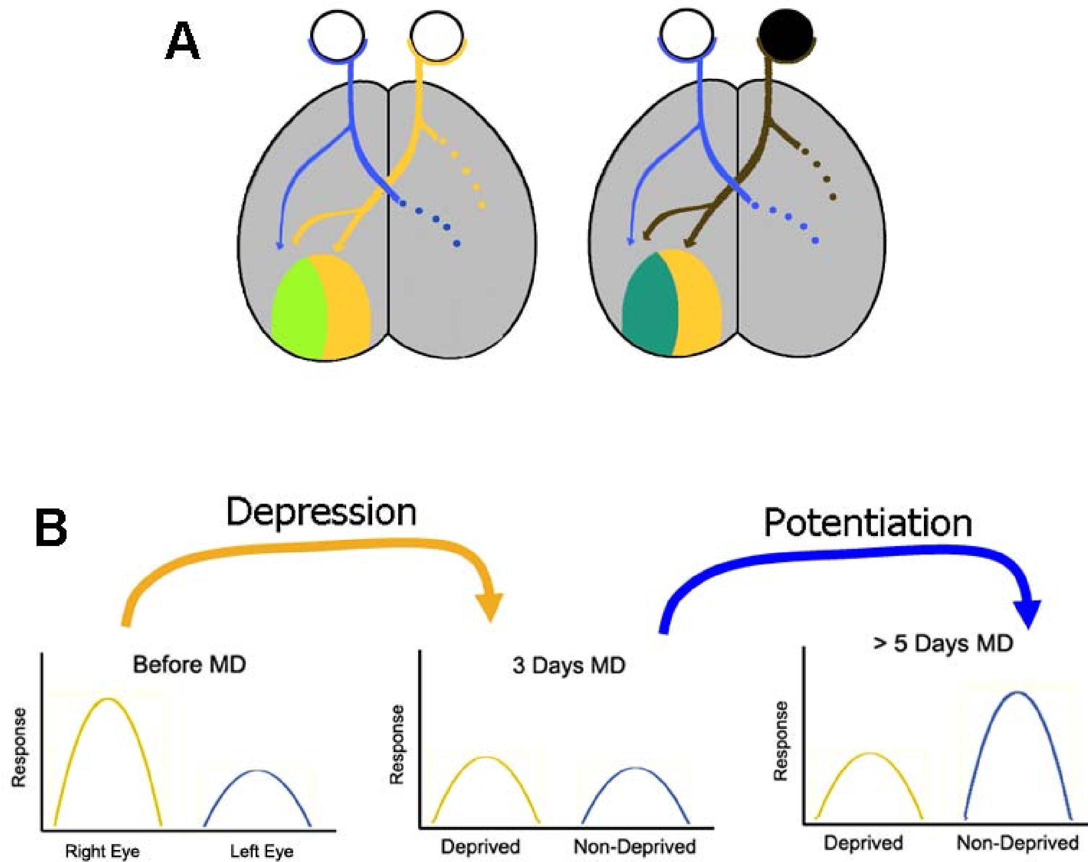

Figure 3. Ocular dominance plasticity, in mice. When a mouse's eye is monocularly deprived for a period of days (A), and ocular dominance shift occurs. This is traditionally measured in the hemisphere contralateral to the monocularly deprived eye. This shift in eye dominance in the binocular zone of V1 occurs through two temporally different mechanisms. First, there is a depression of inputs from the deprived eye which occurs after approximately 3 days of MD (B). This is followed by a potentiation of the non-deprived eye inputs, after approximately 5 days of MD.

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The effects of MD can be temporally and mechanistically distinct through cortical layers. After MD, the initial change in visual acuity is relayed to dLGN and then to the visual cortex through thalamocortical connections. These connections synapse on layer 4, which then makes corticocortical connections to layer 2/3. While it was theorized that ODP started in layer 2/3, recent studies have been challenging this idea, raising the possibility that the changes might happen simultaneously (Liu et al., 2008; Smith et al., 2009).

It is quite established that the processes of LTD and LTP underlie many types of activity-dependant neuronal plasticity from establishing and abolishing synapses to sprouting and pruning of terminals (Bear and Malenka, 1994; Malenka and Bear, 2004). While OD plasticity is a complex process that involves multiple synaptic and morphological changes, it is fair to say that LTD and LTP processes are determinant in modifying deprived and experienced eye cortical responses. In fact, blockage of LTP and LTD respectively abolishes the potentiation and depression of OD plasticity (Daw et al., 2004).

The depression component of OD plasticity occurs through different mechanisms in layers 2/3 and 4. Low frequency stimulation of the radial inputs to the visual cortex has revealed that synaptic depression in layer 2/3 is mediated by cannabinoid receptors, while synaptic depression in layer 4 is mediated by endocytosis of AMPA receptors (Crozier et al., 2007).

The potentiation component of OD plasticity occurs after approximately 5d of MD, peaking at 7 days (Frenkel and Bear, 2004). This potentiation of ipsilateral inputs is

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thought to be a response to a decrease in cortical activity after depression of the contralateral inputs (See review (Bear, 2003)). Unlike LTD in the visual cortex, the potentiation of open eye responses appears to be similar across layers. NMDA receptor dependant LTP is induced in both layers 2/3 and 4 after tetanic stimulation of the LGN *in vivo*, increasing the amplitude of Visually Evoked Potential (VEP) responses.(Heynen and Bear, 2001). Experiments using stimulus response potentiation, which cause an increase in VEPs similar to the potentiation of ipsilateral response after MD, have shown it is dependant on AMPA receptor trafficking (Frenkel et al., 2006).

For both the increase and decrease of responses to occur, the MD must occur during the critical period of visual cortex plasticity. (Hubel and Wiesel, 1970); (Gordon and Stryker, 1996). This phase greatly varies between species (see Table 1). The mechanisms underlying opening and closing the critical period for V1 plasticity have been extensively studied. Early studies noted that the closure of the this period coincides with the maturation of astrocytes (Muller, 1990; Engel and Muller, 1989). This theory was bolstered when immature astrocytes were implanted into V1 of an adult cat, and ODP was seen (Muller and Best, 1989). While the mechanisms underlying these results are still unclear, one possibility is that astrocytes release molecules which would affect the rigidity of the extracellular matrix (Faissner et al., 2010). In fact, it was demonstrated that the closure of the critical period also coincides with changes in the extracellular matrix and the formation of a “perineuronal net”. Remarkably, when this net is degraded by infusion of chondroitinase, a complete OD shift is observed in adults (Pizzorusso et al., 2002). In addition to changes in the matrix, the balance of excitatory and inhibitory inputs into the visual cortex may also play a major role to the

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Animal	Critical Period	Citation
Human	birth to 7 years	Lewis, 2005
Cat	4 weeks - 3 months	Hubel, 1970
Ferret	P32 - P70	Issa, 1999
Mouse	P19 - P32	Gordon, 1996

Table 1. Timing of critical period of visual cortex plasticity across species.

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establishment of the critical period. At the onset of the critical period, an increase in the NR1 and NR2A subunits of the NMDA receptor has been seen (Roberts and Ramoa, 1999). This up regulation of can be delayed with dark rearing, effectively retarding the maturation of the critical period of visual cortex plasticity (Chen et al., 2000). While glutamatergic transmission may also play a role in this process, developmental changes in inhibition seem to lead to more dramatic effects. For instance, BDNF- induced early GABAergic maturation of the critical period begins and terminates earlier, along with a precocious maturation of GABAergic innervation (Huang et al., 1999; Hanover et al., 1999). Additionally, when immature GABAergic neurons are implanted into the visual cortex after the closure of the critical period, OD plasticity is restored (Southwell et al., 2010). Computer modeling has indicated that these neurons make wide-spread, weak, local connections within V1, which shift slowly in response to MD (Gandhi et al., 2008). Together these data support the idea that an optimal balance of NMDA and GABA function is necessary for the establishment of the critical period.

Systems for Studying V1 Plasticity

Ocular dominance plasticity can be assessed by different techniques. The most common approach is single unit recordings which consist of recording extracellular responses from isolated neurons while visual stimulation is presented (Hubel and Wiesel, 1970). In this method a glass or tungsten electrode is inserted perpendicularly or tangentially to the cortex surface. For instance, in cats, ferrets and monkeys (animals that have ocular dominance columns) these two insertion methods function to either record from cells in the same column (and similar profile), or cells from distinct columns

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(Hubel and Wiesel, 1970; Hubel et al., 1977). Single unit recordings use different sets of visual stimulation to detect features such as orientation tuning, degree of binocularity, disparity, neuronal responsivity and spontaneous activity (Hubel and Wiesel, 1970; Hubel et al., 1977). While this technique allows the assessment of distinct neural properties, it has its limitations. Due to its invasiveness, adequate levels of anesthesia are required and survival experiments require complex implants. Moreover, given the nature of recording a single cell at a time, these types of preparations can be very time-consuming (generally over 12 hours), requiring multiple electrode penetrations before an adequate number of cells can be sampled. The use of multichannel electrodes has been useful to increase the number of cells sampled as well as reduce the duration of the experiment (Owens et al., 1995).

Another tool to study V1 plasticity is optical imaging of intrinsic signals, a technique pioneered in the last two decades. In this procedure, visual responses are measured as a response of a population of cells, and not single cells such as in single units (Bonhoeffer, 1995). For this technique the animal is anesthetized and a craniotomy is performed (for larger animals; in mice imaging through the skull is generally preferred) (Medina et al., 2003; Cang et al., 2005). The cortex is then illuminated using a green filter to map blood vessels (to use as landmarks) followed by a red light (700 nm), which is kept during the recording (Figure 4). To create cortical activity maps, a visual stimulus consisting of gratings or bars is presented while a camera takes pictures of the cortex. The basis of this type of imaging is that active tissues consume more oxygen, shifting the light red oxy-hemoglobin into a bluish red deoxy-hemoglobin. Therefore, computer software can compare images obtained under

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Figure 4

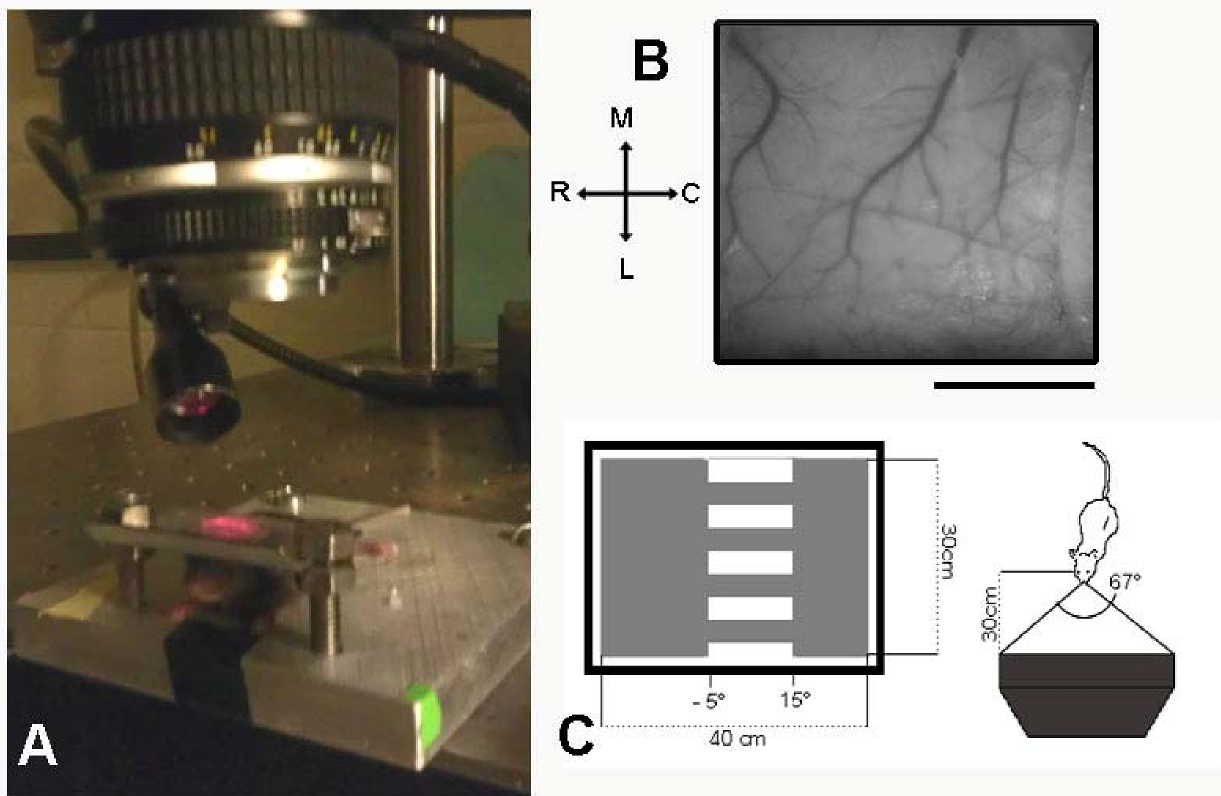

Figure 4. Optical imaging of intrinsic signals. An animal is anesthetized and the scalp is retracted and a metal plate with a small window is attached to the skull. A) The animal and plate are then fixed to a frame, and the skull is illuminated with red (740nm) light. A picture is taken of the b) pattern of cortical vessels through the skull. After the baseline picture is recorded, c) a monitor is placed in front of the animal which displays a binocular field grating. Changes in cortical activation are then recorded in response to the stimuli.

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two different conditions and create a map identifying the cortical areas where a change occurred (Grinvald et al., 1986; Thompson et al., 2003). For instance, when the two conditions are the presence or absence of visual stimulation, the resulting pictures will display differences in the visual cortex, while non-visual areas will remain unchanged. In this case the more active an area is, bigger the differences will be. The activation maps represent the activity of cortical layers 2/3, as the penetration of the cameras focus is limited (Frostig and Chen-Bee, 2009).

Another technique, which has come into favor in the past few years for studying OD plasticity, is chronic visually evoked potentials (VEPs). VEPs recording are set up much like single unit recordings, with the exception that the electrode has lower impedance (0.4 MOhm, compared to 1MOhm) to allow recording from multiple cells and the amplifiers are set with a lower cut off than with single units. This difference arises from the need to record field potentials, which are the sum of the extracellular electrical current flowing from multiple neurons around the recording electrode (Porciatti et al., 1999). Electrodes are surgically implanted in an anesthetized animal, which is then allowed to recover for 48 hours. For habituation and recording, the animal's head is held in place by a post attached to the skull at the time of electrode implantation (see Figure 5). During stimulus presentation, animals are awake, removing any possible confounders created by anesthesia. VEPs are elicited by visual stimulation done by reversing gratings displayed on a computer moniter (Yashiro et al., 2009). The responses recorded represent neuronal responses within layer 4 (Cooke and Bear, 2010). This method allows the recording of visual responses before and after MD in the same animal. Additionally, while the other methods above are based in ratios, the use of

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Figure 5

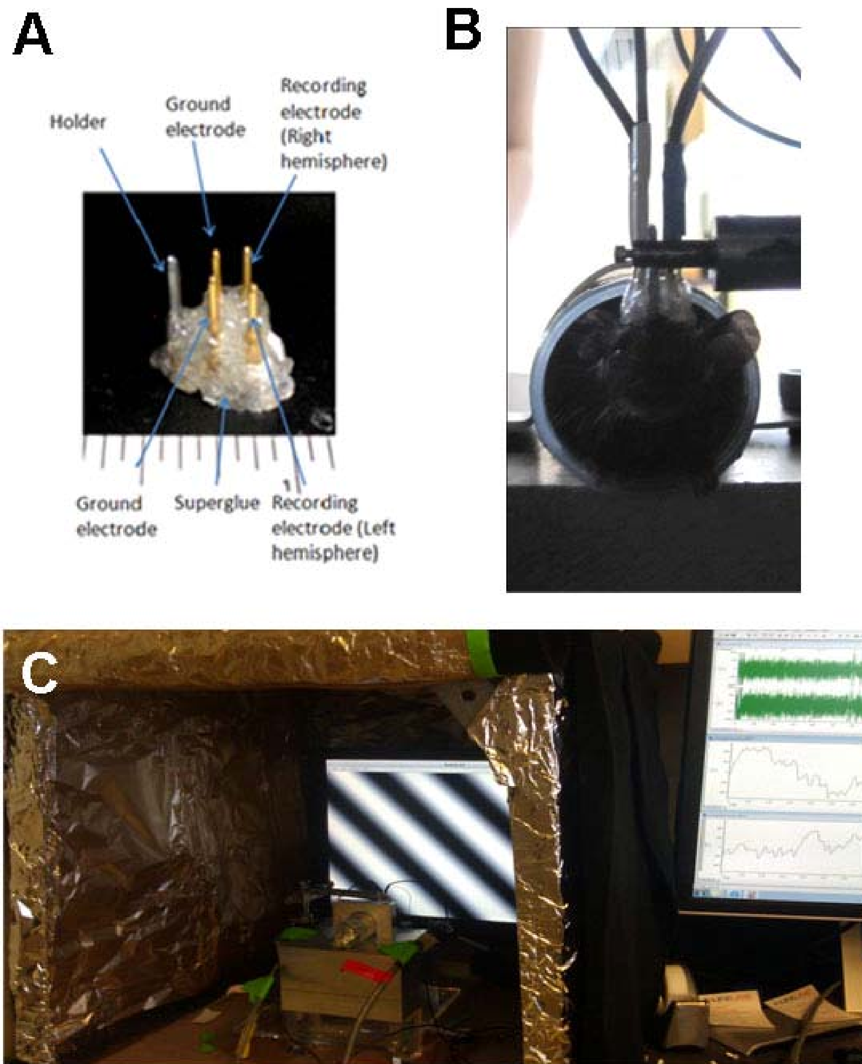

Figure 5. VEP recording. a) Ground and recording electrodes are surgically impanted into the brain of the experimental animal, and a holding post is attached to the frontal skull. After a period of recovery the animals is placed into b) a frame which holds the head fixed. The awake animal then views a series of c) visual stimuli and the resulting cortical activation is recorded.

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VEPs allows the independent measure of contra and ipsilateral eye responses. This feature is important to distinguish the contributions of the depression and potentiation components of ODP (Smith et al., 2009).

V1 Plasticity and Neurodevelopmental Disorders

The mechanisms of ODP have been well established, comprising changes from the molecular (number of receptors, expression of plasticity-related genes), to the cellular (dendrites branching and pruning) and behavioral (loss of vision) level. With this variety of observable effects, ODP has become a model system for the study of disease states associated with disordered neuronal plasticity. So far, ODP have been used to study FASD (Medina et al., 2003), Fragile X (Dolen et al., 2007), neonatal hypoxia-ischemia (Failor et al., 2010), Angelmann syndrome (Yashiro et al., 2009) and Fetal valproate syndrome (Pohl-Guimaraes et al., 2011).

Fragile X is an inherited form of intellectual disability. It is characterized by a mutation in the *Fmr1* gene, causing autistic behavior, anxiety, epilepsy and hyperactivity (Bernardet and Crusio, 2006; Verkerk et al., 1991). *Fmr1* codes for the protein Fragile X Mental Retardation Protien (FMRP). This protein has been shown to be a repressor of mRNA transcription in response to mGluR1/5 activation (See review (Bear et al., 2004)). Using the *Fmr1*-null mouse (The Dutch-Belgian Fragile X Consorthium, 1994), Mark Bear's group showed that these animals have abnormal ODP; after 3d of MD the *Fmr1* knockout show a precocious increase in response to the ipsilateral eye; similar to what is seen in wild-type animals after 5d of MD (Dolen et al., 2007). This increase was accompanied by no change in the responsiveness of the deprived eye. This excessive

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cortical plasticity seems to arise from an increase in transcription of proteins downstream of mGluR5 activation. In fact, crossing the *Fmr1* knockout with an mGlur5 knockdown restored to ODP to normal levels.

In contrast to the excessive plasticity observed in Fragile X, in Angelman syndrome ODP is decreased. First described in 1965, Angelman syndrome is a neuro-genetic disorder characterized by developmental delays, intellectual disabilities and an unusually happy demeanor (Hart, 2008). Angelman syndrome is caused by a deletion of the *UBE3A* gene on the maternal chromosome, which codes for a ubiquitin ligase responsible for protein degradation in the brain (Kishino et al., 1997). *UBE3A* appears to be important in the normal experience-dependant maturation of the cortex, but its mechanism of action is still unknown (Sato and Stryker, 2010). Using VEPs recordings, Benjamin Philpot's group showed that *UBE3A* knockout mice show disrupted ODP, with no shift in the contralateral eye responses after 3d of MD (Yashiro et al., 2009). This lack of shift in the visual cortex was also corroborated by a lack inducible LTD in *UBE3A* knockouts, as well as deficits in LTP induction. These effects mirror LTP deficits seen in the hippocampus in previous studies of this disease model (Weeber et al., 2003).

Finally, the study of neonatal hypoxia-ischemia has also been advanced by the use of the ODP model. Neonatal hypoxia-ischemia occurs when a fetus' brain is deprived of oxygen and blood for a short period of time during development. This restriction causes massive cell death throughout the brain, resulting in developmental delays, intellectual disabilities and visual deficits (Piecuch et al., 1997; Lanzi et al., 1998). In a rat model of neonatal hypoxia-ischemia, the right common carotid artery is

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permanently ligated at P1. After 2 hours of post-surgical recovery, pups are then placed in a chamber with 6% oxygen for 210 minutes (Sheldon et al., 1996). After this procedure animals show progressive cell death of neurons, astrocytes and microglia. More specifically, this treatment leads to wide spread death of subplate neurons, which have been shown to play an integral role in thalamocortical development (McQuillen et al., 2003). Animals subjected to hypoxia-ischemia showed no changes in V1 structure, demonstrating normal contralateral bias as well as normal retinotopic maps, when testing using optical imaging of intrinsic signals (Failor et al., 2010). Yet, when these rats receive a MD for 4 to 7 days, they exhibited a distinct lack ODP compared to sham and non-damaged littermates, retaining contralateral eye dominance. These animals also showed an overall decrease in responses of V1 when tested using single unit recordings.

These neurodevelopmental disorders vary in their causes and severity, yet they all show disruption of ODP. These disruptions also vary with each disease state, but allow a better understanding of how these deleterious conditions affect the brain and its development. Their study also gives insight into the mechanisms behind ODP and the critical period of V1 development.

Restoring Plasticity in FASD

Using optical imaging of intrinsic signals and *in vivo* single unit recordings, in ferrets, our group demonstrated that 3d of MD were does not cause a shift in the ocular dominance columns of alcohol exposed animals (Medina et al., 2003). Additionally, when alcohol exposed animals were MD durring early, middle or late

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portions of the critical period they continued to demonstrate a lack of ODP, indicating that this disruption of ODP is not a result of a delayed development, and remains persistent throughout the established critical period for V1 (Medina and Ramoa, 2005). In addition, the disruption of neuronal plasticity by alcohol is likely to contribute to the altered formation of orientation selectivity columns and neuronal orientation tuning in the ferret model of FASD (Krahe et al., 2009; Medina, 2005).

Early alcohol exposure alters many pathways downstream of the NMDA receptor. Chronic alcohol exposure in utero results in a decrease in PKA, as well as a decrease in levels of pCREB (Naseer et al., 2011). These decreases are accompanied by changes in CaM kinase II subunits and phosphorylation states (Naseer et al., 2011). Finally, early ethanol exposure alters the levels of cGMP after stimulation of NMDA receptors (Butters et al., 2003). These changes appear to be a result of an overall suppression of the NMDA-Nitric Oxide Synthase system (Kimura et al., 2000). Interestingly, these pathways converge to the transcription factors CREB and SRF. Recently, inhibition of PDEs has been studied as a possible pharmacological approach to activate these transcription factors and facilitate the expression of plasticity related genes (Medina, 2011). By inhibiting specific PDEs one can increase intracellular concentrations of cAMP and/or cGMP, by stopping their degradation (Keravis and Lugnier, 2010). Thus, these nucleotides can act as second messengers in cascades that might culminate with the phosphorylation of CREB and SRF (Figure 6).

There are 11 different types of PDEs all functioning to degrade the phosphodiester bond in second messanger cyclic-nucleotides, converting cAMP to AMP and/or cGMP to GMP. However, some PDE types act only on cAMP (PDE4, 7 and 8)

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Figure 6

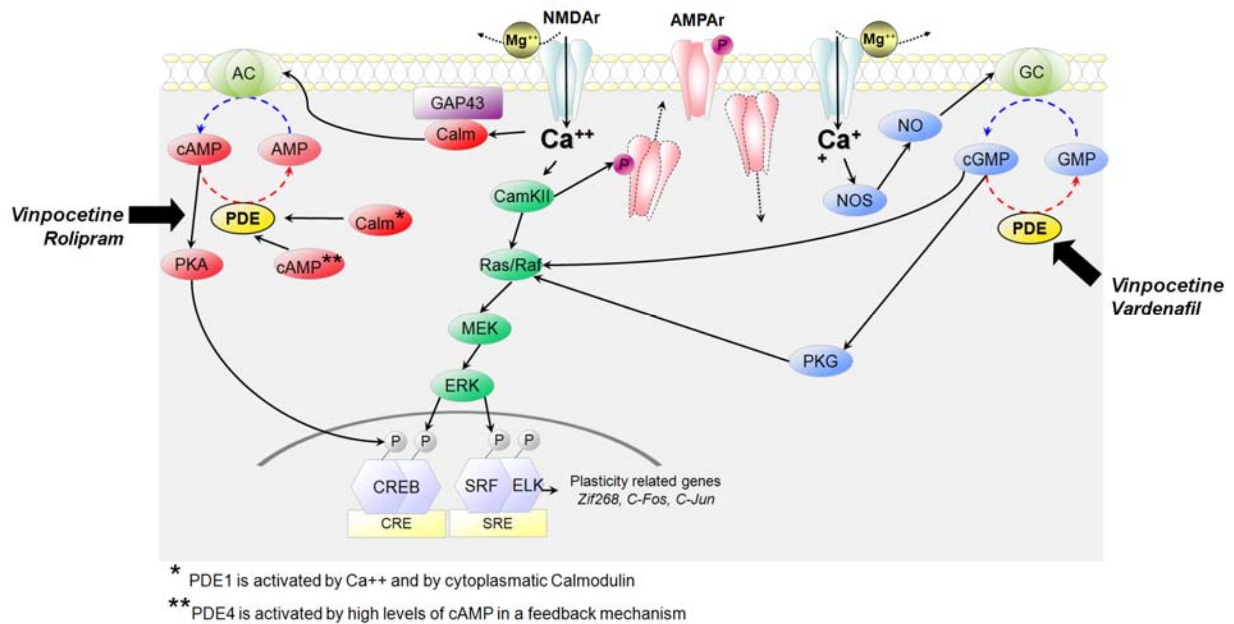

Figure 6. cAMP and cGMP Signaling Casades. Cascades which lead to the activation of CREB and SRF are crucial for many types of neuronal plasticity. It has been shown in previous studies that treatment with vinpocetine, through its action as a PDEi, leads to increases in cAMP and cGMP, and increases in pCREB. Drugs such as vardenafil and rolipram also are PDEis but act only on cGMP and cAMP respectively. Adapted from (Medina, 2011**)**

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others only on cGMP (PDE5, 6 and 9). In addition, there are types that can target both (PDE1, 2,3, 10 and 11)(Keravis and Lugnier, 2010).

Cyclic-nucleotides cAMP and cGMP are second messengers which are both mediated by Ca^{+2} influx after NMDA receptor activation (see Figure 6). Through their actions they are thought to activate two important transcription factors, serum response factor (SRF) and cAMP response element-binding (CREB). Cyclic-GMP is theorized to activate SRF, by activating the Rho-Ras and mitogen-activated protein kinase (MAPK) cascades (Gille et al., 1992; Hill et al., 1995; Miralles et al., 2003; Gudi et al., 2002). Through possible cGMP signaling, SRF is thought to play a large role in actin microfilament dynamics (Miralles et al., 2003). Studies have shown the late stage LTP requires cAMP production via PKA, and gene transcription possibly through pCREB (Frey et al., 1993; Bourtchuladze et al., 1994). CREB exerts its function through neuron survival pathways, and transcription of plasticity related genes such as brain derived neurotrophic factor (BDNF) (Finkbeiner et al., 1997).

Due to the action of PDEis on intracellular levels of cAMP/cGMP, they have been used as plasticity enhancers in various disease states. In fact PDEi1s have been shown to rescue rescues MWM learning and memory, and passive avoidance in an intracerebroventricular streptozotocin model of Alzheimer's type dementia (Deshmukh et al., 2009). Treatment with PDEi4s has also shown promise for plasticity improvement in other disease states. For instance, rolipram restored performance in the MWM and in step-through passive avoidance in models of cerebral ischemia and Alzheimer's (Li et al., 2011); (Wang et al., 2011). Additionally, rolipram treatment as also able to improve LTP in aged mice and a genetic model of Alzheimer's (Gong et al., 2004; Barad et al.,

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1998). Finally, PDEi5s have already proved themseves useful as a treatment for erectile dysfunction (Klotz et al., 2001), but they have also been studied for its ability improve neuronal plasticity. In rats, acute tryptophan depletion causes impairment of object recognition, yet when animals are treated with vardenafil, a PDEi5, their performance returns to control levels (van Donkelaar et al., 2008). Additionally PDEi5 have been shown to enhance object recognition, and memory reconsolidation in normal animals (Rutten et al., 2005; Boccia et al., 2011; Rutten et al., 2009). These enhancements have been shown to be associated with increased levels of cGMP, but not cAMP in hippocampal slice preparations after type 5 PDEi treatment (Rutten et al., 2005).

The potential of phosphodiesterase inhibition in improving plasticity in FASD was recently tested by our group in the visual cortex of the ferret (Medina, 2006). In this study, treatment with the PDEi1, vinpocetine, restored OD plasticity in ferrets exposed to alcohol during the third trimester equivalent of human gestation. In a subsequent study, vinpocetine was shown to increase the phosphorylation of CREB and to rescue orientation tuning (Krahe et al., 2009). In contrast to this treatment the PDEi4 rolipram, which acts specifically on the cAMP cascade, failed to restore OD plasticity in the ferret model of FASD even when tested with different doses (0.5mg/kg, 1.25mg/kg and 2.5 mg/kg). Because of the distinct results obtained with rolipram (acting specifically on cAMP) and Vinpocetine (acting on both cAMP and cGMP) it is possible that the positive effects of PDE1 inhibitors are due to activation of the cGMP cascade. Alternatively, it is also conceivable that the restoration of ODP plasticity by vinpocetine is due to a synergistic effect between cGMP and cAMP cascades.

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Chapter 3

[Manuscript 1]

Reversibility of Effects of Early Alcohol Exposure on Neuronal Plasticity by Treatment with Phosphodiesterase Inhibitors

Introduction

Fetal alcohol spectrum disorder (FASD) is an umbrella term for a variety of conditions affecting the children of women who drink alcohol during pregnancy. The effects of early alcohol exposure are wide ranging and can vary from subtle behavioral changes to severe mental retardation (Guerri, 1998; Riley, 2004; Rasmussen et al., 2006). Recent epidemiological studies have shown that FASD is the leading cause of mental retardation in the western world (May, 2009).

There is growing evidence that deficits in neuronal plasticity underlie many of the neurological problems observed in FASD (Rema and Ebner, 1999; Medina and Ramoa, 2005; Thomas et al., 2007; Medina, 2005; Savage et al., 2002; Medina and Krahe, 2008; Vaglenova et al., 2008). A commonly used model of neuronal plasticity is ocular dominance plasticity (ODP) which is seen in the primary visual cortex of mammals. This type of plasticity has been widely used to study the mechanisms that underlie neural plasticity in general; sharing common mechanisms with learning and memory such as

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dependence on the NMDA receptor (Bear et al., 1990) and the transcription factor cAMP/Calcium-dependent response element binding protein (CREB) (Mower et al., 2002). In rodents ODP plasticity is evident in the binocular zone of the primary visual cortex. In this area, neurons present different degrees of binocularity; from cells that are equally responsive to stimulation of either eye, to cells that respond almost exclusively to one eye (Métin et al., 1988). When both eyes are receiving normal input, the binocular zone is dominated by responses to the contralateral eye. However if the contralateral eye is sutured closed during the critical period of the visual system, which is roughly between postnatal days (P) 19 and 32 in rodents (Cang et al., 2005), the eye dominance of the binocular zone shifts to respond primarily to inputs from the ipsilateral, open eye (Smith et al., 2009). This shift in binocularity is seen in mice after as little as 1 day when a mechanism similar to long term depression (LTD) plays a role in a suppression of contralateral eye inputs (Frenkel and Bear, 2004). After 5 days, mechanisms similar to long term potentiation (LTP) act to increase the strength of the ipsilateral eye inputs (Frenkel and Bear, 2004).

Our group pioneered the use of ocular dominance plasticity to investigate the deficits of neuronal plasticity in FASD (Medina et al., 2003). We showed that exposure of ferrets to alcohol from P10 to P30, a period of development roughly equivalent to the third trimester equivalent of human gestation (and to P4 to P10 in rodents), leads to a persistent impairment of ocular dominance plasticity (Medina et al., 2003; Medina, 2006; Medina, 2005). Importantly, this impairment was reversed if animals were given the phosphodiesterase type 1 inhibitor (PDE1i) Vinpocetine (Medina, 2006). The rationale of using of vinpocetine was to increase cAMP/cGMP levels, which would lead to

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phosphorylation of CREB and other transcription factors such as SRF and ELK-1 (Beavo, 1995; Blokland et al., 2006; Chai and Tarnawski, 2002). The activation of these transcription factors can lead to activity based translation of plasticity related genes (Knoll and Nordheim, 2009).

While the use of the ferret model has proven to be particularly useful to FASD research, the creation of a mouse model would benefit from the growing availability of transgenic animals with mutations in genes related to neuronal plasticity. Here we tested whether exposure to alcohol, in a chronic binge-drinking paradigm, during the third trimester equivalent of human gestation affects ODP in mice. After observing that indeed ODP is disrupted after early alcohol exposure, we tested the efficacy of three different PDEis in reversing this impairment. The choice of PDEis was made based on their capacity to act on cAMP, cGMP or both.

Methods

Animals

Visibly pregnant C57/BL6 female mice were obtained from a commercial supplier (Harlan), and singularly housed in the university animal facility. Pregnant dams were checked twice daily until pups were born. The day of birth was considered P0.

Pharmacological Treatment

Pups received a single injection of 5g/kg of alcohol (25% ethanol in normal saline i.p.) or saline as a control on days P5, 7 and 9. Animals then were allowed an alcohol free period until P20. Beginning at P21 (one day prior to monocular deprivation),

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animals were treated with 20 mg/kg i.p. of vinpocetine (Sigma, St. Louis, MO), 1.25 mg/kg i.p. of rolipram (Sigma, St. Louis, MO), 3 mg/kg oral of vardenafil (Bayer pharmaceuticals) or volume matched vehicle as a control every other day for 10 days, until optical imaging of intrinsic signals was preformed (Figure 7A).

Monocular Deprivation

At P21, 12 days after last alcohol injection, pups were anesthetized using vaporized isoflurane (Baxter, Deerfield, IL). Anesthesia was characterized as absence of the paw withdrawal reflex. Once the animal was anesthetized small portions of the upper and lower right eyelid were trimmed. The eyelids were then sutured together using 4 interrupted sutures of 7.0 Prolene (Ethicon, Inc, Cornelia, GA). The sutured eye was then covered with tissue-glue (CP Medical, Portland, OR. The animal remained monocularly deprived for 10 days, until optical imaging of intrinsic signal experiments were performed.

Optical Imaging of Intrinsic Signals

Optical imaging of intrinsic signals was performed using Imager 3001 VSD+ (Optical Imaging System, Germantown, NY) as previously described, with some modifications (Medina, 2006; Medina, 2005; Antonini et al., 1999). Briefly, mice were anesthetized using 10mg of chlorprothixene (Sigma) and 15g/kg of urethane (Sigma). The scalp was then cut open, and the animal's head attached to a metal plate with a window that allows for the exposure of the skull. The skull was covered with agar (2.5% in normal saline) and a cover slip. The MD eye was then cut open to allow for deprived eye

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Figure 7

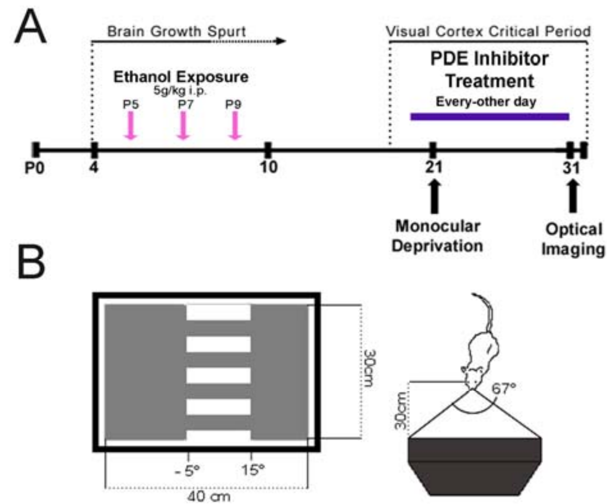

Figure 7. Experimental paradigm. (A) Mouse pups receive a single injection of 5g/kg of ethanol on postnatal days 5, 7 and 9. At P21 animals are monocularly deprived and treated with PDE inhibitors or vehicle every-other-day until P31 when an optical imaging of intrinsic signals is performed. B) Visual stimulus; mice are placed 30cm from a high refresh rate monitor displaying a drifting grating.

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stimulation. An image of the cortical vascular pattern was obtained through the skull by illumination with a green filter (550 nm); this type of image was possible due to the transparency of the mouse skull at this age. Images of intrinsic signals were then obtained by using a red filter (700 nm). Recordings were made using VDAQ (Optical Imaging Inc.) software. Visual stimulation was then performed using a horizontal banding of downward drifting grey and black bars on a 21 inch monitor (Sony Trinitron; Sony) using a SGT+ graphics board and STIM software (Optical Imaging Inc.). Gratings were presented only in the center 12cm of the monitor, which was placed 30cm from the nose of the animal (See Figure 6B). A single trial consisted of this stimulus and a blank screen presented to each eye for 9s in sequence, with data acquisition during the last 8s. Eye shutters (Optical Imaging Inc.) controlled by the acquisition computer were used to stimulate each eye individually. A total of 20 trials were performed for each eye, and the summed images were used to obtain a monocular response map by subtracting responses to a blank screen from responses to visual stimulation of each eye. To obtain a quantitative estimate of intensity, differential maps were mixed with the floating point files clipped at ±2SD from the median. All pixels with a value higher than 133 (256 being white) were excluded as noise. An Ocular Dominance Index (ODI) was calculated for each animal using the average pixel intensity for each eye. Pixel intensity for both the contra- and ipsi- lateral eye signals were calculated by selecting a region of interest based on the summed signal from bilateral eye stimulation. Using this region of interest mean pixel intensity for individual eye input was calculated using the histogram function in Image J (version 1.40g, National Institutes of Health, USA). The following equation

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was used to calculate ODI, where C and I represent average pixel intensities after stimulation of the contralateral and ipsilateral eye, respectively;

$$ODI = \frac{C - I}{C + I}$$

Positive ODI values would indicate a contralateral eye bias (unshifted), and negative values would indicate an ipsilateral eye bias (shifted)(See Figure 8).

Blood Ethanol Concentration

Blood was collected from a separate subset of C57/BL6 pups at P7 via cardiac puncture. Blood was collected at several time points after ethanol injection (0.5h, 1h, 2h, 3h, 4h and 6h), then centrifuged at 4,000 rpm for 5 minutes and serum was collected. BEC was assessed using a commercial kit (333-A diagnostics kit; Sigma, St. Louis, MO).

cAMP and cGMP measures

To prepare samples for the ELISA, P50 mice were injected i.p. with either 3.3mg/kg of sodium nitrite (Fluka AG, Germany), or 1.5mg/kg of forskolin (Sigma, St. Louis, MO) these drugs were used to stimulate production of cGMP and cAMP respectively. The increase of these nucleotides are naturally reduced by the action of phosphodiesterases by converting them to GMP and AMP. In animals given sodium nitrite either 3 mg/kg of vardenafil (Bayer pharmaceuticals), or volume matched vehicle were added to the stimulating drug. In animals given forskolin either 1.25 mg/kg of rolipram (Sigma, St. Louis, MO), volume matched vehicle were added to the stimulating

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Figure 8

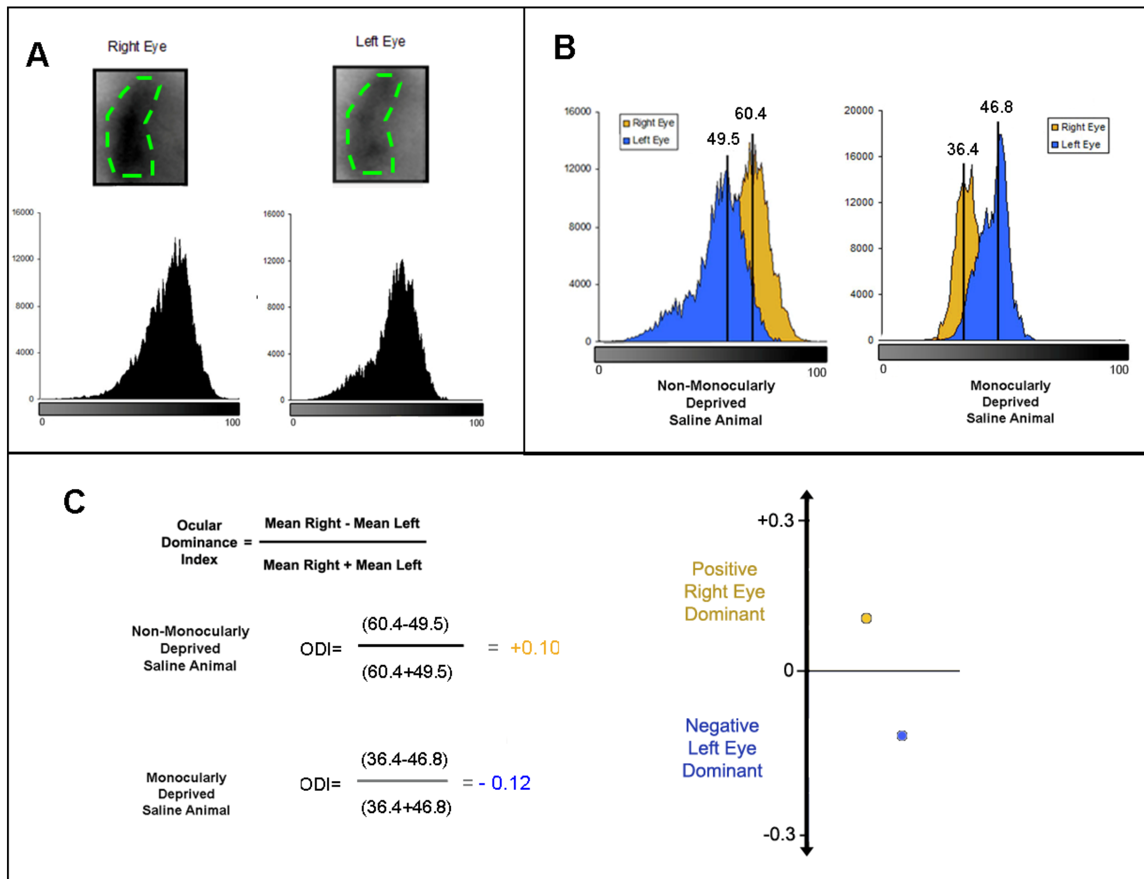

Figure 8. Analysis of optical imaging of intrinsic signals. To analyze the images acquired using OI, first a region of interest (ROI) is created around the binocular zone for each animal. This area is the same for both right and left eye responses (A). Using this ROI, a histogram of the intensity of each pixel is created for each eye. These responses can then be over laid and the mean pixel intensity for each histogram can be calculated (B). These values are then used to calculate an ocular dominance index for the animal. If this value is greater than one there is a bias towards the right eye, if this value is negative (as for an MD animal) there is a bias towards the left eye (C).

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drug. Animals were then sacrificed 1 hour later using vaporized isoflurane (Baxter, Deerfield, IL) and the occipital pole of each brain was collected. One hemisphere was used for cAMP and cGMP measures, while the other was used for Western blot studies. Dection of cAMP and cGMP was performed using a commercially available ELISA kit (Enzo Life Sciences, Farmingdale, NY).

Western Blotting

Tissue was homogenized in an Extraction Reagent (Invitrogen, Camarillo, CA) with a Protenase and Phosphatase inhibitor cocktail (Sigma, St. Louis, MO). Samples were centrifuged and the supernatant was collected. Concentrations were measured using Bradford protein assay with bovine serum albumin as a standard (Bio-Rad, Hercules, CA). 90µg of total protein was resolved by SDS-PAGE (10% Tris-Hcl gel, Bio-Rad, Hercules, CA). Blocks were incubated in blocking buffer (Li-Cor, Lincoln, NE) for 1 hour, then incubated in GAPDH mouse antibody (1:5000, Sigma, St. Louis, MO) for 1 hour at room temperature. Blots were then incubated in phospho-CREB rabbit antibody (1:200, Cell Signalling, Danvers, MA) over night at 4° C. The secondary antibodyes were goat anti-rabbit IRDye 800 IgG and goat anti-mouse IRDye 680 IgG (1:5000, Li-Cor, Lincoln, NE). Blots were detected by Odyssey Imaging System (Li-Cor, Lincoln, NE).

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Results

Blood ethanol concentrations and survival rate

Peak of blood ethanol concentration (BEC) was 411 mg/dl (± 43) at 1 hour after injection. BEC then remained elevated over 300mg/dl for 4 hours (Figure 9). This BEC is consistent with previous studies on maximal neuronal death after postnatal ethanol exposure in mice (Ikonomidou, 2000). The majority of animals survived the ethanol and saline injections with survival rates of 90% (n=64) and 92% (n=33). No animals died during vinpocetine, rolipram, vardenafil or vehicle treatment.

Pup growth

Both treatment groups gained weight during the treatment period, but saline gained significantly more weight, with a weight of 5.5g (± 0.5) on the last day of exposure, than ethanol treated litter mates (4.7g ± 0.75; t-test, $t = -5.26$, $df=98$, $p < 0.0001$) (Figure 9C). At the time of optical imaging there were no difference in weight between ethanol (16.3g ± 1.1) and saline (16.2g ± 0.9) treated groups ($t = 0.47$, $df=14$, $p=0.35$) (Figure 7D).

Ocular Dominance Plasticity in Ethanol Exposed Animals

To quantify the results obtained with optical imaging of intrinsic signals an ODI was created (see methods). An ODI of -1 indicates the animal's binocular zone responds only to simulation of the ipsilateral eye or an ODI of +1 indicactes responses to only the contralateral eye, respectively. An ODI of 0 would indicate the binocular zone responds equally to simulation of the ipsilateral eye and the contralateral eye. The shift

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Figure 9

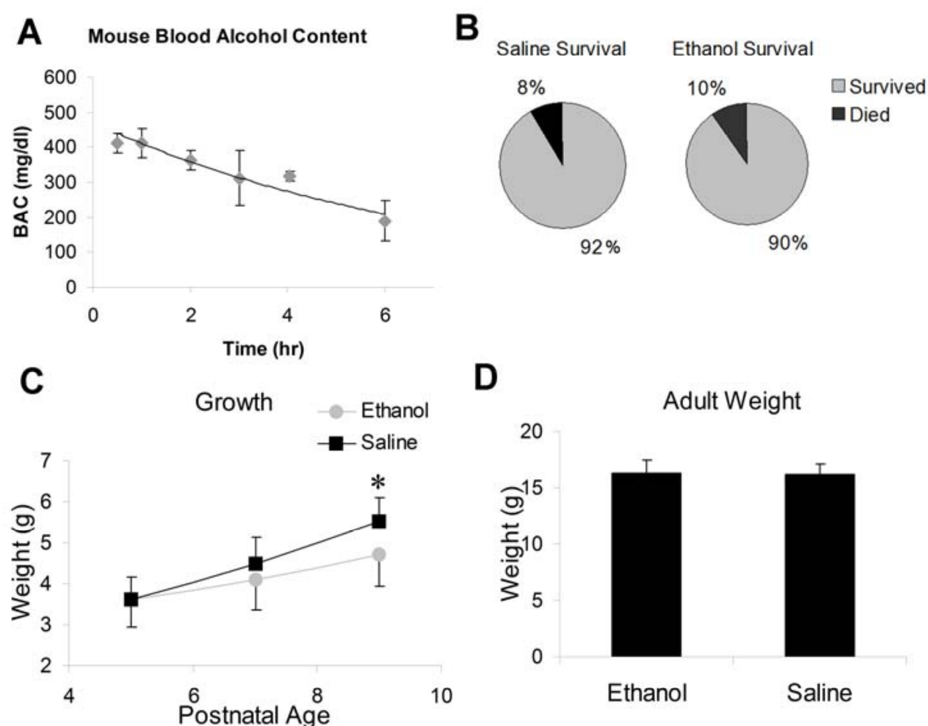

Figure 9. General Effects of Alcohol Treatment. A) Blood alcohol content was measured at various time points post injection in P7 mice. Mice exhibited a sustained BAC above 300 mg/dl for at least 4 hours. B) Saline and ethanol exposed mice exhibited similar mortality rates during the exposure paradigm. C) Mouse pups treated with ethanol gained less weight during the course of exposure than their saline treated littermates (* $p < 0.001$). D) At the time of optical imaging, there was no difference in weight between ethanol and saline treated littermates.

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from positive towards negative values in ODI indicates ODP. As expected non-MD ethanol and saline treated animals demonstrated the typical contralateral bias observed in rodents (ethanol: n = 3, +0.09 ± 0.03; saline: n = 5, +0.08 ± 0.01). An ANOVA showed that there was a significant effect of exposure (saline, ethanol; F= 9.6, df=1, p= 0.007), a trend in the effect of MD (MD, no MD; F= 4.14, df=1, p=0.06) and significant interaction between exposure and MD (F= 7.773, df = 1, p=0.013). The significant effect of exposure indicates that there are significant differences between saline and alcohol treated animals. While, the trend in the effect of MD was most likely due to the lack of OD shift in the ethanol treated animals, and is best shown in the interaction between treatment and MD groups, indicating that MD is necessary to show the effect of ethanol exposure. Post-hoc t-tests showed that while MD lead to significantly lower ODI in saline treated animals (n = 6, ODI = -0.12 ± 0.03, t=5.59, df=7, p < 0.0001), ethanol animals with no MD showed no ODI difference to their MD counterparts (n = 7, ODI = +0.07 ± 0.02, t= -0.39, df=8, p=0.7) (Figure 10).

Rescue of Ocular Dominance Plasticity

After showing that ocular dominance plasticity is impaired in ethanol treated animals, we examined whether treating animals with PDE inhibitors during the period of MD would restore these deficits in. An ANOVA showed an effect of treatment, indicating differences between vehicle and PDE inhibition treatment (F=6.47, df=4, p=0.001). To test what treatments effectively reduced ODI values we used the Dunnett test (< control). Negative ODI values would indicate an OD shift. Animals that received vinpocetine displayed an OD shift (n = 5, -0.06 ± .02), represented by a reduction on

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Figure 10

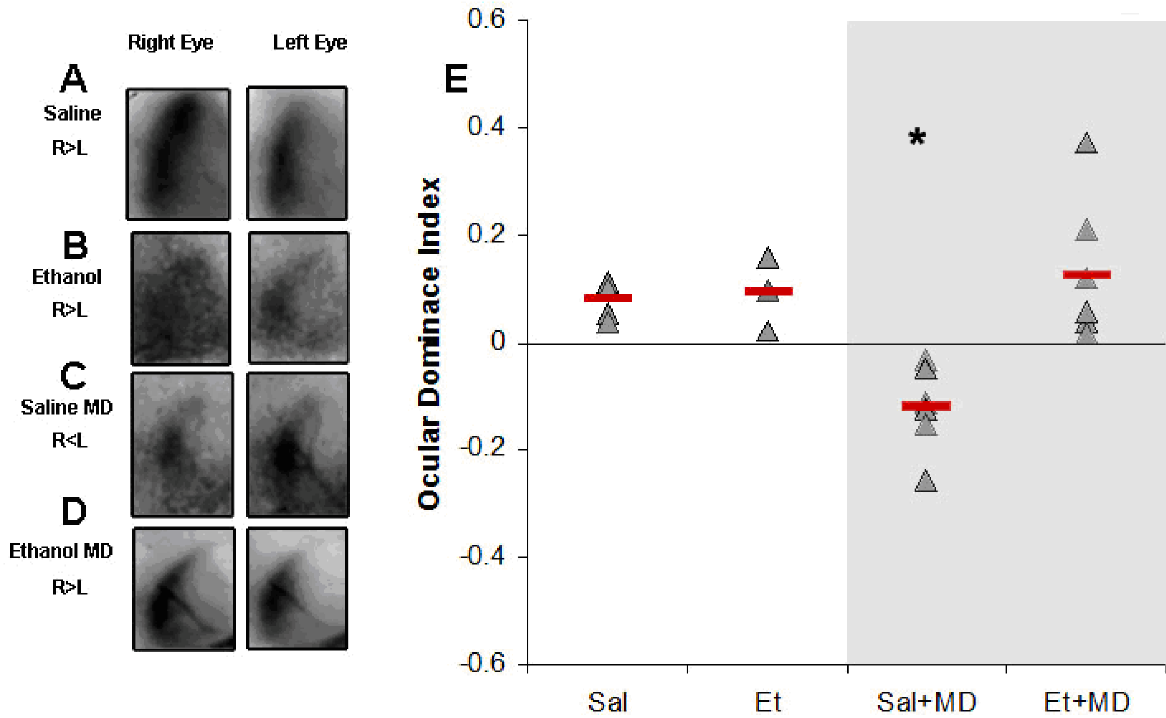

Figure 10. Effect of early alcohol exposure on ODP. A-D) Optical imaging of intrinsic signals from the left visual cortex of representative cases of each group. Darker maps indicated stronger signals after visual stimulation. Note that in non-deprived animals (A and B) stimulation of the right eye leads to stronger signals than left eye stimulation, which is consistent with the contralateral bias seen in the primary visual cortex of rodents. After 10 days of monocular deprivation (MD) an ocular dominance shift is seen; note the stronger signal after left eye stimulation (C). This shift was not seen in ethanol exposed animals (D) which indicates impairment in ocular dominance plasticity. (E) Quantification of optical imaging findings. Ocular dominance indexes were calculated (see methods); positive and negative values represent whether the cortex is more responsive to the contralateral (right) or ipsilateral (left) eye respectively. Red bars represent average ODI values for each group (* $p < 0.0001$) Sal=Saline treated; Et= Alcohol-treated; MD= Monocular deprivation.

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ODI when compared to controls (Dunnet's t = 0.039). In contrast, ethanol exposed animals treated with rolipram showed values not significantly different than controls, indicating persisting contralateral eye dominance (n = 6, +0.07 ± 0.03, Dunnet's t= 0.547). Similarly vardenafil treatment was also unable to restore ODP (n = 5, +0.13 ± 0.07, Dunnet's t= 0.940). Yet, when animals were treated with a combination of vardenafil and rolipram, a significant reduction in ODIs was observed (n = 4, -0.17 ± 0.10, Dunnet's t= 0.001). This result was similar to what was observed with vinpocetine, indicating a requirement of both cAMP and cGMP cascades to restore OD plasticity (see Figure 11). Some saline MD animals were also given vinpocetine treatment during the period of MD, to test for a possible further increase in plasticity, yet they displayed a normal shift in ODI (t-test; t=2.228, df=10, p=0.646; data not shown).

Effect of Vardenafil and Rolipram treatment

To test whether the negative results obtained with rolipram and vardenafil alone were due to failure of PDE inhibition we conducted the following experiment. Animals were given either sodium nitrite or forskolin as stimulators of guanalyl and adenalyl cyclases respectively, along with a PDE inhibitor treatment or vehicle (DMSO). With forskolin stimulation, rolipram treatment was shown to result in a cAMP concentration of 57.60 pM/mL (± 6.9) in the visual cortex; this concentration was higher than that seen in DMSO treated animals (10.99 pM/mL ± 4.5). When sodium nitrite was paired with vardenafil a cGMP concentration of 4.17pM/mL (±0.45) was seen. This concentration was higher than what was seen in DMSO treated animals, which showed a concentration of 2.02pM/mL (±0.23, see Figure 12). Western blotting also confirmed

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Figure 11

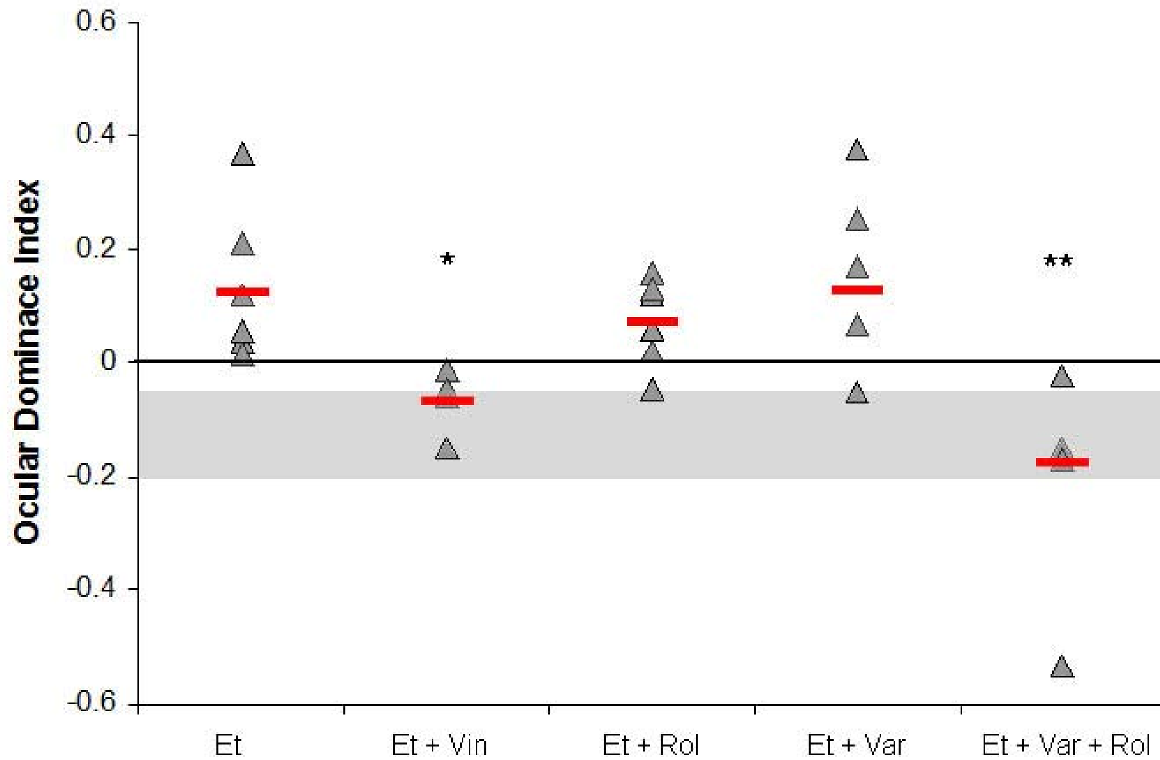

Figure 11. Effect of different types of PDE inhibitors on ODP. Vinpocetine (Vin) restores ODP in alcohol-treated animals (* p = 0.05). This rescue is not achieved by either rolipram (Rol) or vardenafil (Var). However, when vardenafil and rolipram are given together ODP is restored (** p = 0.001). Grey area show range of values obtained from saline-treated animals after a monocular deprivation. Et = alcohol.

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Figure 12

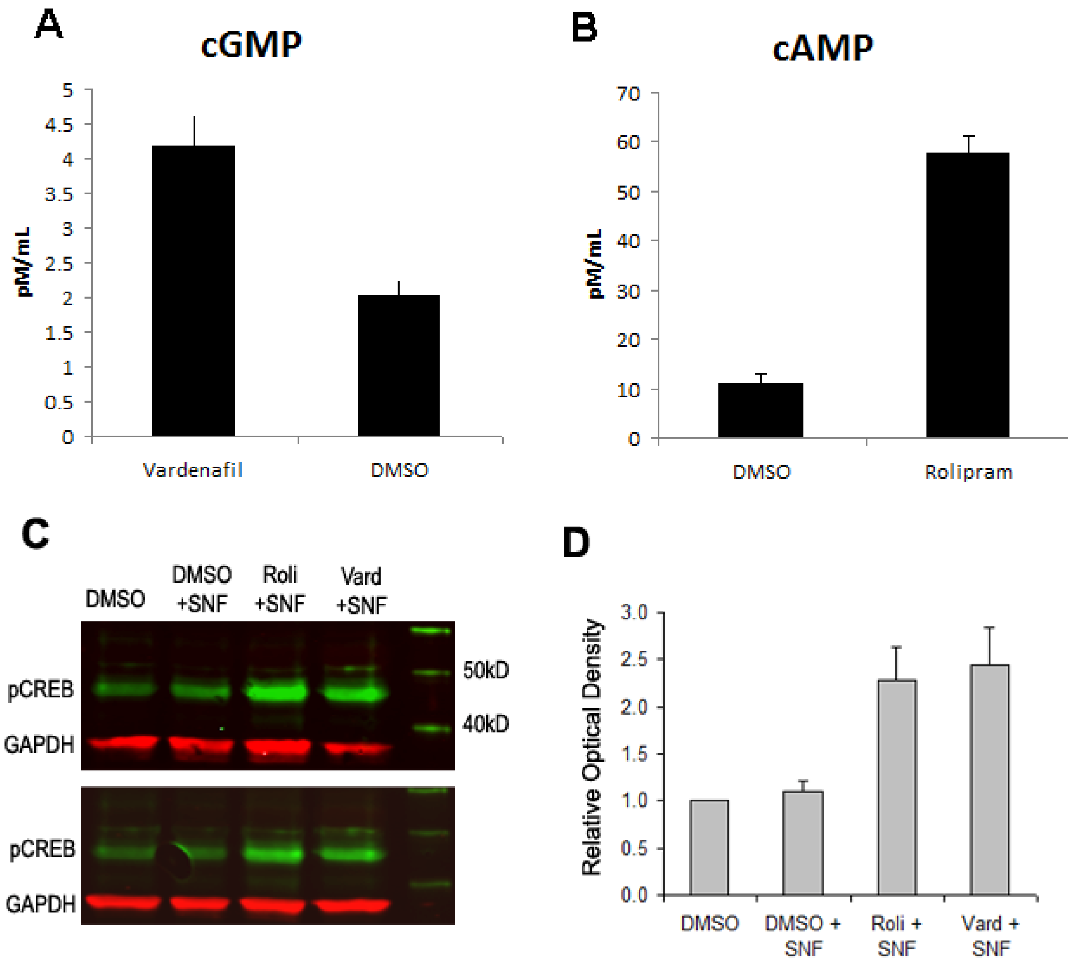

Figure 12. Levels of cAMP, cGMP and pCREB after Rolipram and Vardenafil Treatment. Animals were in injected with either sodium nitrite or forskolin concurrently with a specific PDE inhibitor. Treatment with vardenafil (3mg/kg) (A) or rolipram (1.25 mg/kg) (B) resulted in an increase in cGMP and cAMP in the visual cortex respectively. Western blots show levels of pCREB (green) and GAPDH (red) as loading control (C). Quantification of these two western blots indicates that rolipram and vardenafil both increase levels of pCREB as normalized to the DMSO group.

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these findings (see Figure 12C), demonstrating that both rolipram (2.3 ± 0.35) and vardenafil (2.4 ± 0.39) increase, the levels of pCREB compared to DMSO alone or DMSO with the sodium nitrite/ forskolin cocktail (1.1 ± 0.10). These results indicate that although vardenafil and rolipram treatment alone cannot rescue ODP, they are producing an effect on cyclic-nucleotide concentrations and pCREB levels in V1. In these studies n = 1 for each group, thus a statistical analysis was not performed.

Discussion

Here we have shown that mice exposed to alcohol early in development present impairment in OD plasticity when compared to saline treated controls. We also demonstrated that this impairment in ODP is reversed by a treatment with a PDE1i (vinpocetine). Although this rescue was not seen with a PDE4i (rolipram) or a PDE5i (vardenafil) alone, it was seen with a combination of these treatments. This finding suggests a requirement of the activation of both cAMP and cGMP cascades in order to rescue ethanol induced ODP deficits. But why both of these cascades would be necessary for this effect?

Acting as second messengers in several processes, cAMP and cGMP are particularly important for neuronal plasticity. The cAMP-PKA and the cGMP-PKG pathways have been extensively studied and their importance for neuronal plasticity well documented (Michel et al., 2011; Nugent et al., 2009; Pilz and Broderick, 2005). Interestingly, these pathways can have overlapping functions. For instance, both PKA and PKG can act in parallel to increase CREB phosphorylation and the expression of BDNF and c-Fos (Frank and Greenberg, 1994; Pilz and Broderick, 2005; Lonze and

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Ginty, 2002). In addition, both PKA and the cGMP-dependent enzyme gKII can phosphorylate the GluR1 subunit of the AMPA receptor at serine 845 (Serulle et al., 2007). This phosphorylation contributes to the incorporation of AMPA receptors to the postsynaptic membrane and is crucial for synaptic plasticity (Malinow and Malenka, 2002).

In the present study, while a PDE1 inhibitor successfully restored neuronal plasticity, the use of PDE5 or PDE4 inhibitors did not. It is conceivable that simultaneous activation of the cAMP-PKA and cGMP-PKG cascades leads to a summation of their effects. The restoration of neuronal plasticity observed here may not be accomplished even by using higher doses of PDE5 or PDE4 inhibitors individually due to negative feedback mechanisms (Zhang et al., 2005; Borlikova and Endo, 2009). In fact, it was demonstrated that while increased cAMP and cGMP can facilitate neuronal plasticity, high levels of either one can be ineffective or even disruptive (Barad et al., 1998; Zhang and O'Donnell, 2000). Another possibility to explain our findings is that simultaneous activation of cAMP-PKA and cGMP-PKG lead to the expression of plasticity-related molecules that, while specific for each cascade, might act in synergy.

Despite of not restoring OD plasticity, Rolipram and vardenafil doses used in the present study (1.25 and 3 mg/Kg respectively) were still able to increase cAMP and cGMP levels respectively. Moreover, the doses used here were high when compared with previous studies that tested the effects of PDEis on neuronal plasticity. For instance, Rolipram in doses as low as 0.03 mg/Kg was shown to improve object recognition in rats (Rutten et al., 2006) and object retrieval in monkeys (Rutten et al., 2008). In higher doses (0.5 mg/Kg), rolipram has been shown to improve performance

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in the morris water maze and in the passive avoidance test (Cheng et al., 2010). Studies using vardenafil, observed that doses as low as 1 mg/Kg successfully improved object recognition (Rutten et al., 2009; Prickaerts et al., 2002).

The potential of Vinpocetine to improve neuronal plasticity, have been demonstrated using multiple paradigms. Vinpocetine has been shown to facilitate LTP (Molnar and Gaal, 1992; Molnar et al., 1994), enhance the structural dynamics of dendritical spines (Lendvai et al., 2003), improve memory retrieval (DeNoble, 1987), and enhance performance on cognitive tests in humans (Hindmarch et al., 1991). Vinpocetine was tested in several animal models of FASD. It has been shown to restore neuronal plasticity in the visual cortex of ferrets (Medina, 2006), improve performance in the water maze in rats (Filgueiras et al., 2010), and reduce hyperactivity in mice (Nunes et al., 2011). These findings, allied with our current results suggest that PDE1 inhibition may be useful in improving neuronal plasticity in FASD.

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Chapter 4

[Manuscript 2]

Early Alcohol Exposure Affects the Potentiation but not the Depression Component of Ocular Dominance Plasticity

Introduction

Alcohol consumption during pregnancy can lead to a wide variety of neurologic problems in offspring. These alterations can vary widely, from subtle behavioral changes to severe mental retardation (Hoyme et al., 2005), and are collectively referred to as Fetal Alcohol Spectrum Disorders (FASD). One of the most frequently observed impairments in children with FASD and in animal models of this condition is an alteration in neuronal plasticity (Filgueiras et al., 2010; Kim et al., 1997). For instance, FASD subjects consistently show deficits in learning and memory tests, an impairment that has been also observed in animal models (Engle and Kerns, 2011; Hamilton et al., 2003).

Neuronal plasticity is the capacity of neurons to change their connections in response to environmental or molecular cues, resulting in either a gain or loss of connections. Processes of "gain of function" usually involve synaptic strengthening, long term potentiation (LTP), increase in spine number and sprouting of terminals (De Roo et

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al., 2008; Malinow and Malenka, 2002; Malinow, 2003). In contrast, processes of "loss of function" usually involve synaptic weakening, long term depression (LTD), reduction in spine number and pruning of terminals (Malinow and Malenka, 2002). These two components of neuronal plasticity often act in concert and are essential during development, when circuits are refined (Katz and Shatz, 1996) and throughout our lives, in learning and memory processes (Malinow and Malenka, 2002). A model commonly used to study neuronal plasticity is the manipulation of ocular dominance (OD) in the primary visual cortex using monocular eye lid suture (Hubel et al., 1977; Gordon and Stryker, 1996).

Unilateral eyelid closure for a few days (monocular deprivation; MD) during a period in early development leads to a depression in the responses of cortical cells wired to the deprived eye, as well as a potentiation of responses in cells wired to the experienced (open) eye (Frenkel and Bear, 2004). This type of plasticity, known as OD plasticity, has been widely used to study the general mechanisms that underlie neural plasticity as it involves changes in synaptic strength, spine density and both dendritic and axonal arborization (Smith et al., 2009; Oray et al., 2004). Furthermore, OD plasticity shares common mechanisms with learning and memory such as dependence on the NMDA receptor (Smith et al., 2009) and function of the transcription factor cAMP/Ca^{++} dependent response element binding protein (CREB) (Mower et al., 2002). Another advantage of the use of OD plasticity as a paradigm is the presence of both process of "gain" (potentiation) and "loss" (depression) of function occurring in the same animal as a response to a single manipulation (Frenkel and Bear, 2004). This paradigm has been used as a model to study neuronal plasticity deficits in distinct developmental

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conditions such as FASD (Medina et al., 2003), Fragile X (Dolen et al., 2007), Neonatal hypoxia-ischemia (Failor et al., 2010) and Angelmann Syndrome (Yashiro et al., 2009).

One technique that has been previous used to study plasticity deficits in mice is that of VEPs (Yashiro et al., 2009; Dolen et al., 2007). This technique involves in the implantation of electrodes into the binocular zone of the V1. These electrodes are used to record field potentials evoked in response to a set visual stimulus. The recorded field potential represents the summation of the responses from many neurons, but is primarily composed of responses from layer IV. This layer IV preference has been shown using current density analysis and is thought to be a result of thalamocoritcal connections synapsing on the dendrites of stellate cells (Heynen and Bear, 2001).

In previous studies we demonstrated that mice exposed to alcohol during the third trimester equivalent of human gestation have a permanent impairment in OD plasticity (chapter 3). However, it is unknown whether alcohol affects the potentiation, the depression or both of these components of OD plasticity. To address this question, mice were exposed to alcohol or saline, as a control, during the 3rd trimester equivalent of human gestation. After an alcohol-free period of approximately 2 weeks, visually evoked potentials (VEPs) were recorded before and after MD.

Methods

Animals

Visibly pregnant C57/BL6 female mice were obtained from a commercial supplier (Harlan), and singularly housed in the university animal housing. Pregnant dams were checked daily until pups were born. Day of birth was designated as P0.

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Ethanol Exposure

Pups received a single injection of 5g/kg of alcohol (25% ethanol in normal saline i.p.) or saline as a control on days P5, 7 and 9. According to our previous studies, this protocol leads to blood alcohol levels of 411 mg/dl (± 43) at 1 hour post injection.

Electrode Implantation

Electrodes were implanted in P21 – 22 mice. Mice were anesthetized with i.p. ketamine 120 mg/kg (Bioniche Pharma, Lake Forest, IL) and xylazine 9mg/kg (Akorn, Inc, Decatur, IL). Once anesthetized, 2% lidocaine jelly (Akorn, Inc, Decatur, IL) was applied locally on the scalp at the incision site. Burr holes were drilled at 1.0 mm caudal from bregma, and 2.0 mm lateral from the midline and silver ground electrodes were implanted. Tungsten microelectrodes (FHC, impedance 0.3–0.5 M ohms) were implanted in bilateral burr holes drilled at 3.00 mm lateral of midline and 0.00 mm of lambda, at a depth of 0.43 mm (See Figure 13). Electrodes were secured with cyanoacrylate glue (Elmers, Westerville, OH). To secure the animal's head during recording, a nail (which would be used to fix the animal during recording) was glued over the rostral portion of the skull. After surgery the animal was monitored until recovery of righting reflexes and was then given 0.05mg/kg of bupernorphine (Stokes Pharmacy, Mt. Laurel, NJ) for post-surgical analgesia.

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Figure 13

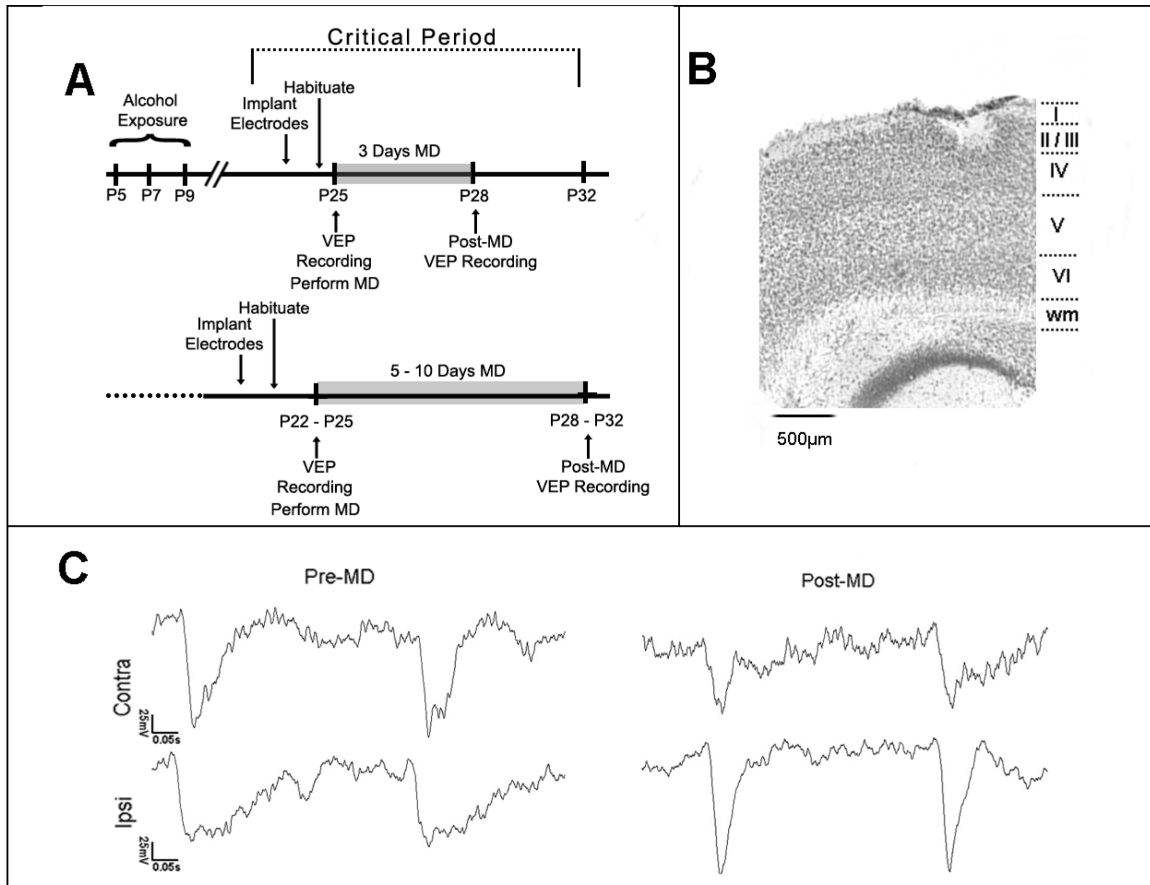

Figure 13. Visually Evoked Potential recordings. Animals used for visually evoked potentials received injections of either alcohol or saline on P5, P7, and P9 (A). Animals were then implanted with recording electrodes on P21, and habituated to the recording apparatus following surgical recovery. Animals were then MD for a short (3 days) period or a long period (5-10 days), after which their MD eye was opened and post-MD VEPs were recorded. Recording electrodes were implanted at a depth of 430 microns, in to layer IV of the visual cortex (B). From these electrodes pre-MD and post-MD VEPs could be recorded after stimulation of the ipsilateral and contralateral eye (C).

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Assessment of Ocular dominance

Awake animals were habituated on the experimental setup for 45 minutes one day prior to the experiment. VEPs were recorded using XCell-3 amplifiers (FHC inc., Bowdoin ME; one for each recording electrode), a 1401 digitizer (CED, Cambridge, England) and Spike 2 software (Cambridge Electronics Design, Cambridge, UK). Xcell-3 amplifiers were set with a low cut-off of 0.1 Hz, and a high cut-off of 100Hz. Visual stimulations were presented to each eye individually using a monitor placed 18 cm from the nose of the animal (mean luminance 27cd/m2, area of 15x31cm) and controlled by a custom program using MATLAB (MathWorks, Natick, MA). Stimuli consisted of full field ordinal sin wave 2 Hz reversing gratings, at 0.05 cycles per degree with 100% contrast. To avoid stimulus response potentiation (Cooke and Bear, 2010), drifting gratings were presented at different angles in the experiments performed before (45°) and after (135°) MD. VEP responses were then averaged from 100 stimulation presentations, and amplitudes were recorded using peak to trough measurements. At the end of the first day of recording, animals were anesthetized using vaporized isoflurane (Baxter, Deerfield, IL) and small portions of the upper and lower right eyelids were trimmed. The eyelids were then sutured and then covered with tissue-glue (CP Medical, Portland, OR). The animal was then returned to the animal colony and remained monocularly deprived for 3, or 5 -10 days, until post-MD VEPs were recorded. Eyelids were checked daily for any sign of opening during the period of MD. After this period, animals were briefly anesthetized using vaporized isoflurane (Baxter, Deerfield, IL), the sutured eye was opened and animals were placed in the experimental setup. Post-MD VEP recordings were done immediately after recovery from light anesthesia. Animals that

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had their eye lids partially opened during the period of deprivation were discarded. We also discarded animals where responses from both eyes decreased or increased more than 2 fold from the pre-MD day of recording.

Acuity Measurements

Acuity measures were recorded 10 days after the assessment of ocular dominance. Six randomized full field reversing sine-wave gratings of 0.5 to 0.02 cycles per degree and an equal luminance grey screen were presented binocularly for spatial frequency acuity. Spatial frequency acuity measures were based on average VEP amplitude of 100 trials. For contrast sensitivity six randomized full field reversing sine-wave gratings with equal luminance and contrasts from 100% to 0% were presented. Contrast sensitivity acuity measures were based on average VEP amplitude of 100 trials.

Results

VEP Amplitude

Pups were exposed to 5g/Kg of alcohol or saline on P5, P7 and P9, mimicking binge alcohol drinking during the third trimester equivalent of human gestation. At P25 ocular dominance was assessed by calculating peak to trough measures of VEPs resulted from stimulation of each eye individually.

Saline treated animals exhibited the expected contralateral eye dominance with average contralateral bias indexes (CBIs, ratio of contralateral/ipsilateral response amplitude of each animal) of 1.6 (± 0.09, S.E.M., n = 12; Figure 14A). In this group, the

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average amplitude of contralateral and ipsilateral eye responses were 197.16mV (± 19.5) and 127.82mV (± 18.46) respectively. Animals exposed to early alcohol exposure demonstrated an average CBI value of 1.5 ± 0.07 (n = 12, Figure 12A) similar to saline controls. Interestingly, early ethanol exposure affected the strength of VEPs as alcohol-treated animals showed significantly lower amplitudes than controls in response to either contralateral (120.17mV ± 8.61, p = 0.001) or ipsilateral (83.31mV ± 7.09, p = 0.013) eye stimulation (Figure 14B).

Acuity

To assess whether our results on ocular dominance plasticity could be influenced by poor visual acuity we recorded VEPs elicited by different spatial frequencies and contrasts. Figure 6A show responses to gratings from 0.02 to 0.50 cycles per degree (cpd). Responses were normalized to the amplitude of the response to 0.02 cpd. Saline and ethanol treated animals demonstrated similar spatial frequency acuity curves with maximal responses at 0.02 and 0.05 cpd, which decreased until responses could not be detected above noise at 0.50 cpd (Figure 15 A). In fact, a repeated measures ANOVA showed no differences between-groups for saline (n = 6) and ethanol (n = 6) exposed animals (F= 0.636, df = 1, p = 0.434), but there was a significant linear effect with-in subjects (F= 331.24, df = 1, p <0.001), indicating differences in response amplitude compared to changes in the cpd for each stimulation. These results are compatible with the spatial frequency acuity responses described for mice (Porciatti et al., 1999).

Despite no change in spatial frequency acuity, we decided to explore contrast sensitivity in saline and ethanol exposed animals. Animals were presented with 0.05

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Figure 14

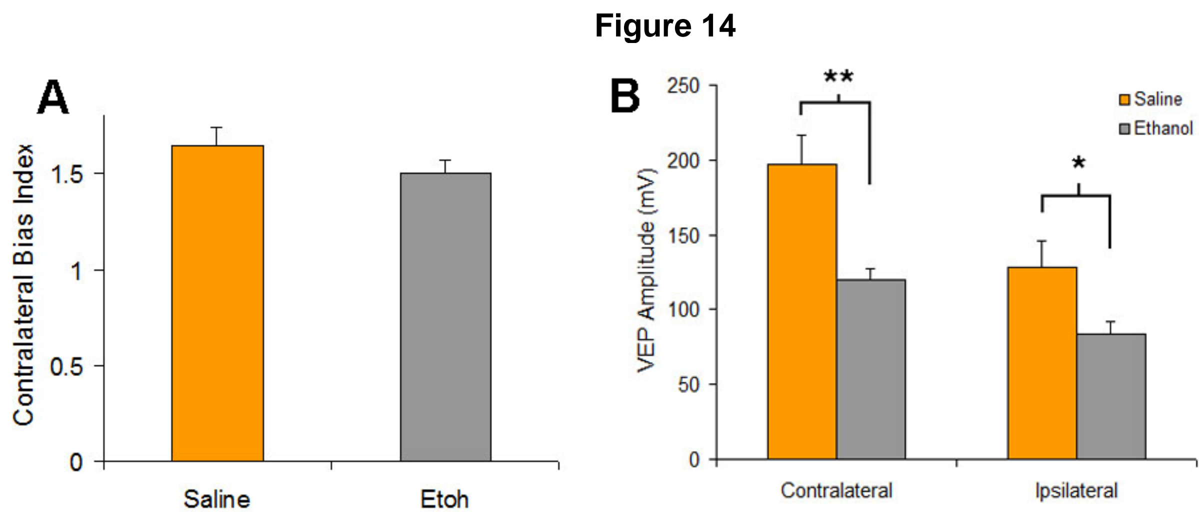

Figure 14. Contralateral Bias Index and Amplitudes of Visually Evoked Potentials. A) Alcohol treatment does not affect the eye dominance in the binocular zone of the visual cortex. Note similarity of CBIs between groups. B) Alcohol treatments affects amplitude of visually evoked potentials. Note that VEPs elicited by either contralateral or ipsilateral eye stimulation resulted in lower amplitude values in the ethanol group (contra, 127.82mV ± 18.46; ipsi, 83.31mV ± 7.09) than the saline group (contra, 197.16mV ± 19.5; ipsi, 120.17mV ± 8.61). * p = 0.013; ** p = 0.001.

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Figure 15

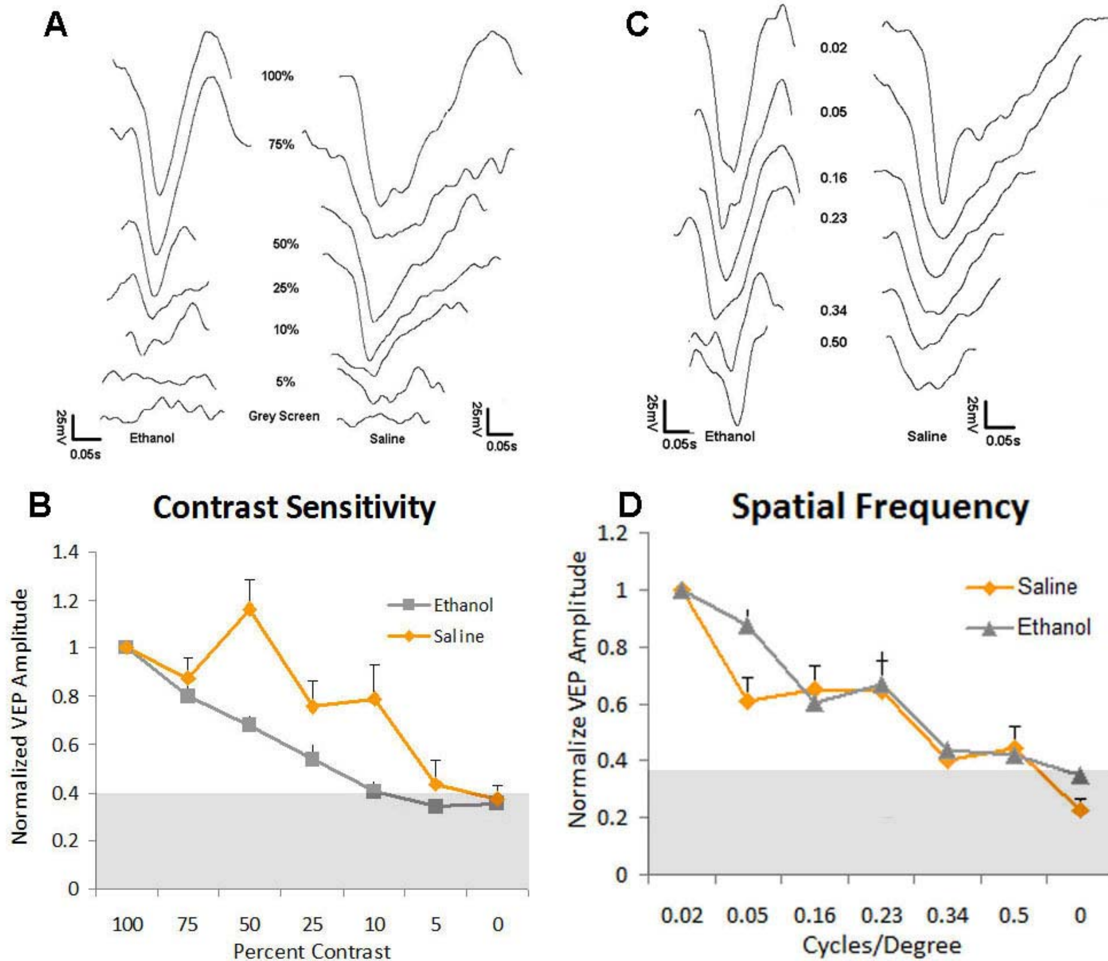

Figure 15. Responses to varying Contrast and Spatial Frequency. (A) Representative VEPs from an ethanol and a saline animal after visual stimulation at different contrasts. As contrast decreases saline treated animals have a slow decrease in responses, showing a small response to 5% contrast, while ethanol treated animals sharply drop off their responses after 100% contrast, with no response occurring at 5% contrast (B). (C) Representative VEP traces from an ethanol and a saline animal after stimulation with different spatial frequencies. Saline and ethanol treated animals showed similar decay in amplitudes after decreasing spatial frequencies were presented. Minimal responses at 0.5 cpd. Grey bar represents noise (D).

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cpd stimuli with different levels of contrast from 100% to 5%. Contrast sensitivity responses were normalized to the amplitude of the response to 100% contrast. Control animals (n = 6) exhibited contrast sensitivity with peak responses occurring at 100% and 75% contrast. Response amplitudes then slowly decreased until there was no response above noise at 5% contrast. In contrast, ethanol treated animals (n = 6) exhibited a precipitous drop off of responses after 75% contrast, with no response detectable above noise at 10% contrast (Figure 15C). When this difference in contrast sensitivity was compared using a repeated measures ANOVA, there was a significant linear effect within subjects ($F= 428.77$, $df = 1$, $p <0.001$), indicating a differences in response amplitude compared to changes in contrast. Moreover, in between groups measures, ethanol exposed animals were shown to be significantly different from their control counter parts ($F= 7.74$, $df= 1$, $p = 0.019$).

Visual Cortex Plasticity

After the initial evaluation of ocular dominance, each saline and alcohol treated animal was monocularly deprived for 5-10 days (see table 2). It is well established that in mice 5 days of MD is sufficient to produce a decrease and an increase in the responses of the deprived and experienced eye respectively (Frenkel and Bear, 2004). At the end of this period, VEPs were recorded to assess the effect of MD on ocular dominance.

After MD saline treated animals exhibited a decrease in CBI values from 1.80 ±0.13 to a CBI of 0.76 ±0.12 (n = 10, $t=7.07$, $df=6$, $p=0.0004$, paired T-tests). This change indicates a shift from contralateral (deprived) to ipsilateral (experienced) eye

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Ethanol

Days of MD	Number of Animals
3 Days	4
5 Days	4
7 Days	1
10 Days	2

Saline

Days of MD	Number of Animals
3 Days	5
5 Days	5
7 Days	1
10 Days	4

Table 2. Number of animals per period of MD use in VEPs

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dominance. A reduction in CBI values was also seen in the ethanol exposed animals, from an average CBI value of 1.46 (±0.10) to 1.04 (±0.07; n = 7, df= 9, t= 3.22, p=0.01). Despite this difference, the average CBI value remained above 1, indicating a continued dominance of the contralateral eye (Figure 16). While MD was able to significantly reduce CBIs in both experimental groups, we wanted to test whether the magnitude of these changes were different. To accomplish this we calculated the percentage of change in CBIs before and after MD for each animal. Figure 16 shows that the reduction in CBI after MD was higher in saline (57% ± 7, S.E.M.) than in alcohol treated animals (26% ± 6; df = 15, t=3.24, p = 0.005).

After examining the changes in CBI, we compared the individual changes in eye responses (see Figure 17). In saline exposed animals both potentiation and depression components of ODP were observed. This can be illustrated by the capacity of MD to reduce contralateral (deprived) eye responses from 156.9mV (±13.8) to 94.21mV (±7.89) and an increase in ipsilateral eye responses from 87.12mV (±4.90) to 131.52mv (±11.37). Paired t-tests indicated that both changes reached statistical significance (contra, t=5.21, df=6, p=0.001; ipsi, t=-3.67, df=6, p=0.01). In the alcohol-treated group we also observed a reduction in contralateral (deprived) eye responses (t=2.96, df=9, p=0.01), with VEPs decreasing from 125.74mV (±13.49) to 96.6mV (± 6.05). However, this change was not accompanied by a significant increase in ipsilateral eye responses (t = -1.16, df=9, p = 0.27) as amplitudes changed only from 89.69mV (± 12.4) to 97.53mV (± 10.26). These findings suggest that early alcohol exposure leads to a remarkable impairment in the potentiation, but not the depression component of ocular

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Figure 16

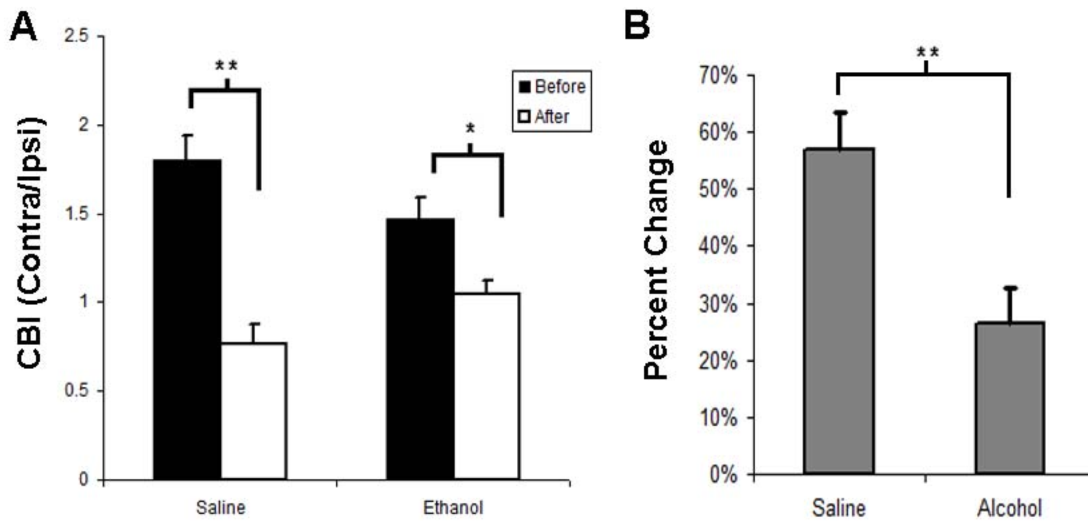

Figure 16. Changes in CBI after 5-10 days of MD. After 5-10 days of MD alcohol and saline treated animals exhibited a decrease in CBI values (A). Yet, saline treated animals exhibited a larger percent change in the CBi from before to after MD (B). * $p = 0.01$ ** $p < 0.001$

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Figure 17

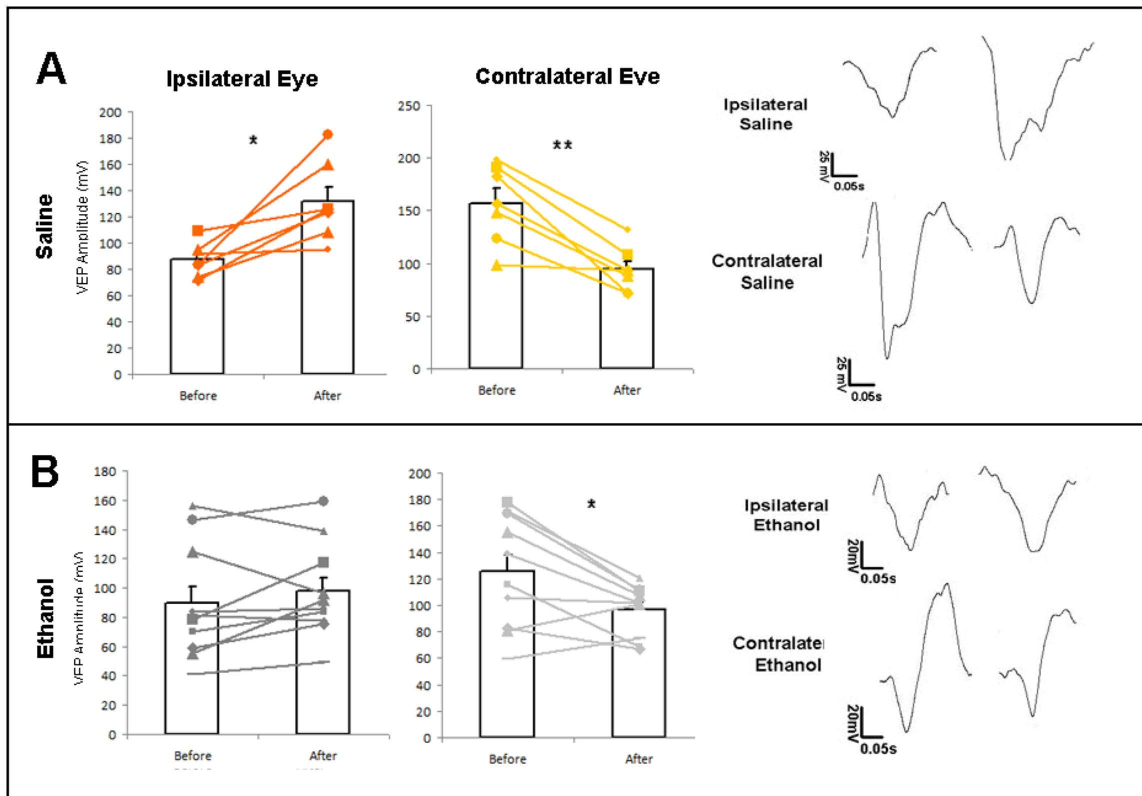

Figure 17. Changes in individual eye responses after 5-10 days of MD. After 5-10 days of MD saline treated animals exhibited a significant increase in ipsilateral eye responses, as well as a significant decrease in contralateral eye responses (A). These bidirectional changes can be seen in traces from a representative animal. In contrast, ethanol exposed animals demonstrated no change in ipsilateral eye responses, yet showed a significant decrease in contralateral eye response (B). * $p = 0.01$ ** $p < 0.001$

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dominance plasticity. This difference appears to be the cause of the smaller CBI changes seen in alcohol exposed animals.

It is well established that the two components of OD plasticity do not have the same time course (Frenkel and Bear, 2004). The potentiation component is observed after 5 days of MD. In contrast, the depression component can start 24 hours after MD and decreased VEPs can be reliably recorded after 3 days (Smith et al., 2009). Therefore, we considered the possibility that alcohol could delay the early stages of the depression component. To test this we recorded from mice after only 3 days of MD. When we examined CBI values for alcohol exposed animals, we observed a significant shift in CBI values in from 1.67 (± 0.09) to 1.07 (± 0.15; $n = 4$, $df=3$, $t=4.71$, $p = 0.01$). This value closely mirrors the CBI changes we see in saline treated animals with 3 days of MD, shifting from 1.62 (±0.21) to 0.94 (±0.08; $n = 5$, $df=4$, $t=4.11$, $p = 0.014$, Figure 18). Indeed when we compare the percent change between pre- and post MD, we saw no significant difference ($df= 8$, $t= -0.46$, $p= 0.65$). These results indicate that after 3 days of MD alcohol and saline treated animals demonstrate a similar magnitude of response changes.

To see if these changes in responses were indeed similar, we looked at the effect of 3d of MD on the individual eye responses. This period of MD caused a decrease in contralateral eye responses in alcohol animals with a shift in contralateral eye amplitudes from 126.5mV (±7.56) to 90.7mV (±4.44; $df=3$, $t=4.15$, $p = 0.02$). On the other hand, the ipsilateral (experienced) eye responses showed no change increase in VEP amplitude, from 77.43mV (±6.09) to 85.01mV (±10.09; $df=3$, $t=-0.04$, $p= 0.96$) after 3d MD (Figure 19). Curiously, in saline treated animals this period of MD resulted in no

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Figure 18

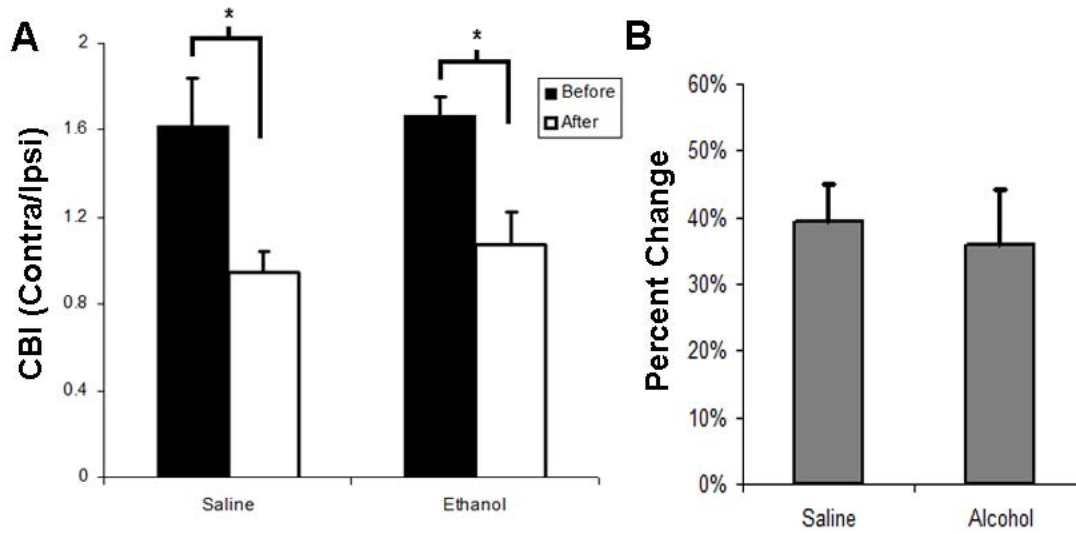

Figure 18. Changes in CBI after 3 days of MD. After 3 days of MD alcohol and saline treated animals exhibited a decrease in CBI values (A). This CBI shift was the similar in both alcohol and saline treated animals (B). * p = 0.01

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Figure 19

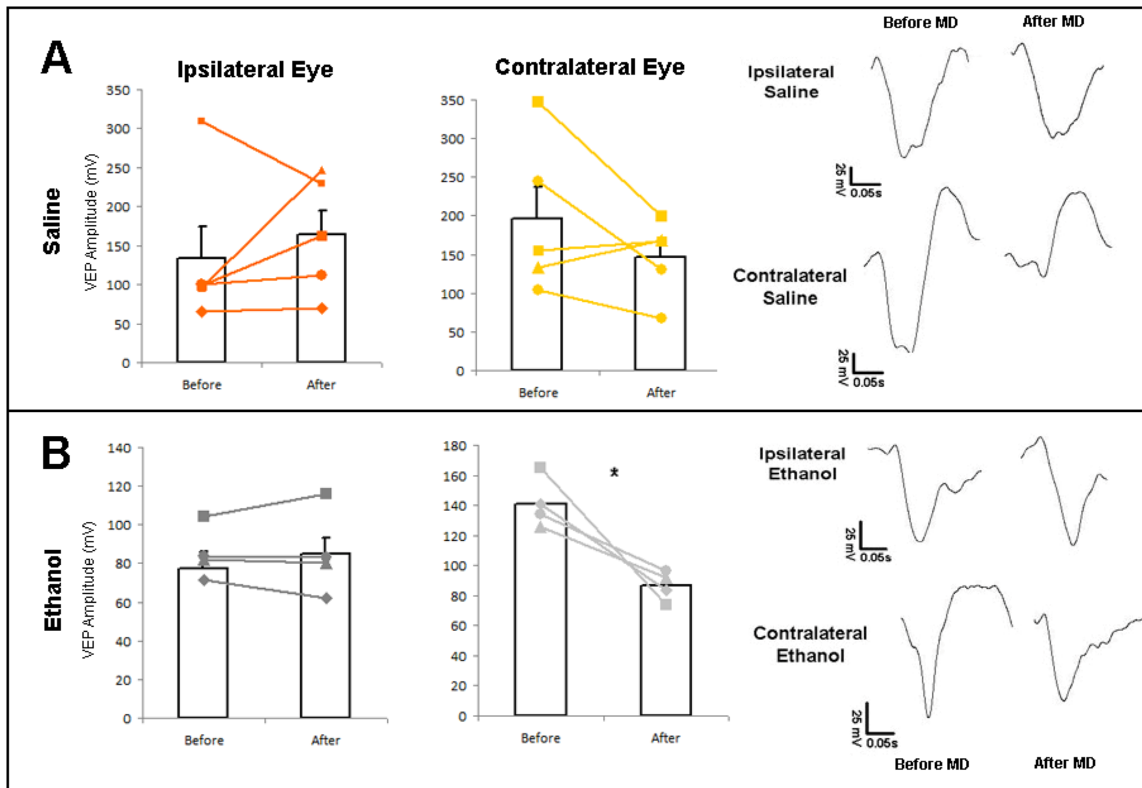

Figure 19. Changes in individual eye responses after 3 days of MD. After 3 days of MD saline treated animals exhibited no change in response amplitude of the ipsilateral eye, yet there was a trend of a decrease in the contralateral eye responses (A). This change in contralateral eye response can be seen in VEP responses from a representative animal. Ethanol exposed animals showed a significant decrease in their contralateral eye responses after MD, and no change in the ipsilateral eye responses (B). Again, this change can been seen in VEP traces from a representative animal. * p = 0.01

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significant change in ipsilateral eye VEP amplitudes, from 133.73mV (±44.35) to 163.75mV (±33.71; df= 4, t= -0.78, p =0.47), or contralateral eye VEP amplitudes, from 169.51mV (±44.3) to 146mv (±22.6; df = 4, t= 1.42, p = 0.22). After analyzing animals individually we observed that the lack of significance was probably due to animals in which both responses increased or decreased. This change in general responsiveness is thought to affect more the 3 days of MD paradigm since this manipulation produces less robust OD shifts than 5 or more days. In fact most studies using 3 days of MD normalize contralateral responses to the ipsilateral ones (Frenkel and Bear, 2004). This normalization is based on the assumption that the ipsilateral eye responses do not change after short periods of MD. This type of normalization cannot be done in the 5 days of MD paradigm as both eye responses change. Figure 20 shows the responses of saline treated animals after normalization, note that all animals presented a decrease in contralateral responses. In summary, our results indicate that early ethanol exposure results in a disruption of the potentiation, but not the depression compoant of OD plasticity, as assessed by VEP recordings.

Discussion

Here we showed that early alcohol exposure affects OD plasticity. This finding, obtained in awake animals, is consistent with our previous results obtained with optical imaging of intrinsic signals in an anesthetized preparation of the same mouse strain (chapter 3). Moreover, we observed that alcohol affects the potentiation, rather than the

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Figure 20

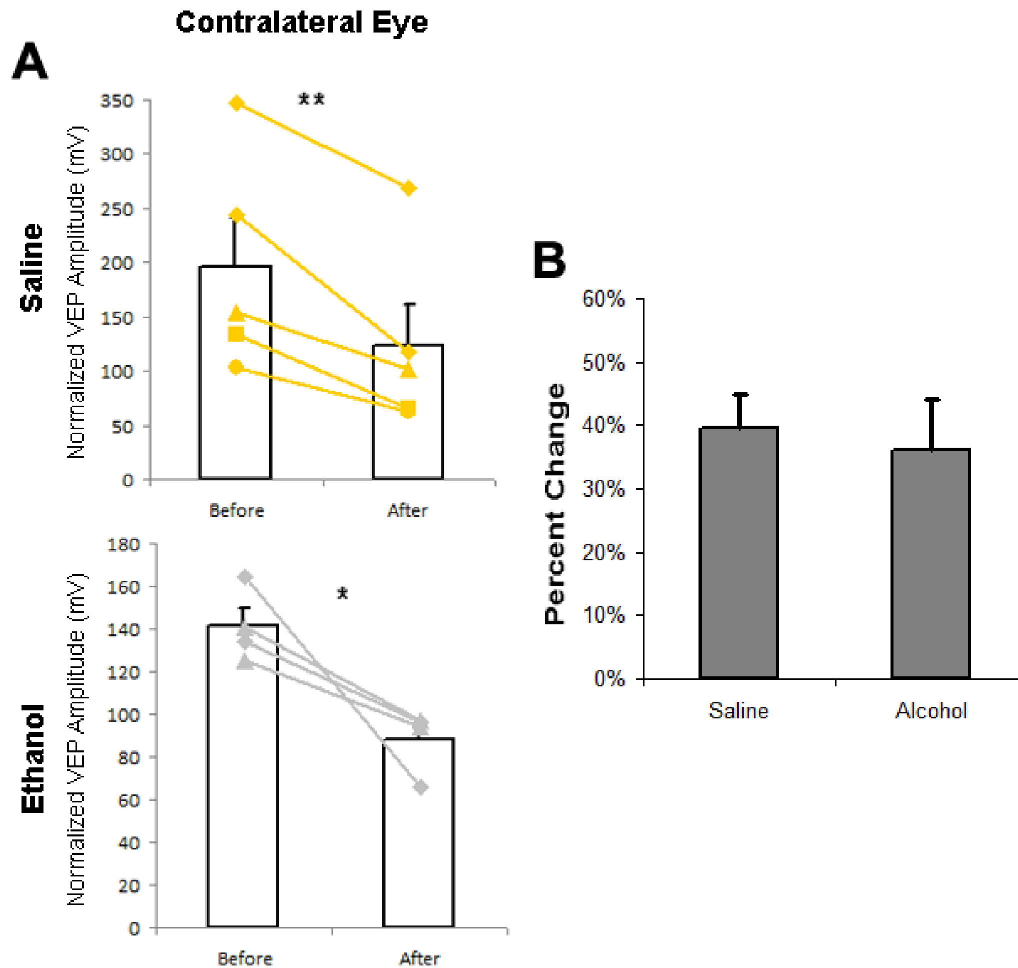

Figure 20. Normalized contralateral eye responses after 3 days of MD. When contralateral eye VEPs are normalized to the ipsilateral eye responses, the decrease in contralateral eye responses for all groups becomes apparent (A). With this data transformation, saline and alcohol treated animals continue to exhibit a similar percent change in VEP amplitude from before to after MD (B). * p = 0.04, ** p = 0.007

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depression component of OD plasticity. This finding may be explained by the different mechanisms underlying each one of these components.

The depression component, which relies on LTD (Smith et al., 2009) begins during the first 24hrs after MD, and is clear after 3 days (Frenkel and Bear, 2004). This effect is temporally and mechanistically different according to cortical layers. For instance, the weakening of deprived eye responses is first observed in layer 2/3, then in layer 4 and lastly in infragranular layers. In layer 2/3, the depression component relies on cannabinoid receptors and is independent of AMPA receptor internalization (Liu et al., 2008). In contrast, in layer 4 the depression component is independent of cannabinoid receptors, and appears to rely on traditional LTD mechanisms, involving clathrin dependent AMPA receptor internalization (Smith et al., 2009; Crozier et al., 2007).

The potentiation component relies on LTP and is consistently detected after 5 days of MD (Frenkel and Bear, 2004). Differently than the depression component, potentiation of the non-deprived eye responses appear to be similar between layers 2/3 and 4, relying on AMPA receptor insertion at the synapse (Heynen and Bear, 2001).

In our findings we observed that early alcohol exposure affects the potentiation but not the depression component of OD plasticity. The fact that VEPs recordings are derived mostly from layer 4 (Cooke and Bear, 2010), where potentiation and depression rely respectively in increased and decreased glutamatergic transmission allow us to hypothesize that our findings might be explained by effects of alcohol on NMDA or AMPA receptors (Rema and Ebner, 1999; Bellinger et al., 2002; Savage et al., 1991).

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Most studies on the effects of early alcohol exposure in LTP and LTD were done in rats using hippocampal slice preparations. Studies using a variety of alcohol doses, regimens and time periods showed unequivocally that alcohol can disrupt LTP in hippocampus (Puglia and Valenzuela, 2010; Izumi et al., 2005; Richardson et al., 2002; Sutherland et al., 1997). Interestingly, in this region, pharmacological potentiation of AMPA receptor responses reverses this disruption (Vaglenova et al., 2008). Surprisingly, few studies looked at the effects of alcohol in LTD. Using the rat hippocampal slice preparation, Izumi and collaborators demonstrated that exposure to alcohol at P0 or P7 (2 injections of 2.5g/Kg s.c.; BEC=~500mg/dl) can disrupt LTD in rats at P30 (Izumi et al., 2005). In contrast, exposure to moderate levels of alcohol (BEC= ~200mg/Kg) during the whole rat gestation did not affect LTD *in vivo* (Titterness and Christie, 2008).

While we demonstrated here with VEPs that early alcohol exposure affects OD plasticity, its effect seems to be less robust than what we previously observed using optical imaging of intrinsic signals. In our previous study, 10 days of MD did not result in any distinguishable change in CBIs in alcohol exposed mice. However, the great difference between the techniques precludes a direct comparison. Nonetheless, the fact that optical imaging of intrinsic signals and VEPs register responses from layers 2/3 and 4 respectively, allow us to speculate that alcohol might affect more the former than the latter. This possibility is also based on the fact that the depression component of OD plasticity has different mechanisms in layer 2/3 (dependent on canabinoids) and in layer 4 (dependent on AMPA receptor internalization) (Crozier et al., 2007).

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We also observed a decrease in VEPs amplitudes in alcohol treated animals when compared to controls. This is the first time that early alcohol exposure has been shown to decrease over-all VEP amplitude in an animal model. Previously, this affect of alcohol has been reported on in a few case studies of humans with FAS, but does not appear to be well studied (Ribeiro et al., 2007). These decreased field potential amplitudes might be a consequence of weak responses of individual neurons or due to a reduced number of cells. The latter seems to be more likely due to dramatic effect of alcohol exposure in triggering neuroapoptosis, especially when exposure is done during the first two weeks after birth (Ikonomidou, 2000). Moreover, previous studies from or lab showed that early alcohol exposure does not change visual responsiveness of individual neurons in the primary visual cortex of the ferret (Medina et al., 2003; Medina, 2005).

Finally, we observed that alcohol-treated animals presented less contrast sensitivity than controls. Saline-treated animals displayed robust VEPs at 10% contrast. On the other hand, in alcohol treated animals VEPs decreased gradually at lower contrasts and at 10% were indistinquishable from noise. Because rod deterioration has a strong correlation with contrast sensitivity (Kolesnikov et al., 2010), it is possible that these findings are due to an effect of alcohol on the retina. Importantly, when stimulus were presented at the optimal contrast (the same used to assess OD plasticity) alcohol-treated animals exhibited normal spatial frequency acuity.

In conclusion, we showed here that early alcohol exposure impairs OD plasticity in layer IV by disrupting the potentiation of the experienced eye responses while sparing the depression of the deprived eye responses. This lack of potentiation was

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accompanied by an over-all decrease in VEP amplitude in alcohol exposed animals. These findings contribute to our understanding of neuronal plasticity deficits in FASD.

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Chapter 5

Conclusions and Future Directions

Here we have successfully translated our ferret model of FASD to the mouse. This translation will give us more options in the investigation of the neuronal plasticity deficits caused by early alcohol exposure. The lower cost of the mouse in comparison to ferrets facilitates using larger samples enabling us to test multiple experimental groups. For instance, the mouse model can be used to test dose-responses curves to assess the minimal alcohol dose that can lead to a similar OD plasticity deficit. Additionally, we could evaluate the minimal effective dose of vinpocetine for the restoration of OD plasticity. This mouse model will also allow us to explore the effects of first and second trimester ethanol exposure. In addition to dosage and timing studies, the use of mice also allows for the study of transgenic animals in order to better tease out the molecular mechanisms behind the damaging effects of early ethanol exposure.

Ethanol and layer specific affects

VEPs have been shown to be comprised primarily of responses from layer 4 neurons (Frenkel et al., 2006; Sawtell et al., 2003). Interestingly, the mechanisms behind the depression of contralateral eye responses varies between cortical layer, with

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layer 2/3 relying on cannabinoid receptors and layer 4 on AMPA receptor trafficking (Liu et al., 2008; Crozier et al., 2007). Therefore it will be important to study the effects of early alcohol exposure on the depression of deprived eye responses in layers 2/3. Our results with OI suggest that the depression component might be impaired in layer 2/3 This hypothesis is based on the two facts: a) optical imaging records mainly from supragranular layers and b) the disruption of OD plasticity by alcohol was much more evident in our study using optical imaging than with VEPs. To test this hypothesis we should conduct single-unit recordings. The use of a multichannel electrode would be particularly important to be able to reliably record from different layers at the same time.

Dissection of PDEi Mechanisms

In these studies, we found that treatment with vinpocetine rescued disrupted ODP in ethanol exposed animals. Yet, treatment with PDEi4 and PDEi5 inhibitors (rolipram and vardenafil) alone had no effect. Interestingly, when rolipram and vardenafil were given concurrently there was a rescue of ODP, similar to vinpocetine treatment. We hypothesize that this recovery is due to the dual increases in intracellular cAMP and cGMP affected by vinpocetine treatment, and the dual treatment with rolipram and vardenafil. Although we were able to demonstrate that rolipram and vardenafil increase levels of cAMP and cGMP, respectively, we do not know whether vinpocetine or the concurrent treatment of rolipram and vardenafil can produce a higher increase of these nucleotides. For these studies it will be necessary to do dose-response curves to establish timing of the peak of cAMP and cGMP levels, a study only possible using a great number of animals. In addition to the timing of peak cAMP and cGMP levels in

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each treatment, the use of mice will also allow us to explore the effect of PDEis on the phosphorylation of transcription factors downstream of cAMP and cGMP such as CREB and SRF.

The use of mice will also make it possible to determine if the rescue of ODP by the dual treatment is simply the result of a sum of the dosages, which could be achieved by increasing the individual dosage of rolipram or vardenafil alone or if the recovery is due to a synergistic affect of both cAMP and cGMP cascades being activated. Supporting the later hypothesis, previous studies of these drugs have shown a ceiling effect of vardenafil and rolipram treatment, where increased dosages result in no increases in learning behavior (Barad et al., 1998; Prickaerts et al., 2002). This ceiling effect is most likely due to intrinsic feedback mechanisms (Zhang et al., 2005; Borlikova and Endo, 2009).

Another promising PDEi1 to be tested is caffeine. Unlike vinpocetine, caffeine has been used extensively in premature infants to improve respiration, ameliorate cognitive deficit and increase survival rate (Stevenson, 2007; Schmidt et al., 2007). In these studies, caffeine's inhibition of the adenosine receptor was only able to account for half of the effects seen on respiration in these children. These additional effects point to the ability of caffeine to act as a PDEi1 when given in higher concentrations (Fredholm et al., 1999). It would then be interesting to explore the use of caffeine as treatment for the rescue of early ethanol induced deficits in OD plasticity, a study that could be accomplished with the use of our mouse model of FASD.

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How does ethanol disrupt ODP?

One of the many advantages of changing to a mouse model is the ability to use transgenics. Through the use of transgenics we could further explore how ethanol is acting to disrupt ODP. One such line of inquiry would be to investigate if alcohol induced alteration of the NR2A/NR2B ratio in NMDA receptors is responsible for the plasticity deficits. The ratio between NR2A and NR2B subunits within the cortex is an important as the two subunits have different kinetic properties. While the NR2A subunit is associated with short duration NMDA mediated postsynaptic currents, while the NR2B subunit is associated with longer lasting currents (Medina et al., 2001). It is the ratio between NR2A and NR2B subunits that have proved to be important during development as well as learning. Previous research has showed that early alcohol exposure results in an increased expression of the NR2A subunit of the NMDA receptor (Honse et al., 2003). Using a mouse which over expresses NR2A we could study if it is the alcohol induced increases NR2A which are responsible for the ODP deficits. Additionally, we could use a knock-out, or knock-down, of NR2A to see if the decreased expression is protective against the deleterious effects of alcohol.

Another transgenic animal that would be interesting to investigate is the BAX knockout mouse. BAX is a pro-apoptotic protein which when activated can trigger the release of cytochrome-c from the mitochondria to trigger apoptosis (Gross, 1998). The BAX knockout mouse lacks the wide-spread neuroapoptosis that is associated with third trimester alcohol exposure (Tehranian et al., 2008). Using this model we could explore if this alcohol induced apoptosis contributes in some way to the ODP deficits, or to the decreased VEP responses.

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The use of VEPs to assess other types of plasticity

Finally, our mouse model allows us to record chronic VEPs, in awake animals. This technique enables us to tease out the individual ipsilateral and contralateral eye responses, as well as allowing for relatively fast (1 hour) assessments of acuity and contrast sensitivity. Using awake animals for these recordings removes anesthesia as a confound which may suppress visual responses or even mask effects of early alcohol exposure.

The use of VEPs could answer many remaining questions about OD plasticity after early alcohol exposure. First, mice have been shown to have OD plasticity outside of the critical period. This adult ODP is limited to only a potentiation of the ipsilateral eye responses, occurring after 5 days of MD (Heynen and Bear, 2001; Frostig and Chen-Bee, 2009). It would be interesting to test if the potentiation component, which was disrupted in young ethanol mice, is intact in adult animals, or if this deficit is persistent throughout life.

Additionally we would like to test whether animals could recover from short periods of MD, if the deprived eye is opened during the critical period. When a normal animal's deprived eye is opened after a short MD, the animal quickly recovers binocularity, a process that does not require protein synthesis (Krahe et al., 2005). As this process involves gain of function in a similar fashion as the potentiation of the experienced eye after MD, we hypothesise that alcohol treated animals will fail to present binocular recovery.

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Another of V1 plasticity that has recently been the subject of study in the mouse V1 is that of stimulus response potentiation. This type of plasticity is characterized by a potentiation of visual responses after repeated presentations of specific visual stimuli (Cooke and Bear, 2010). Using this paradigm we could study if the disrupted potentiation of responses in V1 of alcohol exposed animals is limited to potentiation triggered by MD or if it extends to different types of potentiation.

In conclusion, our creation of a chronic binge -drinking model of FASD in mice has allowed us to further demonstrate that exposure to alcohol during early development causes dramatic changes in neuronal plasticity. The use of this model should contribute to our understanding of how early alcohol exposure disrupts plasticity and what is the best treatment to ameliorate these effects.

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