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Overexpression of Serum Response Factor in Astrocytes Improves Neuronal Plasticity in a Model of Fetal Alcohol Spectrum Disorders

Arco P. Paul
Virginia Commonwealth University

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

By

Arco P. Paul
Bachelor of Physiotherapy, Kolkata, India, 1999
PGDM, Goa, India, 2003

Director: Alexandre E. Medina, Ph.D.
Assistant Professor
Department of Anatomy and Neurobiology

Virginia Commonwealth University
Richmond, VA
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List of contributors

1. Dr. Alexandre E. Medina

   He trained me in performing different kinds of techniques, including alcohol injections, animal surgeries, monocular deprivations, Optical Imaging of intrinsic signals and Single Unit recordings.

2. Fernanda Pohl-Guimaraes

   She helped me get trained with the technique of Optical Imaging of intrinsic signals.

3. Dr. Raymond J. Colello

   He taught me the technique of making primary astrocyte cultures.

4. Weili Wang

   She was involved in performing some of the immunohistochemistries.

5. Crystal Lantz

   She was involved in the manual counting of colocalizations from the z-stacks obtained from the confocal microscope.

6. Dr. Scott Henderson

   He trained me in using the Leica laser confocal microscope

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARBD	Alcohol-Related Birth Defects
ARND	Alcohol-Related Neurodevelopmental Disorder
BDNF	Brain Derived Neurotrophic Factor
bpm	beats per minute
cAMP	cyclic Adenosine Monophosphate
CaMK	Ca^{2+}/Calmodulin-Dependent Protein Kinase
CBI	Contralateral Bias Index
cDNA	complementary Deoxyribonucleic Acid
cGMP	cyclic Guanosine Monophosphate
CNS	Central Nervous System
CREB	cAMP Response Element-Binding
CSPG	Chondroitinsulphate Proteoglycans
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
FAS	Fetal Alcohol Syndrome

FASD	Fetal Alcohol Spectrum Disorder
GABA	Gamma-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GDNF	Glial cell line-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
GFAP	Glial fibrillary Acidic Protein
HBSS	Hanks' Balance Salt Solution
HSV	Herpes Simplex Virus
IEG	Immediate Early Gene
KO	Knock-Out
LGN	Lateral Geniculate Nucleus
LPS	Lipopolysaccaride
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MAP kinase	Mitogen-Activated Protein Kinase
MD	Monocular Deprivation
MMP	Matrix Metalloproteinases
MRTF	Myocardin Family of Transcriptional Cofactor
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
OD	Ocular Dominance
ODI	Ocular Dominance Index

ODP	Ocular Dominance Plasticity
OI	Optical Imaging
P	Postnatal day
PBS	Phosphate Buffered Saline
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
PNN	Perineuronal Nets
RER	Rough Endoplasmic Reticulum
SRF	Serum Response Factor
SVZ	Sub-Ventricular Zone
TCF	Ternary Complex Factor
TNFα	Tumor Necrosis Factor alpha
tPA	tissue Plasminogen Activator
V1	Primary Visual Cortex (area 17)
V2	Secondary Visual Cortex (area 18)
VEP	Visually Evoked Potentials

Abstract

OVEREXPRESSION OF SERUM RESPONSE FACTOR IN ASTROCYTES IMPROVES NEURONAL PLASTICITY IN A MODEL OF FETAL ALCOHOL SPECTRUM DISORDERS

By **Arco P. Paul**
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Major Director: Alexandre E. Medina, Ph.D.
Assistant Professor, Department of Anatomy and Neurobiology

Neuronal plasticity deficits underlie many of the neurobehavioral problems seen in Fetal Alcohol Spectrum Disorders (FASD). Recently, we showed that third trimester alcohol exposure lead to a persistent disruption in ocular dominance (OD) plasticity. For instance, few days of monocular deprivation results in a robust reduction of cortical

regions responsive to the deprived eye in normal animals, but not in ferrets exposed early to alcohol. This plasticity deficit can be reversed if alcohol-exposed animals are treated with a phosphodiesterase type 1 (PDE1) inhibitor during the period of monocular deprivation. PDE1 inhibition can increase cAMP and cGMP levels, activating transcription factors such as the cAMP response element binding protein (CREB) and the Serum response factor (SRF). SRF is important for many plasticity processes such as LTP, LTD, spine motility and axonal pathfinding. Here we attempt to rescue OD plasticity in alcohol-treated ferrets using a Sindbis viral vector to express a constitutively active form of SRF during the period of monocular deprivation. Using optical imaging of intrinsic signals and single unit recordings we observed that overexpression of a constitutively active form of SRF (Sindbis SRF+), but neither its dominant negative (SRF-) nor GFP, restored OD plasticity in alcohol-treated animals. Surprisingly, this restoration was observed throughout the extent of the primary visual cortex and most cells infected by the virus were positive for GFAP rather than NeuN. Hence, we further tested whether overexpression of SRF exclusively in astrocytes is sufficient to restore OD plasticity in alcohol-exposed ferrets. To accomplish that, first we exposed cultured astrocytes to the SRF+, SRF- or control GFP viruses. After 24h, these astrocytes were implanted in the visual cortex of alcohol-exposed animals or saline controls one day before MD. Optical imaging of intrinsic signals showed that alcohol-exposed animals that were implanted with astrocytes expressing SRF, but not SRF- or GFP, showed robust restoration of OD plasticity in all visual cortex. These findings suggest that overexpression of SRF exclusively in astrocytes can improve neuronal plasticity in FASD.

Chapter 1 - Background and Significance

I. Mechanisms of Ocular Dominance plasticity

Neuronal plasticity is the lifelong ability of the brain to make and break connections. This neuronal feature is crucial for the refinement of circuits that occur during development, for recovery after lesions and for learning and memory processes. Plasticity can be elicited by molecular cues or by activity (Shen and Cowan, 2010; Katz and Shatz, 1996). Activity-dependent plasticity is particularly important in the development of sensory maps (Inan and Crair, 2007; White and Fitzpatrick, 2007; Strotmann and Breer, 2006; Jones, 1990; Feldman, 2009). Alterations in visual, auditory of somatosensory experience during development can have a striking impact in the formation of sensory systems. A good comprehension of the mechanisms underlying activity-dependent neuronal plasticity is important not only for a better understanding of brain function, but also for the development of therapeutic interventions to ameliorate neurological problems.

Among the different sensory areas of the brain, the visual cortex is probably studied the most. Its circuitry has been well described and mechanisms of plasticity in the visual system are extensively investigated (see reviews in (Rauschecker, 1991;

Gordon, 1997; Berardi et al., 2003; Tropea et al., 2009; Prasad and Galetta, 2011). In the visual cortex of higher mammals such as the ferret, cats, monkeys and humans, eye specific afferents arrive in layer IV in a segregated fashion, forming the ocular dominance (OD) columns (Wiesel et al., 1974; Shatz and Stryker, 1978). These columns are anatomically and functionally dominated by projections from each eye. An overview of the visual system and OD columns is presented in Figure 1A. Accordingly, the center of a column has neurons that are responsive to a stimulus from a particular eye. However, in the border between columns, neurons can be driven by stimulation of either eye. Alteration of visual experience can dramatically affect the formation of such columns. For example, a misalignment of the eyes during infancy (i.e., exotropia) can lead to OD columns with sharper edges and a dramatic reduction in the number of binocular neurons (Lowel, 1994; Hohmann and Creutzfeldt, 1975; Distler and Hoffmann, 1991). If this misalignment is corrected at a later age, and after a “critical period" (which is approximately between birth and seven years of age in humans (Berardi et al., 2000)), corrective surgeries will not result in an increase in number of binocular cells (Raina and Wright, 1995). This condition is called strabismus and results in deficits in stereopsis and depth perception (Distler and Hoffmann, 1991; Steinbach, 2003). If instead of a misalignment, an eye is completely deprived of visual experience the results can be even more dramatic. One example is the case of unilateral congenital cataract (Ellemberg et al., 2000; Ejzenbaum et al., 2009; Chan et al., 2012; Timney, 1983). The monocular deprivation (MD) of visual inputs can make the columns connected to the deprived eye to shrink and the ones connected to the experienced eye to expand (Figure 1B). If the cataract is not removed during the critical period, the vision

of that eye will have poorly developed acuity resulting in a condition called amblyopia (Lloyd et al., 2007; Ostrovsky et al., 2006).

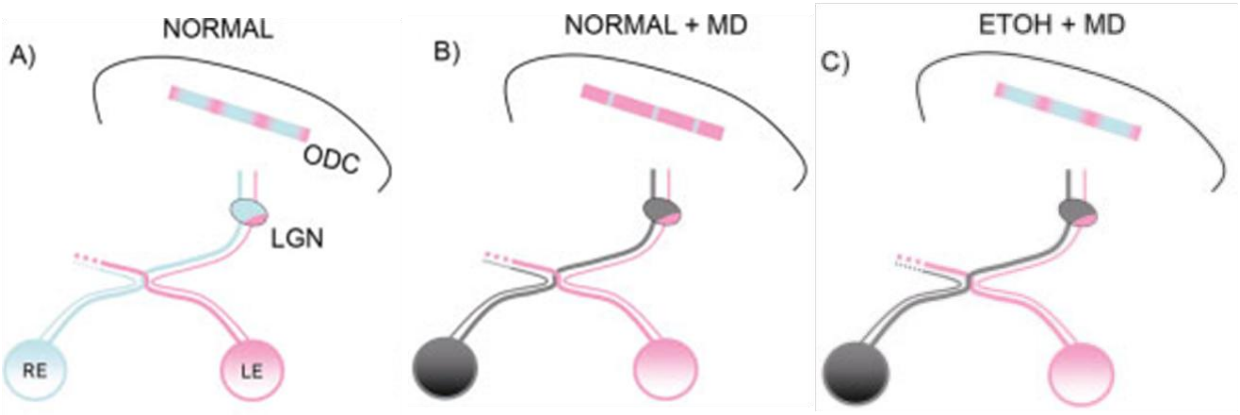


Figure 1. The visual system and Ocular Dominance (OD) plasticity. **A**, In normal ferrets, eye-specific afferents go from the right eye (RE) and left eye (LE) via the lateral geniculate nucleus (LGN) into the primary visual cortex forming OD columns (ODC). **B**, A few days of MD (dark circle) results in shrinkage of the deprived eye (blue) columns and expansion of the experienced eye (pink) columns. **C**, Alcohol exposure (ETOH) during the third trimester-equivalent of human gestation impairs this plasticity effect preventing loss of responses from the deprived eye.

In the sixties Hubel and Wiesel established the experimental model of OD plasticity in cats and monkeys using eyelid sutures to mimic the alterations in visual experience (Hubel and Wiesel, 1970; Hubel et al., 1977). Since then, OD plasticity has been demonstrated physiologically and anatomically in different mammals including ferrets, tree shrews and rodents (Issa et al., 1999; Florence and Casagrande, 1986; Hofer et al., 2006). Although rodents do not have OD columns, they still display OD plasticity since a monocular deprivation in rats and mice lead to an increase and decrease of visual cortex responses after stimulation of the experienced and deprived

eye, respectively (Frenkel and Bear, 2004; Guire et al., 1999; Tropea et al., 2006). The prevailing theory to explain activity-dependent plasticity suggests that mechanisms exist to strengthen synapses, the activity of which coincides with postsynaptic depolarization beyond some threshold level, and to eliminate synapses, the activity of which is not correlated with postsynaptic activation (Katz and Shatz, 1996; Stent, 1973; Spatz, 1996). According to this feedforward Hebbian theory, a competition between right and left eye inputs would regulate synaptic strength. However, studies using rodents suggested that this theory cannot account for all changes observed during OD plasticity. The OD plasticity seen in adult animals occurs solely due the potentiation of responses to open eye and there is hardly any reduction of deprived-eye responses (Sawtell et al., 2003). Also, the binocular neurons maintain their original level of excitability following MD and monocular neurons receiving inputs only from the deprived eye increased their excitability (Mrsic-Flogel et al., 2007). Additionally, the deprived weak eye gets stronger once binocular vision is restored during the critical period (Malach et al., 1984). According to the Hebbian theory, persistent reduction or increase in visual activity should lead to unrestrained weakening or strengthening of neuronal drive, respectively. Since these later findings could not be explained just by simple feed-forward processes, additional homeostatic mechanisms needed to be uncovered. Homeostatic plasticity is a negative feedback mechanism that neurons use to offset excessive excitation or inhibition in order to keep the cellular and network excitability in check for appropriate information processing. These homeostatic mechanisms have been observed in various *in-vivo* and *in-vitro* models (Bienenstock et al., 1982; Bienenstock et al., 1982; Kirkwood et al., 1996; Philpot et al., 2003; Turrigiano and Nelson, 2004). As per current

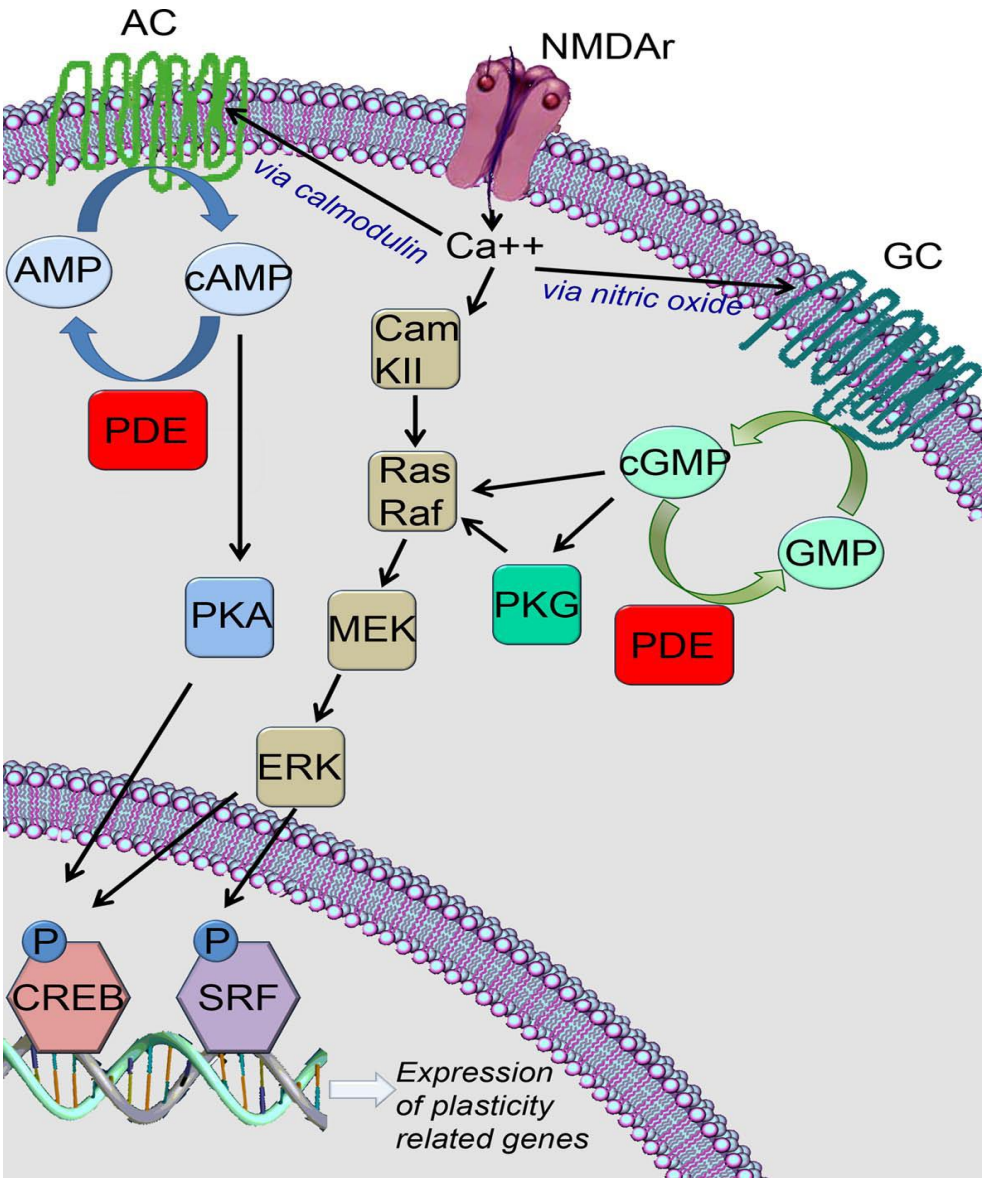
knowledge, OD plasticity is the manifestation of a combination of feed-forward Hebbian and feed-back homeostatic mechanisms working together.

Feed-forward excitatory mechanisms

Numerous studies have suggested that the MD-induced depression of the deprived-eye responses and potentiation of the open-eye responses are due to LTD and LTP, respectively, occurring *in- vivo* (Frenkel and Bear, 2004; Heynen et al., 2003; Kirkwood et al., 1995; Yoon et al., 2009). It has already been shown that ocular dominance of neurons can be modified artificially by pairing visual stimulus with neuronal activation, just like LTP and LTD can be induced *in vitro* by synchronous and asynchronous stimulation of pre- and post-synaptic neurons (Fregnac et al., 1988). This model has gained strength as both OD plasticity and LTP/LTD share similar molecular pathways, such as the activation of NMDA receptors (Sawtell et al., 2003; Roberts et al., 1998; Kirkwood and Bear, 1995). The ability of the NMDA receptors to induce visual plasticity depends on the state of excitatory currents and calcium influx through it, which is determined by the developmental stage of its subunit composition. The peak of the NR2A/NR2B ratio coincides with the end of the critical period for juvenile OD plasticity and it can be reopened by manipulating visual experience in adults which lowers the ratio back to the developmental stages (Quinlan et al., 1999; Chen and Bear, 2007).

The molecular signals that mediate this experience-dependent plasticity are very complex. The calcium influx following the activation of NMDA and AMPA receptors triggers many signaling cascades. A number of studies have demonstrated that the activation of many calcium-induced protein kinases is critical for the OD shift. Some of

the kinases that are essential for visual plasticity are cAMP-dependent protein kinase A (PKA), extracellular signal-regulated kinase (ERK) and calcium/calmodulin-dependent protein kinase II (Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002). These activated kinases can act in two ways - they can either induce rapid short-lived changes in synaptic efficacy by directly phosphorylating receptors in the cytoplasm near synapses or promote late long-lasting changes in neuronal circuitry by signaling to the nucleus to mediate plasticity-related gene transcription and protein synthesis. There is considerable overlap and cross talk among the various pathways involving these kinases (Figure 2) and many of these converge to activate a transcription factor, cAMP response element binding protein (CREB) (Impey et al., 1996; Impey et al., 1998; Sgambato et al., 1998). CREB is now well known to be important for the cellular processes of plasticity and memory (Alberini, 2009; Silva et al., 1998). In fact, blockade of CREB disrupts OD plasticity in the ferret (Mower et al., 2002). Another transcription factor that is present downstream of these plasticity pathways is Serum Response Factor (SRF) (Chai and Tarnawski, 2002; Knoll and Nordheim, 2009). Although CREB and SRF have been known to share many neuronal functions including activity-induced gene expressions, SRF has only recently gained importance as a factor important for plasticity (Knoll and Nordheim, 2009; Etkin et al., 2006).



(Courtesy Medina AE, 2011)

Figure 2. The plasticity-related signaling pathways. The cascades that lead to the expression of many plasticity-promoting genes. Influx of calcium following activation of NMDA receptors triggers cAMP and cGMP cascades, which in turn activate transcription factors like CREB and SRF via various kinases.

Along with visual activity, neurotrophins are also known to contribute to normal visual development and plasticity (Huberman and McAllister, 2002; Berardi et al., 1999; Caleo et al., 1999). Both overexpression and blocking experiments have shown that neurotrophins like nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are important for development and plasticity of the visual system (Huberman and McAllister, 2002; Cabelli et al., 1997; Berardi et al., 1994; Kovalchuk et al., 2002). When coupled with electrical activity, neurotrophins have also been shown to exert both fast actions (by depolarizing neurons directly) and slow actions (by regulating gene expression) (Berardi et al., 2003; Kafitz et al., 1999; Poo, 2001). Interestingly, localized BDNF application, when coupled with weak synaptic activity, leads to strong CREB-mediated gene expression and long-lasting changes in synaptic efficacy, suggesting neurotrophins probably activate molecular pathways similar to those that are present downstream of the glutamate receptors (Berardi et al., 2003; Hu et al., 1999).

Feed-forward inhibitory mechanisms

In addition to the excitatory connections, intracortical inhibition plays a pivotal role in shaping the development and plasticity of the visual cortical circuitry. A certain level of inhibition has been shown to be necessary for the development of the columnar architecture in the developing visual cortex (Hensch and Stryker, 2004). The critical period for OD plasticity also seems to be controlled by GABA-mediated inhibition. An inhibitory threshold needs to be surpassed to start the OD plasticity during the onset of the critical period, as shown by reduced plasticity in the transgenic mice lacking the GABA-synthesizing enzyme GAD65 (Hensch et al., 1998). Premature development of

inhibition by overexpressing BDNF or by administering benzodiazepines results in early opening and closure of the critical period, suggesting the existence of another higher inhibitory threshold that causes critical period closure (Huang et al., 1999; Fagiolini and Hensch, 2000). But even in adulthood, when the cortical inhibition is at its matured state, OD plasticity can be re-induced by fluoxetine treatment or environmental enrichment, which brings the level of GABAergic inhibition down (Maya Vetencourt et al., 2008; Baroncelli et al., 2010). Finally, fast-spiking basket cells, a type of inhibitory neurons, have been shown to induce a form of LTP of inhibition following MD, suggesting a feed-forward mechanism of deprived-eye response depression (Maffei et al., 2006).

Feedback homeostatic mechanisms

In order to better explain the mechanism of the OD shift, the BCM model was developed (Bienenstock et al., 1982; Clothiaux et al., 1991). According to this theory, the open-eye response potentiation following MD is a form of feedback mechanism dependent on the feed-forward depression of the deprived-eye responses. The reduced cortical activity caused by eye-lid closure elicits a downward adjustment in the threshold to elicit LTP in the post-synaptic neuron. This theory proposes that a neuron tries to maintain homeostasis of its firing rate in the face of changing synaptic drives by sliding the threshold for LTP up or down. So reduced or increased level of activity makes a neuron, respectively, more or less plastic (metaplasticity). Various groups have suggested that a reduction in the NR2A/2B ratio of NMDA receptors following reduced

activity is responsible for this downward displacement of the threshold for LTP (Philpot et al., 2007; Monyer et al., 1994).

An additional mechanism of feedback homeostatic plasticity, demonstrated both *in vitro* and *in vivo* in various areas of the brain, is synaptic scaling (Turrigiano and Nelson, 2004; Turrigiano, 2008). It also states that cortical neurons have a preferred average firing rate, and any change in network activity that perturbs this firing rate causes the neuron to regulate the strengths of all its synapses up or down proportionally without changing the relative synaptic weights. This brings the firing rates back to the preferred levels, thus providing a mechanism for network stability during developmental and learning-related plasticity processes. In contrast to the traditional BCM concept, which is based on homosynaptic changes, there is a global (heterosynatic) change in responsiveness in scaling. Current evidence suggests that neurons can detect changes in their own firing rates by sensing changes in calcium influx using calcium-dependent kinases (like the CaMK family), which can then regulate the trafficking of glutamate-receptors (specially the AMPA receptors) at synapses (Thiagarajan et al., 2002; Lissin et al., 1998). Many different classes of molecules have been shown to influence synaptic scaling, including BDNF, TNFalpha (a cytokine) and Arc (an immediate early gene) (Rutherford et al., 1998; Stellwagen and Malenka, 2006; Shepherd et al., 2006). It remains to be seen if they work independently or are part of a complex network of signaling pathways interacting with each other.

Structural changes during plasticity: the role of extracellular environment

As mentioned before, studies in different sensory areas have shown that sensory deprivation dramatically affects neuronal function and morphology. For instance, in rodents whisker trimming reduces protrusive spine motility in deprived regions of the barrel cortex (Lendvai et al., 2000). In the visual cortex, the dendritic spines were imaged *in-vivo* during the critical period following MD of the contralateral eye (Oray et al., 2004). Here, spine motility (measured as µm/min) was found to be significantly higher than in non-deprived animals. Additionally, robust pruning of dendritic protrusions was also observed in the binocular visual area following MD during the critical period (Mataga et al., 2004). These spine dynamics are to a great extent regulated by factors that are present in the extracellular matrix. Tissue plasminogen activator (tPA) is an extracellular protease whose activity increases during MD (Mataga et al., 2004; Mataga et al., 2002). tPA has many targets including the extracellular matrix proteins and cell-adhesion molecules (Gravanis and Tsirka, 2005; Endo et al., 1999; Nagai et al., 1999). Attenuation of MD-induced OD shift following tPA inhibition (Mataga et al., 2002) suggests that tPA degrades the extracellular matrix structures to permit structural reorganization of the synaptic connections. Chondroitin-sulfate proteoglycans (CSPGs) are a class of extracellular matrix proteins that form perineuronal nets (PNNs) around neurons in the adult CNS and hinder neurite outgrowth (Karetko and Skangiel-Kramska, 2009). Additionally, the maturation of PNNs coincides with the closure of the critical period, suggesting the role of PNNs in restricting OD plasticity in adults (McRae et al., 2007). This possibility is bolstered by the fact that degradation of PNNs by using

enzyme chondroitinase ABC reactivates the OD shift following MD in adult animals (Pizzorusso et al., 2002).

Role of Glia: Astrocytes, the new cellular processors of plasticity

A final consideration needs to be given to the emerging role of glia in neuronal plasticity. Astrocytes have become very important in this regard, showing that they not only process synaptic information, but also regulate synaptic plasticity by releasing neuroactive factors called 'gliotransmitters' (Perea et al., 2009; Fellin, 2009). Astrocytes have been shown to affect synaptic plasticity by releasing various kinds of neuroactive molecules, such as glutamate, D-serine, GABA, TNFalpha and thrombospondins (Stellwagen and Malenka, 2006; Jourdain et al., 2007; Henneberger et al., 2010; Liauw et al., 2008b). Notably, release of NMDAR coagonist D-serine is essential for LTP at nearby excitatory synapses (Henneberger et al., 2010) and release of thrombospondins is necessary for structural plasticity and functional recovery after stroke (Liauw et al., 2008a). In the developing visual cortex, the closure of the critical period of OD plasticity coincides with the maturation of cortical astrocytes. The relationship of these events was investigated by Muller and Best (Muller and Best, 1989). They showed that implantation of immature astrocytes into the visual cortex of an adult cat reopens the critical period. However, the mechanisms underlying this effect remain unknown.

In conclusion it can be said be that OD plasticity is a complex phenomenon resulting from different forms of plasticity working together at the molecular, physiological, morphological and circuit levels, including synapse-specific/cell-wide

biochemical adjustments, LTP/LTD-like changes and pruning/sprouting of cellular processes.

II. Techniques of assessing OD plasticity

There are various methods for visualizing OD plasticity both anatomically and functionally. One of the ways to anatomically observe changes in OD columns is by doing anterograde tracer injections in the visual pathways following visual deprivation (LeVay et al., 1980). But it does not provide any information about changes in the physiological properties of the neurons or functional architecture of the visual cortex. In order to understand the functional changes in the cortex in response to visual manipulation, *in-vivo* electrophysiological recording and functional imaging techniques are often used. Some of the physiological techniques of studying OD plasticity are Single Unit recording, Optical Imaging of intrinsic signals and Visually Evoked potentials (VEP). In the following sections the prior two techniques are described.

Single Unit electrophysiological recordings

Single Unit recordings is a method of recording extracellular responses of single neurons. The activity-induced response can be visualized as spikes registered by a microelectrode system (Figure 3). A single unit is defined as a distinct firing neuron whose action potentials are isolated from nearby neurons based on the shape of the spike (Green, 1958). As an action potential propagates through the cell, it creates a measurable, changing voltage potential within and outside of the cell. When a microelectrode is close to the cell surface, it measures this voltage change in response to activity, giving spiking information. The microelectrodes are fine-tipped high impedance conductors which are primarily glass micropipettes or metal microelectrodes

made of platinum or tungsten (Wiesel And Hubel, 1963). This technique can be used to receive information about various response properties of neurons, such as degree of responsiveness and spontaneous activity, receptive field size, orientation and direction selectivity, spatial and temporal frequency selectivity and ocular dominance preference (Shatz and Stryker, 1978; Hubel And Wiesel, 1962; Sherk and Stryker, 1976; Yu et al., 2010).

One of the biggest advantages of this technique is that it provides very precise recordings at the level of single neurons. So it provides high spatial and temporal resolutions. On the other hand, it does not provide an instant visualization of the response properties of the whole cortical area. It requires multiple penetrations before an adequate number of cells can be sampled, which makes it a time-consuming procedure. In this regard, the use of multichannel single unit recording systems has been useful to increase the number of cells sampled as well as reduce the duration of the experiment (Rousche et al., 1999). Multichannel systems have several electrode shanks, each containing multiple recording sites. So it can not only furnish a better sampling of cells in a shorter time, it can also provide information about the neuronal circuitry of the recording area.

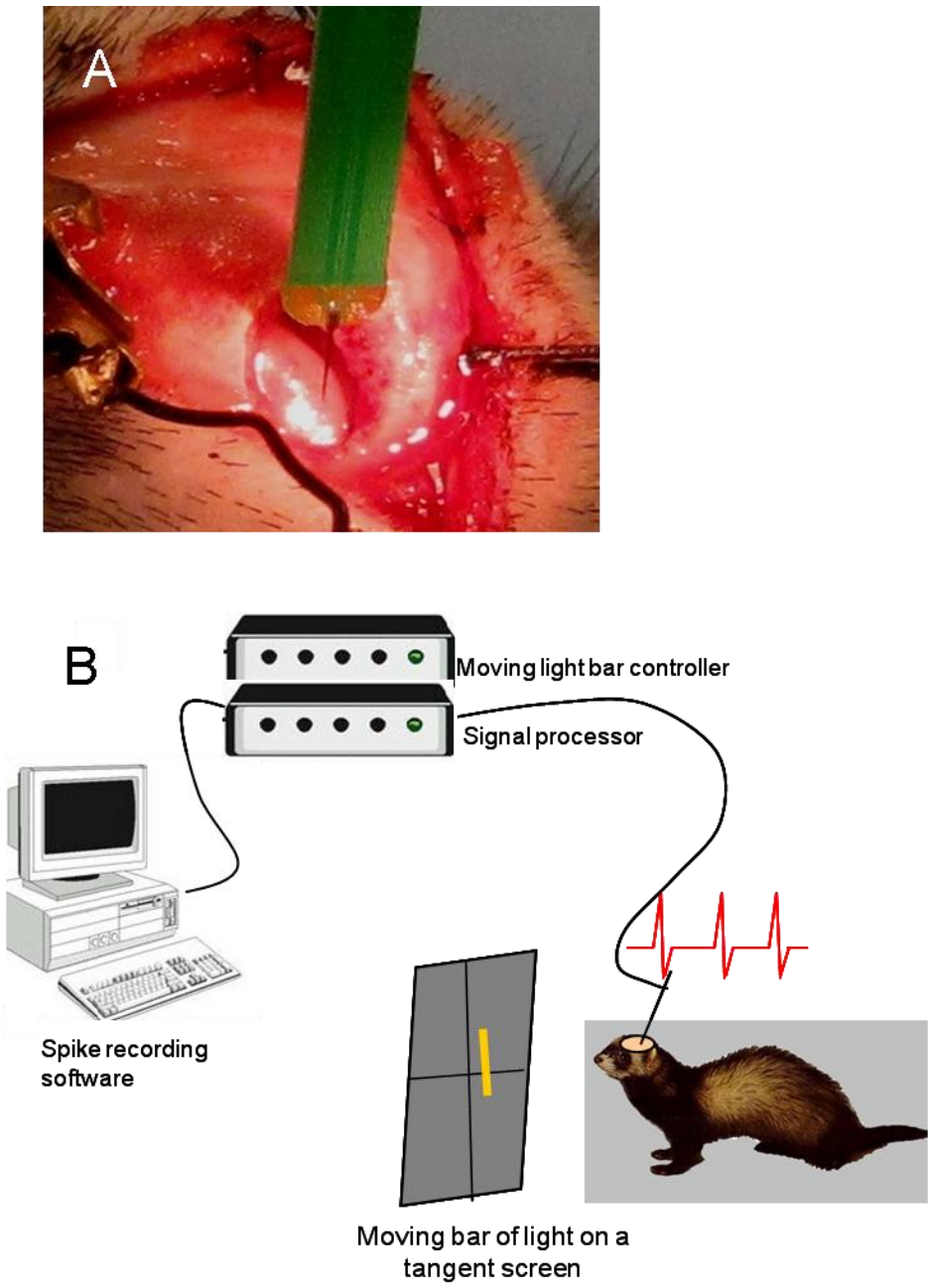


Figure 3. **Single Unit recording**. **A**, An anesthetized animal is kept in a stereotaxic frame. Its visual area is exposed and a microelectrode is inserted into the primary visual area. **B**, The electrode is connected to a recording software, which registers spikes in response to visual stimulation by a bar of light to each eye alternately.

Optical Imaging of intrinsic signals

Even though multichannel electrode systems offer better sampling and resolutions, they are not ideal for studying the functional properties of neuronal assemblies over a large cortical area. One of the fastest and most effective techniques of visualizing the functional architecture of a whole cortical area is by Optical Imaging of intrinsic signals (Figure 4). This technique is based on the changes in the intrinsic optical properties of areas of cortical tissue that respond to sensory activity versus those areas that do not respond (Ts'o et al., 1990).

It has been known for decades that there is strong coupling between neuronal activity, local oxidative metabolic activity and blood flow, which results in small highly localized changes in the optical properties of the brain tissue (Vanzetta and Grinvald, 1999; Frostig et al., 1990). It was shown recently that this spatially restricted coupling between neuronal activity and the hemodynamic changes is brought about by the activation of astrocytes (Schummers et al., 2008). Blocking activity of the astrocytes, that are present nearby visually-stimulated neurons, disrupted the hemodynamically generated optical signals. There can be several sources of these activity-dependent intrinsic optical signals. One component of the signal originates from the activity-dependent changes in the oxygen saturation of hemoglobin (Vanzetta and Grinvald, 1999; Malonek and Grinvald, 1996). This change in oxygenation itself has two parts - an early increase in the deoxy-hemoglobin resulting from elevated oxygen consumption by the activated neurons (which causes a darkening of the cortex), and a delayed increase in local blood flow causing a decrease in the deoxy-hemoglobin concentration. A second component of signal originates from changes in blood volume in the local

capillaries. These blood-related components dominate the intrinsic signals at optical wavelengths of around 600nm (Mayhew et al., 2000). Another significant component of intrinsic signals arises from changes in light scattering accompanying electrical activation of cortical tissue (Arnoldussen et al., 2000). This is caused by ion and water movement, expansion and contraction of extracellular spaces, capillary expansion or neurotransmitter release. This scattering component becomes prominent at higher wavelengths and dominates the intrinsic signal at around 800nm (Frostig et al., 1990). Although these intrinsic signals have different sources at different wavelengths, it has been shown that functional maps obtained at different wavelengths are similar (Frostig et al., 1990). We use a red light (~700nm) for our optical imaging experiments. This longer wavelength also provides deeper penetration into the tissue, and so can sometimes image from the granular layers of the cortex. In order to relate activity maps to anatomical landmarks, a picture of the blood vasculature pattern is obtained at the beginning. This pattern is can be seen particularly well if the cortex is illuminated with a green light (~550nm).

As action potentials in neurons create oxygen demand and change blood flow and light-scatter properties, it has been shown that the intrinsic optical signals primarily measures spiking activity (Toth et al., 1996). The amplitude of intrinsic signals has been found to be well correlated with spike rates in the visual (Shmuel and Grinvald, 1996) and somatosensory areas (Grinvald et al., 1994). Some reports suggest that they also reflect subthreshold synaptic potentials (Das and Gilbert, 1995).

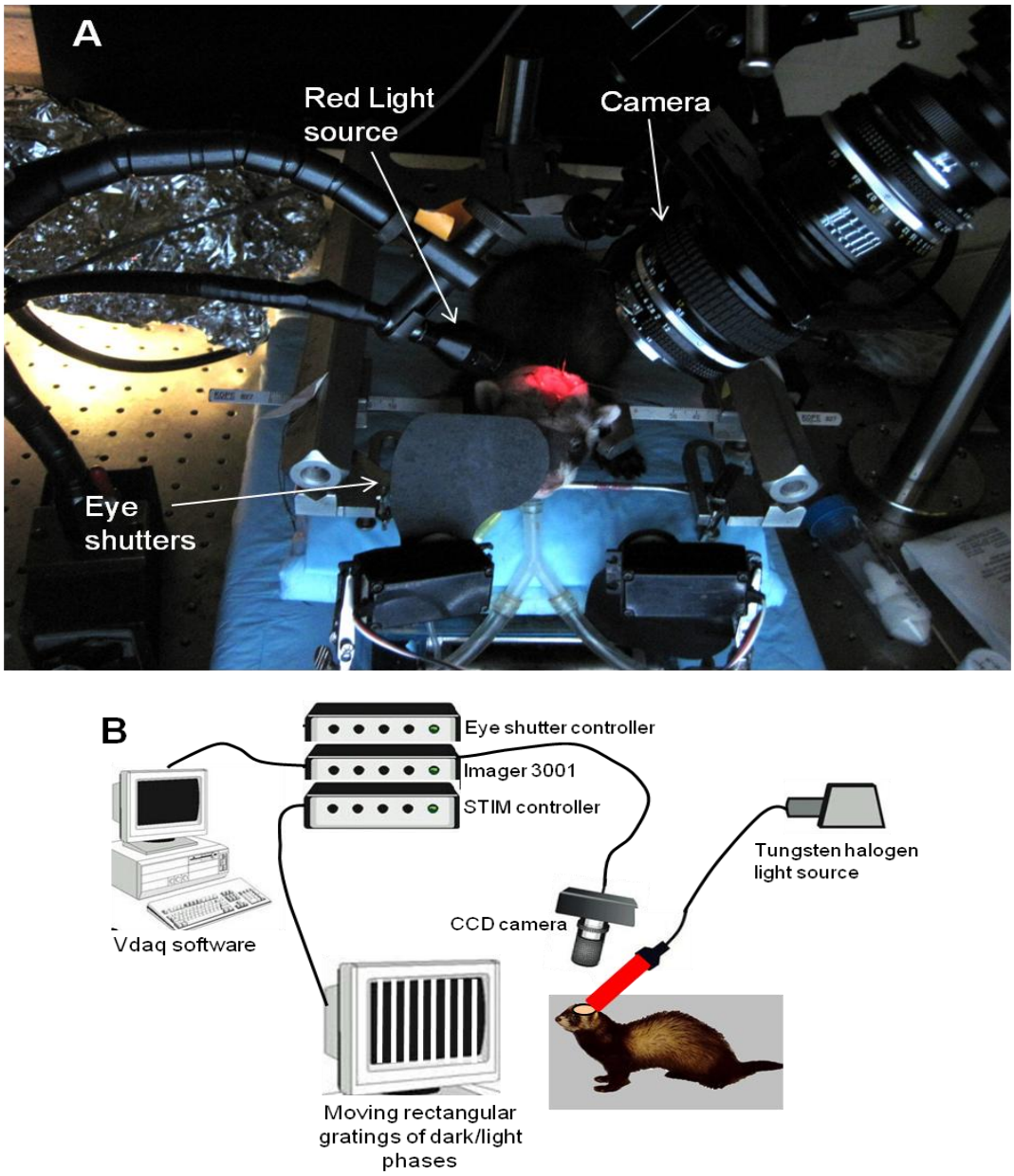


Figure 4. Optical Imaging of intrinsic signals. **A**, An anesthetized animal is kept in a stereotaxic frame. A camera takes pictures of the exposed visual cortex that is illuminated with a red light (~700nm) when the animal is presented moving gratings of light to each eye alternately using eye shutters. **B**, As the animal is shown moving gratings of light at different orientations using a monitor, the camera takes pictures of intrinsic optical signals of the visual cortex and transfers to the Vdaq software, which creates the monocular response maps.

Finally, it can be said that while Single Unit recording directly assesses spiking activity at the level of single neurons, Optical Imaging provides an indirect measurement of the activity of a population of neurons simultaneously in a whole region of cortex. The methodologies for performing each technique of measuring OD plasticity has been discussed in detail in the subsequent chapters.

III. Early alcohol exposure impairs neuronal plasticity

The detrimental effects of alcohol abuse have been a matter of concern and study for a long time. Some of the most profound effects of alcohol are seen when the exposure occurs early in life.

Fetal Alcohol Spectrum Disorders (FASD)

When alcohol is consumed during pregnancy, the offspring may exhibit signs of what is now known as Fetal Alcohol Spectrum Disorders (FASD). FASD encompasses a continuum of effects observed in children exposed to alcohol during prenatal stages, ranging from minor behavioral problems to major malformations, growth deficiency and CNS dysfunctions.

Although this condition is preventable, it remains a leading cause of mental retardation and other neurobehavioral problems in the western world. Prevalence estimates of FASD vary greatly depending on the period of study, the method used and the region reported. But the most quoted figure is 9.1 cases per 1000 live births in US, which makes it approximately 40,000 new cases of FASD each year (May et al., 2009; Klug and Burd, 2003). This prevalence rate far surpasses the combined figures of two other major conditions that present similar problems, namely, Spina bifida and Down's syndrome (Parker et al., 2010; Shin et al., 2010). This high prevalence also adds a huge cost to the healthcare system (Lupton et al., 2004).

Since the time Jones and Smith first described the main features of Fetal Alcohol Syndrome (FAS), many terms have been used to describe the different effects of fetal

alcohol exposure. FAS is the most classical form of FASD in which children present with stunted pre- or postnatal growth, deficient brain growth and some characteristic facial anomalies including small palpebral fissures, indistinct philtrum, thin upper lip, flat mid-face and low nasal bridge (May et al., 2009; Hoyme et al., 2005). Some children also show other associated features like epicanthal folds, short upturned nose, ear anomalies giving a 'railroad track' appearance, 'hockey stick shaped' palmar crease, etc. (Wattendorf and Muenke, 2005; Sampson et al., 1997). Partial FAS (pFAS) is a less severe form in which individuals show at least two facial features and either growth deficits or complex pattern of behavioral or cognitive abnormalities. Both FAS and pFAS do not require confirmation of maternal of alcohol consumption for the establishment of the diagnoses. Another less defined class is Alcohol-related neurodevelopmental disorders (ARND), in which a child must have documented prenatal exposure and display neurological and complex behavioral problems. A yet another category of diagnosis is Alcohol-related Birth Defects (ARBD), in which a child presents with at least two of the characteristic facial anomalies and congenital structural defects, along with evidence of maternal alcohol consumption. Since many prenatally alcohol-exposed children lack the discriminatory facial anomalies, and present with only a range of neurobehavioral problems, it becomes difficult to confirm FASD in them by the process of elimination of other congenital neurological disorders having overlapping signs. This is a reason why many FASD children remain undiagnosed.

The neurological deficits in FASD can range from extreme physical changes in the CNS to minor behavioral problems. Some of the more consistent changes in the brain structure are reduction in the size of the overall cranial vault and brain

(microcephaly) (Archibald et al., 2001), agenesis of the corpus callosum and anterior commissure (Swayze et al., 1997), cerebellar and brainstem anomalies (Mattson et al., 1992). Even in the absence of gross brain defects, FASD presents with a wide range of neuropsychological deficits, including impairments in overall IQ, learning and memory, language, attention, reaction time, executive functioning, fine and gross motor skills, and social and adaptive functioning (Mattson and Riley, 1998; Steinhausen and Spohr, 1998). There is good evidence showing that some of these deficits are caused by impairments in sensory processing, including visual, verbal and somatosensory deficits (Franklin et al., 2008; Manji et al., 2009; Stromland, 2004). Since age-appropriate cognitive functions rely on proper integration of stimuli from the environment, altered sensory modulation in children with FASD may explain the mal-adaptive behaviors and neuropsychological problems mentioned above (Carr et al., 2010; Whaley et al., 2001).

Plasticity deficits in FASD

There is growing evidence showing that deficits in neuronal plasticity underlie the sensory and neurobehavioral problems observed in the FASD (Hamilton et al., 2010; Wheeler et al., 2011; Roebuck-Spencer and Mattson, 2004; Jacobson et al., 2008; Harris et al., 1995; Ferrer et al., 1989). Numerous studies from different groups have shown that various forms of neuronal plasticity are impaired in animal models of FASD, including LTP, LTD, learning and memory and barrel cortex plasticity (Puglia and Valenzuela, 2010; Su et al., 2010; Petkov et al., 1991; Rema and Ebner, 1999; Servais et al., 2007; Toso et al., 2006). Although alcohol exposure at any time during the gestation period can have various detrimental effects on development, the effects of

alcohol on developmental plasticity are particularly severe during the third trimester, when the neocortex is starting to develop and major anatomical and functional refinement is taking place (Katz and Shatz, 1996). Alcohol exposure can disrupt several critical processes, including neuronal differentiation and migration, synaptogenesis, neuroglial interactions, neuronal circuit formation and remodeling, that characterize this period of gestation (Guerri, 1998; Goodlett et al., 2005).

OD plasticity is disrupted in a ferret model of FASD

Ferrets are particularly good models for studying effects of insults during the third trimester-equivalent of human gestation because in these animals this phase occurs after birth in a period roughly corresponding to postnatal days (P) 10 to P30 (Clancy et al., 2001). For instance, the thalamocortical synapses start to form in the layer IV of the cortex during the second postnatal week (Herrmann et al., 1994). Some neurogenesis and migration (in layer II-III) continue to occur until P21 (Jackson et al., 1989). Ocular dominance columns start to form during the third postnatal week (Crowley and Katz, 2000). Importantly, neurons in the visual system start showing properties like spontaneous activity (Maffei and Galli-Resta, 1990), response to visual stimulation (Krug et al., 2001) and orientation selectivity (Chapman and Stryker, 1993) before eye opening (around P32) through closed eyelids.

In order to study the effects of alcohol during the third trimester-equivalent of human gestation, our group exposed ferret neonates to alcohol (3.5 g/Kg, i.p. in 25% of saline) from P10 to P30 and then assessed OD plasticity. They found that OD plasticity is impaired during the peak of critical period (P40 to P60) (Medina et al., 2003), as in

these animals, three days of monocular deprivation (MD) had minimal effect and most of the visual cortical neurons as they continued to respond to both eyes comparably. This is in contrast to the normal animals, where 3 days of MD produced a significant shift in responses towards the undeprived (experienced) eye. Interestingly, this impairment in plasticity occurs only when alcohol was administered during a specific period of development for a sufficient period of time. When ferrets were exposed to alcohol either at a different period (P20 to P40) or for a shorter duration (P10 to P25), they did not show impairment in OD plasticity. Importantly, this impairment was not caused by a decrease of visual responsiveness since the number of spikes after a visual stimulus as well as the spontaneous activity of the cortical neurons was similar between groups. This selective effect on plasticity, while sparing general visual responses, indicated that early alcohol exposure disrupts a specific mechanism.

Over the years, various studies have shown that alcohol exposure can affect many steps of the plasticity-related pathways mentioned previously (Figure 2). For instance, NMDA receptor levels remain reduced for several weeks after fetal alcohol exposure (Savage et al., 1992; Valles et al., 1995) and show delayed developmental switch of its subunits (Nixon et al., 2002; Snell et al., 2001). Alcohol also disrupts the activation of various protein kinases that are present downstream of NMDA activation, including MAP kinase (Samudio-Ruiz et al., 2009) and CaM kinase IV (Misra et al., 2001). The effect of alcohol on the second messenger cyclic GMP has also been reported (Butters et al., 2003). Moreover, the activity of CREB is also found to be severely attenuated after chronic alcohol exposure (Misra et al., 2001).

In order to rescue OD plasticity in ferrets subjected to early alcohol exposure, our group initially used phosphodiesterase (PDE) inhibitors to activate transcription factors that are involved in the expression of plasticity-related genes. PDE inhibition can increase intracellular concentrations of cAMP and/or cGMP by stopping their degradation. These nucleotides can act as second messengers in cascades that result in phosphorylation of the transcription factors CREB and SRF (refer to Figure 2). One example of a PDE inhibitor that is involved with plasticity enhancement is the alkaloid vinpocetine. Vinpocetine is an inhibitor of the PDE1, which acts on the turnover of both cAMP and cGMP. It has been shown to facilitate LTP (Molnar and Gaal, 1992) and enhance structural dynamics of dendritic spines (Lendvai et al., 2003). Recently, our group showed that deficits in OD plasticity caused by early alcohol exposure can be reversed if vinpocetine is given during the period of MD (Medina et al., 2006). Interestingly, if the treatment is done with inhibitors of PDEs type 4 or 5, which are specific for cAMP and cGMP respectively, plasticity is not restored (Krahe et al., 2009b), (Lantz, Wang and Medina under review). The effectiveness of vinpocetine in restoring plasticity can be attributed to a simultaneous increase in cAMP and cGMP (Lugnier, 2006; van Staveren et al., 2001; Marte et al., 2008). It is conceivable that this increase activates both the transcription factors CREB and SRF (Pilz and Casteel, 2003).

IV. Improving neuronal plasticity by targeting Serum Response Factor (SRF)

SRF was named so because it was first found to be responsible for the transcriptional activation of immediate early genes (IEGs) like *c-fos* following stimulation by serum (Treisman, 1985). Many of these IEGs like *c-fos*, *egr-1*, and *junB* are linked to activity-induced gene expression in neuronal plasticity (Cole et al., 1989; Ramanan et al., 2005; Wisden et al., 1990). SRF-dependent transcription occurs when this transcription factor binds to a promoter region known as Serum Response Element (SRE) (Norman et al., 1988). The SRE is an A/T rich core flanked by an inverted repeat, CCATATTAGG, also referred to as the CArG box (Treisman, 1992). To date, about 30 genes have been identified to contain the CArG box sequence and they include many IEGs, neuronal genes, growth and neurotropic factor genes, muscle genes and other cytoskeletal genes (Chai and Tarnawski, 2002; Etkin et al., 2006; Knoll et al., 2006).

The SRF protein is a member of the MADS box superfamily of transcription factors and the human SRF contains 508 amino acids. It is a ubiquitous nuclear protein containing three major domains: 1) a SRE DNA binding and dimerization domain, 2) a transactivation domain, and 3) several phosphorylation sites (Chai and Tarnawski, 2002). Deletion analysis showed that the 90-amino acid domain (between amino acids 133 and 222) is sufficient for DNA binding, dimerization and interaction with other partner proteins necessary for transcriptional activation (Norman et al., 1988; Johansen and Prywes, 1994). The best studied SRF partner proteins are the Ternary Complex

Factors (TCFs) like Elk-1 and members of the myocardin family of transcriptional cofactors (MRTFs) like MRTF-A/MAL/MLK1, MRTF-B.

Activation and Regulation of SRF

Besides serum, SRF can be activated by a variety of agents including mitogens, cytokines, lipopolysaccharide (LPS), agents that elevate intracellular calcium, tumor necrosis factor-α (TNFα) as well as by extracellular stimuli like antioxidants and UV light (Chai and Tarnawski, 2002).

In the nervous system, SRF has been shown to respond to synaptic activity, triggered by neurotransmitters like glutamate (Bading et al., 1993; Johnson et al., 1997; Xia et al., 1996) and kainate (Herdegen et al., 1997) via activation of NMDA receptors or by KCl via activation of voltage-sensitive calcium channels (Misra et al., 1994). These events lead to entry to Ca^{2+} into the cytosol. From here the Ca^{2+} signal can activate SRF either directly by Ca^{2+}/ calmodulin-dependent kinases (Misra et al., 1994; Miranti et al., 1995) or indirectly by activating SRF cofactors like TCFs (Kalita et al., 2006) or MRTFs (Posern and Treisman, 2006) via the MAP kinase pathway (refer to Figure 2). Neurotrophic factors like BDNF and NGF also activate SRF-mediated transcription (Kalita et al., 2006; Chang et al., 2004; Wickramasinghe et al., 2008) and enhance SRF expression (Kalita et al., 2006) via the indirect MAP kinase pathway. G proteins target SRF via the Rho-GTPase/actin and /or the Rho-MAP kinase pathway. In the Rho-GTPase/actin pathway, cell stimulation activates the conversion of G-actin into F-actin through polymerization in the cytoplasm. The depletion of G-actin liberates MRTF-A for entry into nucleus, followed by SRF/MRTF interaction and transcription of SRF-

controlled cytoskeletal genes (Miralles et al., 2003; Sotiropoulos et al., 1999; Vartiainen et al., 2007). There are additional kinases that have been reported to target SRF directly, i.e., MAPK-activated kinase 2 (MK2) (Heidenreich et al., 1999), pp90RSK (Rivera et al., 1993), PI3-kinase (Chang et al., 2004) and serum- and glucocorticoid-inducible kinase (SGK) (Tyan et al., 2008).

Role of SRF in the nervous system

SRF in neurons is responsible for various cellular activities including migration, differentiation and plasticity. Genome-wide transcriptomics analysis in neurons revealed many genes that are known to be important for motility and plasticity that are directly targeted by SRF. These include IEGs (*c-fos, Egr-1* to *Egr-3, Junb,* etc.), actin cytoskeletal genes (*Actb, Actg1, Gelsolin, Arc,* etc.), axonal guidance genes (*Sema3a, Epha4, Epha7,* etc.), neurotrophin genes (*Bdnf, Ngf*) and genes involved in synaptic plasticity (Arc, Psd95, etc.) (Knoll and Nordheim, 2009; Etkin et al., 2006; Knoll et al., 2006; Wickramasinghe et al., 2008).

In the developing nervous system, SRF plays an important role in orchestrating neuronal migration and morphological differentiation. SRF gene control and actin treadmilling are linked by regulatory feedback mechanisms whereby actin dynamics modulates SRF-directed gene expression and the latter influences actin polymerization by both transcriptional and post-translational inputs (Posern and Treisman, 2006; Miralles et al., 2003; Sotiropoulos et al., 1999; Miano et al., 2007). Accordingly, all neuronal functions relying on actin-based motility were severely impaired in conditional neuron-restricted *srf* mutants (Knoll et al., 2006). Conditional SRF ablation in late-

prenatal and early-postnatal stages resulted in severe neurological phenotypes including ataxia, hydrocephalus and loss of body weight, leading to death at around P21 in rodents (Knoll et al., 2006; Alberti et al., 2005). The first *in vivo* defect described in *srf* mutant brain was impaired tangential cell migration along the rostral migratory stream, resulting in ectopic accumulation of progenitor cells in SVZ (Alberti et al., 2005). Subsequently, neurite outgrowth, axon versus dendrite differentiation and axon guidance were likewise found to be affected in *srf* mutants (Knoll et al., 2006; Wickramasinghe et al., 2008). In addition, an entire set of genes associated with oligodendrocyte development/myelination was found to be downregulated in neuronal *srf* mutants, suggesting that neuronal SRF affects development of neighboring glial cells by a paracrine mechanism (Stritt et al., 2009).

SRF ablation in the adult revealed crucial roles of SRF in neuronal-activity-induced gene expression, synaptic plasticity, learning and memory (Etkin et al., 2006; Ramanan et al., 2005; Tyan et al., 2008; Dash et al., 2005; Lindecke et al., 2006; Nikitin and Kozyrev, 2007). Adult *srf* mutants have normal basal excitatory synaptic transmission, but LTP (Ramanan et al., 2005) and LTD (Etkin et al., 2006) are shown to be impaired. It is not yet fully understood how neuronal activity, mediated by SRF-directed IEG responses, is converted into persistent structural synaptic modifications. However, SRF influences actin dynamics by regulating genes encoding actin-binding proteins (*Actb, Actg1, Gelsolin, Arc,* etc.), which can provide a cytoskeletal machinery to explain the morphological synapse modifications (Dillon and Goda, 2005; Matus, 2000).

Chapter 2 - Overexpression of Serum Response Factor Restores Ocular Dominance Plasticity in a Model of Fetal Alcohol Spectrum Disorders

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Arco P. Paul[1] , Fernanda Pohl-Guimaraes[1,2] , Thomas E. Krahe[1], Claudio C. Filgueiras[1,2], Crystal L. Lantz[1], Ray J Colello[1] and Weili Wang[1] and Alexandre E. Medina[1]

[1] Department of Anatomy and Neurobiology, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298-0709

[2] Department of Physiology, Universidade do Estado do Rio de Janeiro

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Correspondence should be addressed to Alexandre E Medina, Department of Anatomy and Neurobiology, Box 0709, Virginia Commonwealth University, 1101 East Marshall Street, Room 12-044, Richmond, VA 23298-0709. E-mail: amedina@vcu.edu

Abstract

Neuronal plasticity deficits underlie many of the neurobehavioral problems seen in Fetal Alcohol Spectrum Disorders (FASD). Recently, we showed that third trimester alcohol exposure lead to a persistent disruption in ocular dominance (OD) plasticity. For instance, few days of monocular deprivation results in a robust reduction of cortical regions responsive to the deprived eye in normal animals, but not in ferrets exposed early to alcohol. This plasticity deficit can be reversed if alcohol-exposed animals are treated with a phosphodiesterase type 1 (PDE1) inhibitor during the period of monocular deprivation. PDE1 inhibition can increase cAMP and cGMP levels, activating transcription factors such as the cAMP response element binding protein (CREB) and the Serum response factor (SRF). SRF is important for many plasticity processes such as LTP, LTD, spine motility and axonal pathfinding. Here we attempt to rescue OD plasticity in alcohol-treated ferrets using a Sindbis viral vector to express a constitutively active form of SRF during the period of monocular deprivation. Using optical imaging of intrinsic signals and single unit recordings we observed that overexpression of a constitutively active form of SRF, but neither its dominant negative nor GFP, restored OD plasticity in alcohol-treated animals. Surprisingly, this restoration was observed throughout the extent of the primary visual cortex and most cells infected by the virus were positive for GFAP rather than NeuN. This finding suggests that overexpression of SRF in astrocytes may reduce the deficits in neuronal plasticity seen in models of FASD.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) is considered the leading cause of mental retardation in the western world with as many as 40,000 cases per year in the United States (Klug and Burd, 2003). The sensory cortex is one of the most affected areas in FASD and children with this condition present altered somatosensory, auditory and visual processing. There is growing evidence indicating that these sensory problems may be related to poor cortical map refinement, organization and plasticity (Rema and Ebner, 1999; Medina et al., 2003; Medina et al., 2005; Powrozek and Zhou, 2005; Margret et al., 2006). We have developed a ferret model of FASD that provides a novel approach to test mechanistically how early alcohol exposure can impair sensory cortex function. We showed that ferrets exposed to alcohol during the third trimester equivalent of human gestation present disorganization of orientation selectivity columns and impaired ocular dominance (OD) plasticity (Medina et al., 2003; Medina et al., 2005; Medina and Ramoa, 2005; Medina and Krahe, 2008). Importantly, both of these cortical features can be rescued by vinpocetine, a phosphodiesterase type 1 inhibitor, even several weeks after alcohol exposure (Medina et al., 2006; Krahe et al., 2009a). The efficacy of vinpocetine can be attributed by an increase in cAMP and cGMP levels, which in turn, would result in activation of the transcription factors cAMP/Ca2+ response element-binding protein (CREB) and Serum response factor (SRF) (Chai and Tarnawski, 2002; Kornhauser et al., 2002). Importantly, while cross talks between the cascades exist it is safe to say the CREB and SRF can be preferentially activated by cAMP and cGMP respectively. Recently, we discovered that, contrary to what was

observed with vinpocetine, Rolipram, a PDE type 4 inhibitor failed to restore OD plasticity in the ferret model of FASD (Krahe et al., 2009b). Since Rolipram increases only cAMP levels and not cGMP, one may suggest that the restoration of plasticity seen earlier is due to SRF activation. While the association of CREB with neuronal plasticity has been in the spotlight for decades (Silva et al., 1998; Frank and Greenberg, 1994; Shaywitz and Greenberg, 1999; Lamprecht, 1999), much less is known regarding SRF. This is surprising, given that many genes necessary for plasticity-related events require activation of both of these transcription factors (Chai and Tarnawski, 2002; Montminy et al., 1990; Platenik et al., 2000). Recently many studies have shown the importance of SRF for LTP, LTD, spine motility and axonal pathfinding (Etkin et al., 2006; Ramanan et al., 2005; Knoll et al., 2006; Alberti et al., 2005). In fact SRF can mediate the expression of many plasticity-related factors such as neurotrophins, immediate early genes, cytoskeletal proteins and thrombospondins (Chai and Tarnawski, 2002; Ramanan et al., 2005; Knoll et al., 2006; Stritt et al., 2009; Morris et al., 1999; Schratt et al., 2002; Christopherson et al., 2005). Here, we use viral-mediated gene transfer to overexpress a constitutively active form of SRF in visual cortex, in order to reverse the OD plasticity deficits seen in a ferret model of FASD.

Materials and Methods

All procedures described in this paper were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University. For a summary of our experimental design see supplemental figure 5A. Briefly, ferrets were treated with 3.5

g/kg alcohol i.p. (25% in saline) or saline as control every other day between postnatal days (P) 10 to P30. This alcohol treatment led to a blood alcohol level of approximately 250 mg/dl for 1-5 h after injection (Medina et al., 2003). At the peak of critical period of the OD plasticity (P38-P45) animals were injected in V1 with a Sindbis virus carrying a constitutively active form of SRF, a dominant negative (SRF-), or a control GFP. Viruses were made and kindly provided by Drs Amit Etkin and Eric Kandel (Columbia University, New York, NY). The construct was made by fusing the dimerization and DNA binding domains of SRF (aa 90-222) with the HSV VP16 transactivation domain (aa 363-490). The resulting fused construct was inserted in a Sindbis vector. One day after the viral transfection, animals were monocularly deprived by eye lid suture for 4 d. For the virus injection, a small hole was drilled in the skull and the tip of a 31 gauge Hamilton Syringe (Hamilton Company, Reno, Nevada) containing the Sindbis virus (titer = 4.0×10^7 infectious units/mL) was stereotaxically positioned at an angle of 15-22° from the midline and lowered 1mm into the binocular region of the primary visual cortex. A volume of 2µl was injected at a rate of 0.1µl every 30 s. Each animal received two injections ~1mm apart. After the period of monocular deprivation (MD), the animals were anesthetized, the deprived eye opened and either an optical imaging of intrinsic signals or a single unit recording experiment was conducted. After the physiology experiments animals were perfused, the visual cortex collected and overexpression of the virus was assessed by immunohistochemistry.

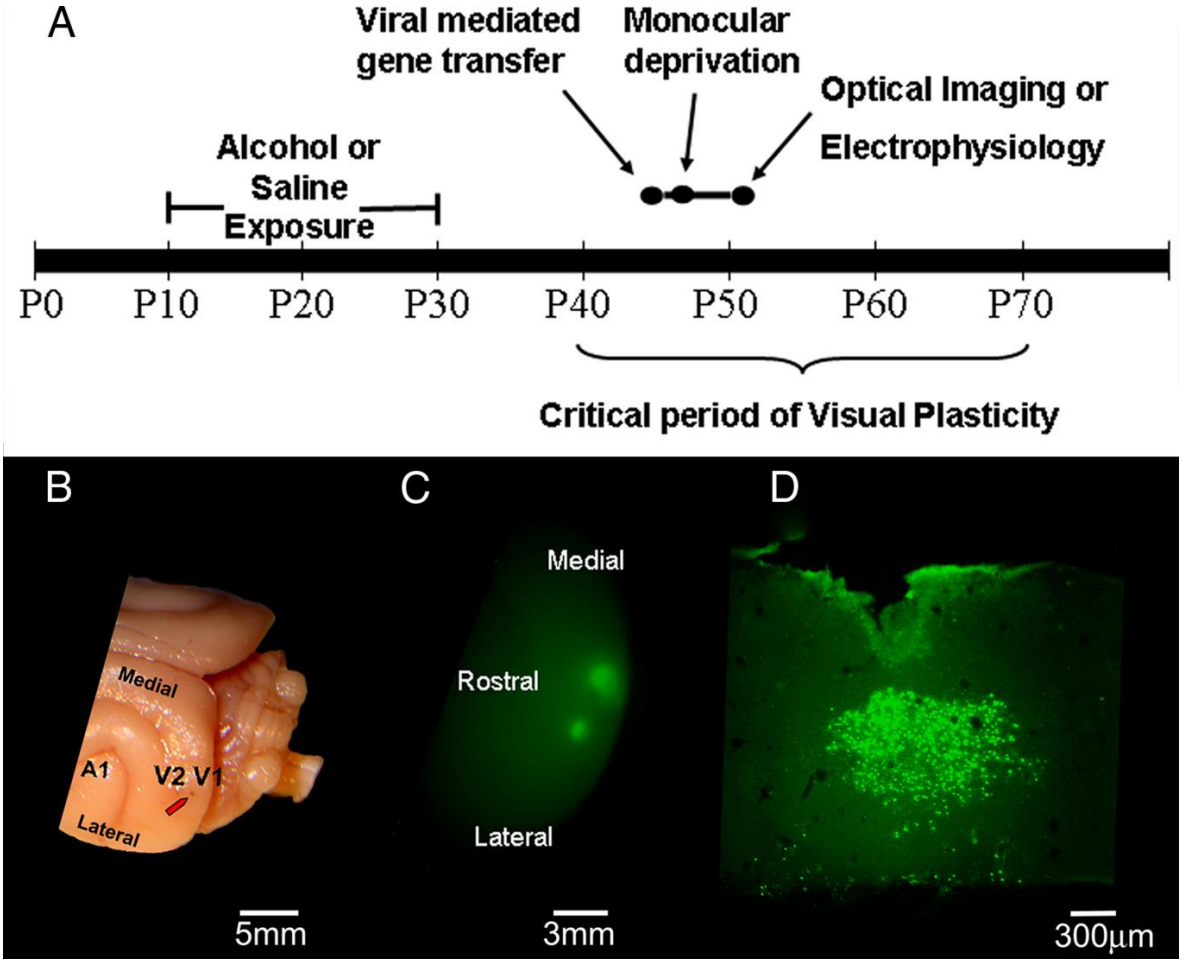

Figure 5. A, **Experimental design.** Ferrets were exposed to alcohol or saline every other day between P10-P30. After a prolonged alcohol-free period animals were injected with Sindbis/SRF+; Sindbis/GFP or Sindbis/SRF-. One day after virus injection animals, were monocularly deprived by eye lid suture for 4 d. After this period, the deprived eye was opened and either optical imaging of intrinsic signals or single unit recordings were conducted. **B**, The red arrow indicates the typical location of a virus injection. **C**, Epifluorescence revealed expression of GFP in the primary visual cortex 4 d after injections with Sindbis/GFP. The lateral and medial injection had volumes of 1.5 and 3 µl respectively. **D**, Site of a Sindbis/SRF+ injection. Cells were stained for VP16 transactivation domain.

Preparation for optical imaging of intrinsic signals or single unit recordings

Animals were premedicated by subcutaneous injection of a tranquilizer (acepromazine, 1 mg/kg), and a muscarinic antagonist (methyl atropine bromide, 0.2 mg/kg) to reduce bronchial secretion, as well as dexamethasone sodium phosphate (0.5 mg/kg) to reduce inflammation. Animals were then anesthetized using sodium pentobarbital (35 mg/kg, Abbott Laboratories, North Chicago, IL) and placed in a stereotaxic frame. No procedures were started until the animal was sufficiently anesthetized, as ascertained by the loss of withdrawal and cornea-blink reflexes. A tracheal cannulation was performed, and the animal was placed on a ventilator and paralyzed using pancuronium bromide (0.2 mg/kg, i.p.). To comply with NIH guidelines for use of paralytic agents and to certify that the animals were maintained at an appropriate level of anesthesia, use of muscle relaxants was omitted in some experiments and withdrawal reflexes were monitored in these animals. Similar procedures have been previously described and shown to be appropriate for visual physiology studies conducted in ferrets (Medina et al., 2003; Medina et al., 2006; Medina et al., 2005; Krahe et al., 2005). Heart rate, expired CO_2 and arterial blood oxygen saturation (SpO_2) were monitored continuously and maintained at approximately 270 bpm, 4.0% and above 90%, respectively. Body temperature was maintained at 38^{o}C using a homeostatic blanket. Supplemental doses of pentobarbital (12 mg/kg) were given every hour throughout the experiment or when heart rate or expired CO2 increased, a procedure previously shown to preserve visual responses over time (Medina et al., 2003). Nictitating membranes were retracted using phenylephrine

hydrochloride (2.5%), the pupils were dilated with atropine sulfate (1%) and contact lenses were placed on the corneas. Subcutaneous injections of 10% dextrose and 0.9% saline were given to prevent dehydration.

Optical imaging of intrinsic signals

Optical imaging of intrinsic signals was performed with Imager 3001 VSD+ (Optical Imaging System) by using imaging methods slightly modified from those described previously (Medina et al., 2003; Medina et al., 2006; Medina and Ramoa, 2005; Krahe et al., 2005). An image of the vascular pattern was first obtained by illuminating the cortical surface with a green filter (approximately 550 nm) using a tungsten-halogen light source. Next, images of intrinsic signals were obtained using a red filter (~ 700 nm). Visual stimulation consisted of high-contrast square wave gratings (8.75º dark phase/1.25º light phase) generated by a 21-inch monitor (Sony Trinitron) using SGT+ graphics board and STIM software. Gratings were presented to each eye at an angle of 0º, 45º, 90º or 135º and drifted (22.5º/s) in both directions along the axis orthogonal to the orientation of the grating. A single trial consisted of these four gratings and a blank screen presented to each eye for 9 s in a pseudorandom sequence, with data acquisition during the last 8 s. A total of 20 trials were performed for each eye and the summed images were used to obtain single condition maps by subtracting responses to each eye from responses to a blank screen. In these images, dark areas correspond to regions that are visually responsive. In addition, differential maps (or ocular dominance images) were obtained by subtracting left eye from right eye single condition maps. In this images dark and light regions correspond to areas

responsive to right and left eye stimulation, respectively. Unfiltered ocular dominance images were first clipped at 2 SD, then a mean filter used to minimize high frequency noise. Then, a region of interest (ROI) outlining the primary visual cortex (V1) was drawn manually by an investigator who was blind to the animal's treatment. The contralateral eye band, which marks the anterior border of V1 and lies caudal to V2 ipsilateral big domains (Medina et al., 2006; White et al., 1999), was used as an anterior reference to draw the ROI. The posterior reference was the caudal pole. The approximate location of the border between V1 and V2 was still distinguishable after 4 days of monocular deprivation (MD). After defining the ROI, we created ocular dominance histograms based on the scale of gray values of 0 –255 that was divided into five class intervals, where 0–50 and 204–255 correspond to classes containing the darkest and lightest pixels, respectively.

In vivo electrophysiology

Single-unit recordings were conducted using a glass-coated tungsten microelectrode with a 5 µm exposed tip lowered into the primary visual cortex at ~20º to the vertical axis. To minimize sampling bias, single-units used in this study were separated by at least 100 µm along the electrode track. After the isolation of a single-unit, the receptive field was mapped and the optimal stimulus orientation, direction and velocity were determined qualitatively using a moving bar of light projected onto a tangent screen. OD, spontaneous activity and number of spikes per stimulus were then quantitatively determined for each cell by presenting a computer-controlled bar of light to each eye. Each stimulus presentation consisted of the bar of light moving across the

receptive field at the optimal orientation in one direction and back across in the opposite direction. To assess OD, the moving bar of light was presented to each eye separately at the optimal orientation. Spikes were collected during the 10 stimulus presentations by a computer using Spike 2 software (Cambridge Electronics Design) and peristimulus histograms were generated. Spontaneous activity was determined by recording activity in the absence of stimulation. At the conclusion of the experiment, ferrets were sacrificed with Euthasol, (0.6 ml/kg, i.p.; Delmarva).

Immunohistochemistry and Confocal microscopy

Ferrets were perfused with cold saline followed by cold 4% paraformaldehyde solution. After dissection of the occipital cortex, 50µm sections were obtained with a vibratome. For immunohistochemistry the following primary antibodies were used at a concentration of 1:200: NeuN (Neuronal marker, mouse; Millipore Bioscience Research Reagents); GFAP (Astrocytic marker, rabbit, Dako); VP16 (Sindbis/SRF+ expression marker, mouse, Santa Cruz Biotechnology), VP16 (Rabbit, Sigma-Aldrich); Flag (Sindbis/SRF- expression marker). The secondary antibodies were goat anti-rabbit (1:400, Alexa Fluor 594) and goat anti-mouse (1:200, Alexa Fluor 488). Tissue sections are alternately stained for VP16/GFAP and VP16/NeuN. Sections were analyzed in a Leica TCS-SP2 AOBS confocal laser scanning microscope (inverted) with a spectrophotometer scan head, a high resolution Märzhäuser MCX-2 motorized XY stage and three confocal detectors. The system has five lasers: blue diode (405 nm), Argon (458, 476, 488, 514 nm), green HeNe (543 nm), orange HeNe (594 nm) and red HeNe (633 nm). Double staining (colocalization) was evaluated by two mutually

reinforcing techniques: Particle analysis and Z-Stack counting. To perform particle analysis we first selected the two adjacent sections (VP16/GFAP, VP16/NeuN; one pair of sections for each animal; 3 animals used) that presented the greater number of infected cells. Confocal microscope tile scan images (20 X magnification) were taken and analyzed using the IPLab software. To confirm the results obtained with the particle analysis, two independent observers evaluated 14 Z-Stacks (seven VP16/GFAP; seven VP16/NeuN) from three animals. A typical Z-Stack had the dimensions of x = 170 µm, y = 170 µm, z = 30 µm. The stack was covered by a 10 x 7 grid and each independent observer assessed whether a VP16 positive cell had double labeling (with GFAP or NeuN) or not. The total number of VP16 positive cells in VP16/GFAP and VP16/NeuN Z-Stacks were 433 and 293 respectively.

Results

We investigated whether overexpression of SRF by Sindbis virus would improve OD plasticity in ferrets exposed to alcohol during the third trimester equivalent of human gestation, which is approximately P10-P30 in the ferret (Clancy et al., 2001; Medina and Ramoa, 2005). A summary of our experimental design can be seen in Figure 5. After a prolonged alcohol-free period animals were injected with Sindbis/SRF+; Sindbis/GFP or Sindbis/SRF in V1/V2 (Fig. 5B, C). We used optical imaging of intrinsic signals to create differential maps to assess changes in OD after monocular deprivation. Fig. 6A shows the left visual cortex of representative cases from each group analyzed. Dark and light areas in OD (differential) maps represent regions that respond preferentially to

stimulation of the right and left eye respectively. In monocular response (single condition) maps, dark regions represent areas that display strong responses to visual stimulation. Note that nondeprived animals (both saline and alcohol-treated) (Fig. 6A) present the typical contralateral bias observed in ferrets (Issa et al., 1999; Medina et al., 2003; White et al., 1999).

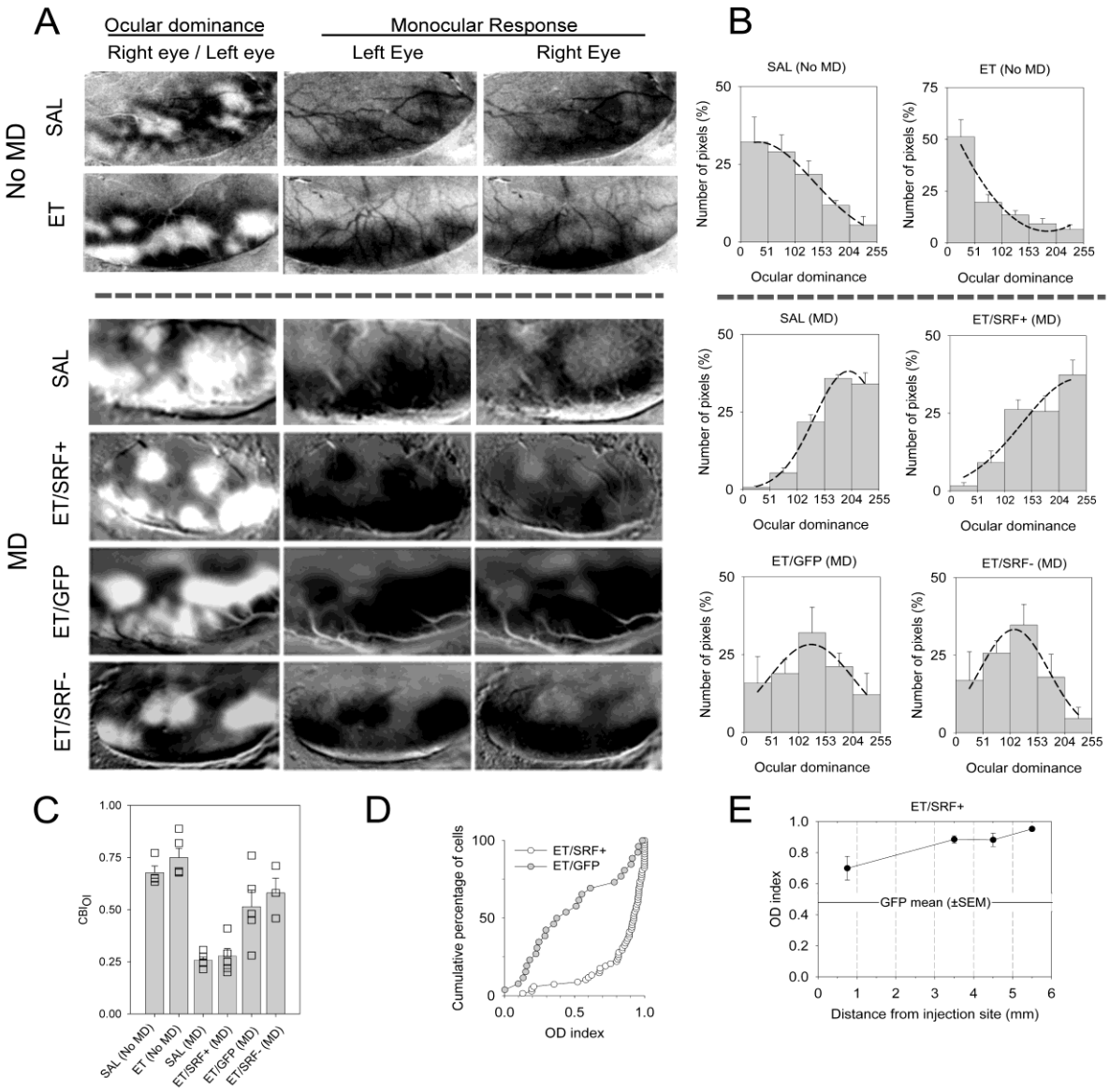

Figure 6. Optical Imaging and Single Unit recording results. Optical imaging of intrinsic signals shows that SRF overexpression restores plasticity in alcohol-exposed animals. **A**, First column shows ocular

dominance maps of representative cases of each experimental group. Dark and light areas represent regions dominated by the right and left eye respectively. In animals overexpressing SRF, monocular deprivation of the right eye resulted in a shift in dominance towards the left eye. In contrast, in both animals overexpressing either GFP or a dominant negative form of SRF, the deprived eye remained responsive. **B**, Histograms display the percentage of pixels fitting in a grayscale (0, black; 255, white). Note the predominance of lighter pixels in animals infected with the virus carrying the constitutively active form of SRF (SRF+), which indicates left (experienced) eye dominance. In contrast, note the predominance of black and gray pixels in animals infected with the virus carrying either GFP or a dominant negative form of SRF (SRF-), which indicates that the right (deprived) eye is still responsive. Saline (SAL) (no MD), n = 4; ethanol (ET) (No MD), n = 5; SAL (MD), n = 5; ET/SRF+ (MD), n = 5; ET/GFP (MD), n = 5; ET/SRF- (MD), n = 3. **C**, Mean contralateral bias indexes for all groups. Squares indicate individual animals. SRF+ animals presented significantly lower CBI_{oi}s than GFP and SRF- (one-way ANOVA followed by Bonferroni, $p<0.05$). These findings indicate that SRF+ rescues OD plasticity in alcohol-treated animals. **D**, Single-unit recordings show that SRF+ overexpression restores plasticity in alcohol-exposed animals. Cumulative number of cells (as percentage) plotted as a function of the OD index for animals that received either SRF+ or GFP. Note that in SRF+ animals, the curve was skewed to the right, indicating an OD shift (most cells respond better to left eye stimulation). In contrast in a GFP animal many cells were still responsive to the right (deprived) eye. ET/SRF+, n = 68 neurons from three animals. ET/GFP, n = 26 neurons from one animal. **E**, Single unit recordings reveal that SRF+ expression rescued ocular dominance plasticity even far away from the injection site.

As expected, 4 d of MD in saline-treated animals lead to a striking shift in ocular dominance towards the experienced eye (Fig. 6A). Remarkably, after the same period of MD, alcohol-treated animals that received the Sindbis/SRF+ virus presented a similar shift (Fig. 6A), which indicates that OD plasticity was restored. Surprisingly, while the virus infected area was around a radius of ~2 mm, the shift in OD was observed along

all the caudal extension of the visual cortex (V1, ~6-10 mm medial to lateral) (Figs. 5B, C, 6A). In contrast, alcohol-treated animals that received either Sindbis/GFP or Sindbis/SRF- presented only a very mild change in OD (Fig. 6A), which confirm our previous findings that early alcohol exposure impairs OD plasticity (Medina et al., 2003; Medina and Ramoa, 2005). To quantify OD (differential) maps we calculated the pixel distribution along a 256 gray scale, divided in five class intervals, where 0-50 and 204-255 correspond to classes containing the darkest and lightest pixels, respectively. Accordingly, nondeprived animals (either saline or ethanol treated) presented histograms skewed to the left, which indicates contralateral eye dominance (Fig. 6B). In contrast, after 4 d of contralateral eye lid suture, saline-treated animals, as well as alcohol-treated animals that received Sindbis/SRF+, presented OD profiles skewed to the right, indicating the dominance of the ipsilateral eye (Fig. 6B). In contrast, alcohol-treated animals that received either Sindbis/GFP or Sindbis/SRF- presented only modest changes in the OD profile. To further quantify these changes we created a contralateral bias index (CBI) (Fig. 6C). This index is defined as follows: $[(P_{0–50} - P_{204–255}) + [(P_{51–101} - P_{153–203})/2] + 100\}/200$, where $P_{A–B}$ denotes the percentage of pixels with gray values between A and B. A CBI close to 1.0 indicates a prevalence of darker pixels and right eye dominance. A CBI close to 0.0 indicates a prevalence of lighter pixels and left eye dominance. An ANOVA showed a significant ($F = 10.09$, $df = 2$, $p = 0.001$) effect of treatment (SRF+, GFP or SRF-). *Post hoc* analysis showed that alcohol-treated animals that received Sindbis/SRF+ had significantly smaller CBIs than animals that received GFP (Bonferroni, $p=0.01$) or SRF- (Bonferroni, $p = 0.004$). To further assess the effects of SRF in improving OD plasticity we conducted single unit

recordings experiments. The virus injection sites were easily identified in each animal and it was used as a landmark to place the electrode. Successful expression of the virus was confirmed by immunohistochemistry after the experiment. We created an OD index for each cell recorded using the following equation: LE/(LE+RE), where LE and RE stand for number of spikes elicited by stimulation of left and right eye, respectively. An OD index of 1.0 indicates a neuron that is responsive only to the left eye whereas an OD index of 0.0 indicates responses only to the right eye. Figure 6D shows the cumulative distributions of neurons from alcohol-treated animals that were injected with Sindbis/SRF+ or Sindbis/GFP. Note that the curve of SRF+ animals is completely skewed to the right when compared to GFP controls. This finding confirms our results with optical imaging showing that SRF restores OD plasticity in alcohol-treated animals. Interestingly, these results were observed even when recordings were made far from the injection site (Fig. 2E). A dominance of the experienced (ipsilateral) eye was present even when cells were recorded 3.5-5.5 mm from the injection site.

To confirm the expression of the virus we conducted immunohistochemistry in tandem with confocal laser microscopy. Figure 7A shows the typical pattern of expression of the Sindbis/SRF+ virus. Note that cells positive for the VP16 transactivation domain can be seen in both gray and white matter (Fig. 7A). The presence of VP16 positive cells was observed in several sections and we calculated that the virus infection had an average diameter of 2300 µm (SEM ± 220, n = 3 animals). Interestingly, most VP16 positive cells could be identified as astrocytes due to double labeling with GFAP (Figs. 7B and C). Further quantification using two different techniques showed that most infected cells could be identified as astrocytes (Fig. 7D).

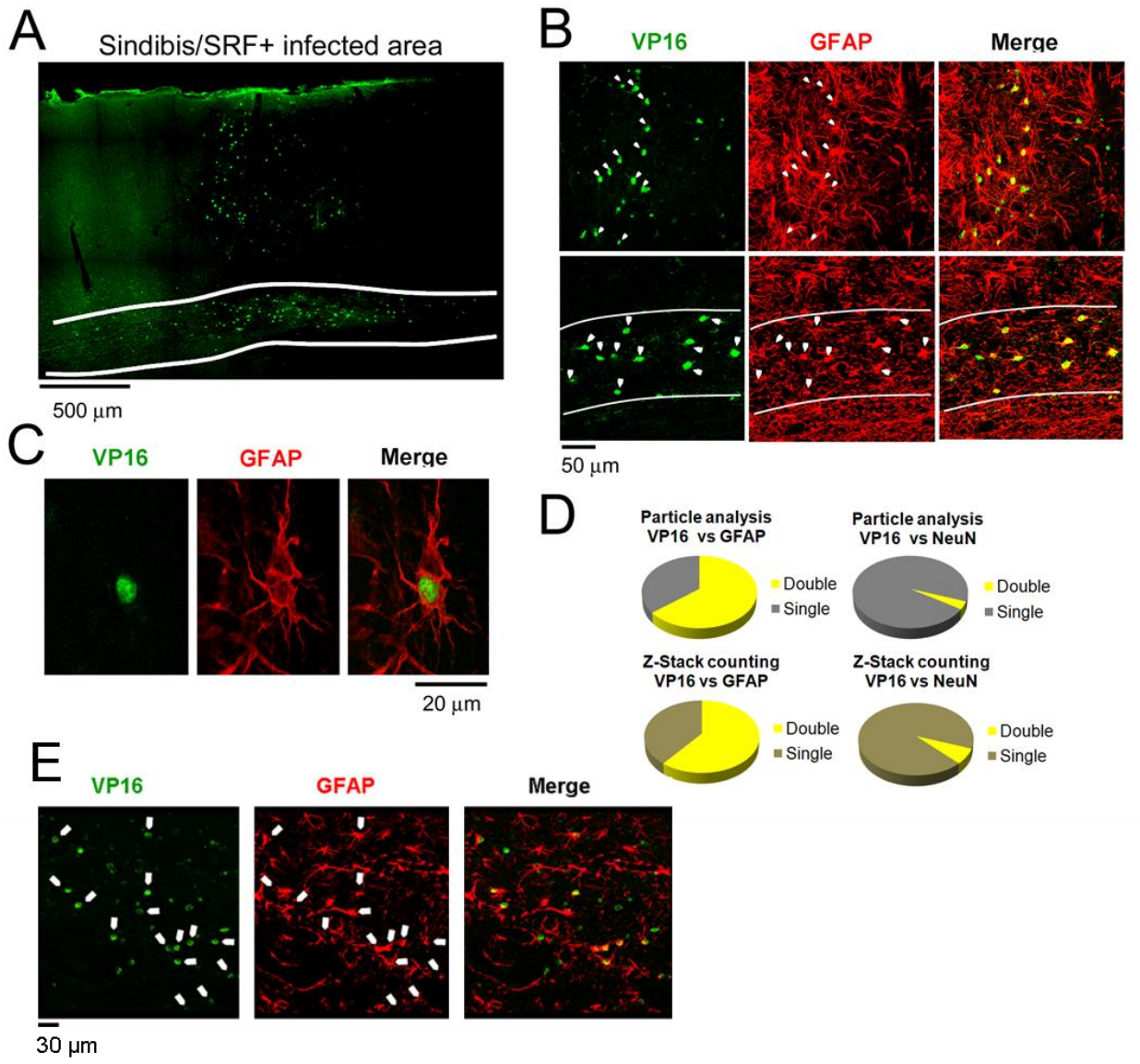

Figure 7. **Laser confocal microscopy**. It reveals that a cortical injection of Sindbis/SRF virus leads to successful expression of the transgenes. Most infected cells are astrocytes rather than neurons. **A**, Typical spread pattern of a Sindbis/SRF virus injection. Staining for the VP16 transactivation domain shows that the infection is present through all the columnar extent of the gray matter. Expression in the white matter (delimited by white lines) seems to be denser and spreads horizontally. **B**, Most cells positive for VP16 are also positive for the astrocyte marker GFAP. This was true for both gray and white matter (first and second rows, respectively). **C**, Image of an infected astrocyte. **D**, Quantification either by particle

analysis (PA) or Z-Stack double-labeling counting (ZS) shows that most of the cells infected by Sindbis/SRF virus are astrocytes rather than neurons. VP16/GFAP colocalization: PA = 64.4%, ZS = 60.8 %. VP16/NeuN colocalization: PA = 3.6%, ZS = 7.1%. **E**, Just 24 h after Sindbis/SRF+ injection, most of the VP16 positive cells are still positive for GFAP.

Surprisingly, only a small proportion of infected cells were positive for NeuN, a typical neuronal marker. One possible explanation for this difference is that the Sindbis virus could have infected and killed neurons during the first days of the infection. However, this possibility is unlikely since a similar pattern of infection (predominance of infected astrocytes rather than neurons) was observed after 24 h of the virus injection (Figure 7E). To evaluate the extension of the "plasticity enhancing" effect of SRF we made the virus injection on the contralateral (right) hemisphere of three alcohol-treated animals. After 4 d of MD, optical imaging of intrinsic signals of the left hemisphere was performed to assess changes in OD. In the animal showed in Figure 8A, no change in the typical ferret OD profile was observed. However, the virus injection was not successful as revealed by immunohistochemistry for VP16. In the second animal, a partial shift in OD and a moderate virus infection was observed (Fig. 8B). Finally, in the third animal, a strong shift in OD and a high degree of infection (in the white matter) was observed (Fig. 8C). These findings indicate a high correlation between Sindbis infection and OD shift, suggesting that overexpression of SRF might improve plasticity in contralateral hemisphere. The higher variability of the virus infection observed in this experiment might be due to the fact that all our equipment had to be adjusted to make the injections on the right hemisphere.

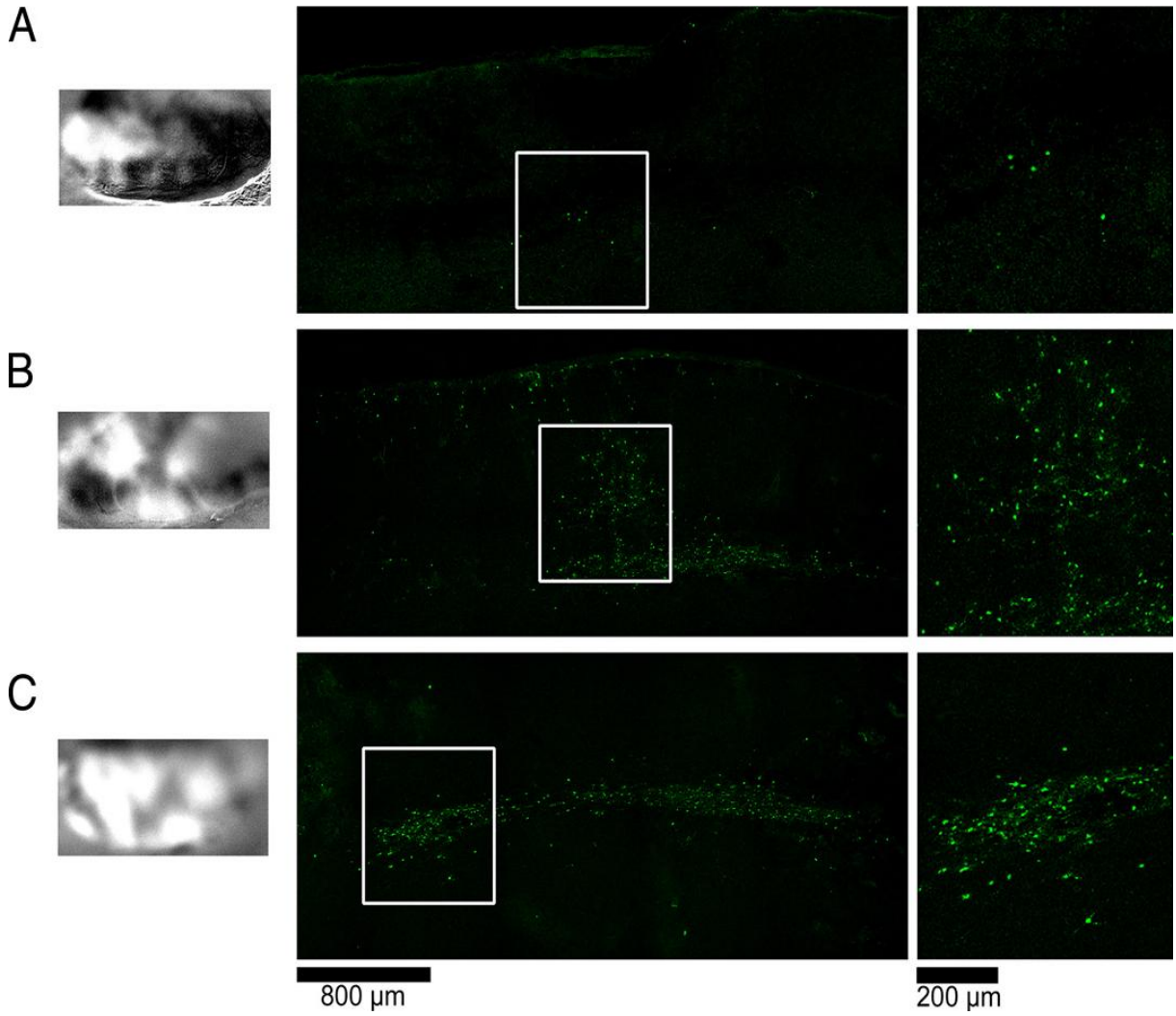

Figure 8. **Effects of Sindbis/SRF injection on contralateral hemisphere**, in three alcohol-treated animals (**A**-**C**), First column shows differential maps obtained with optical imaging of intrinsic signals after 4 d of MD. Second column shows areas of highest virus infection revealed by immunohistochemistry for VP16. Third column shows magnifications of insert boxes. Note the high correlation between infection area and experienced eye dominance (white areas in differential maps).

While it is known that SRF is normally expressed in the brain (Knoll and Nordheim, 2009; Stringer et al., 2002), we decided to assess whether endogenous

levels of this transcription factor could be detected in visual cortex (Figs. 9A-K). To accomplish that, we used a SRF antibody that tags to an amino acid sequence (aa 209-508, see Materials and Methods) that is not present in our SRF construct (Fig. 9G and H). Figure 9A shows strong SRF expression in all cortical layers. While SRF expression was also observed in white matter, staining was drastically reduced. This antibody does not stain our SRF construct, and no colocalization between SRF and VP16 was observed. Endogenous SRF expression was also observed in animals that have not received Sindbis-SRF injection (data not shown). We also confirmed previous observations (Knoll and Nordheim, 2009; Stringer et al., 2002) showing the SRF has a high and low expression in neurons and astrocytes respectively (Fig. 9D-F). Next we wanted to confirm that our Sindbis-SRF virus lead to SRF expression. To accomplish that we used an antibody that tags the amino acids around the lysine 99 phosphorylation sites (aa 97-101). This region is present in our SRF construct (Fig. 9G, H). Figures 9I-K show a high SRF expression in VP16 positive cells. This finding confirms that our construct induces expression of SRF and supports the use of VP16 as a marker in our previous experiments. As Sindbis virus is well known to infect neurons in rodents (Ehrengruber, 2002), we sought to test whether this preference for astrocytes in ferrets is due to species differences. Confirming previous results in rodents, the typical preference of the Sindbis virus for neurons was observed in cortex and hippocampus (Figure 10).

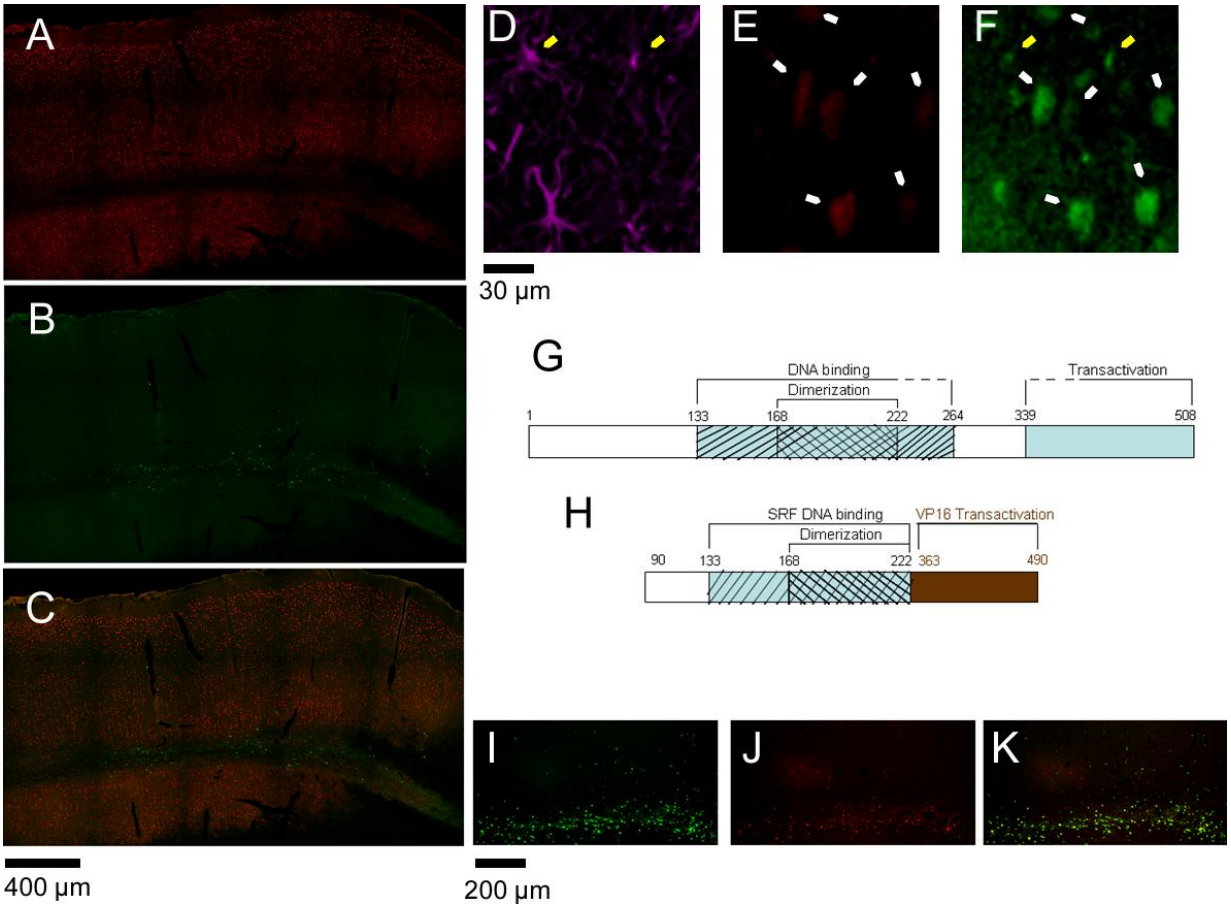

Figure 9. **Expression of endogenous SRF in the brain and the expression of SRF-VP16 by the virus**. **A**, Tile scan of the visual cortex of the ferret using an anti-SRF antibody (Santa Cruz Biotechnology). This antibody attaches to the 209-508 amino acid sequence of the SRF transactivation domain. **B**, Immunostaining for VP16 transactivation domain, which indicates cells infected by the virus. **C**, Merge of the 2 pictures. Note that there is no colocalization of the endogenous SRF with the cells infected. Laser confocal microscopy reveals that SRF is abundant in neurons and is also present in some astrocytes. **D**-**F**, Note that NeuN positive cells (**E**, red) are rich in SRF (**F**, green). White arrows in **E** and **F** indicate same cells. In GFAP positive cells (**D**, magenta) SRF expression is either reduced or absent. Yellow arrows point to same cells. **G** and **H**, Schematic representation of the SRF molecule (**H**) and our constitutively active SRF construct (**H**). The construct was made by fusing the dimerization and DNA binding domains of SRF (aa 90-222) with the HSV VP16 transactivation domain (aa 363-490). The resulting fused construct was inserted into a Sindbis vector. Note that the construct lacks most of the

sequence that is recognized by the SRF antibody used in **B** (aa 209-508). **I**-**K**, Using an SRF antibody that targets a sequence of amino acids that is present in our construct (aa 97-101, Anti-SRF, ABCam), we were able to demonstrate that overexpression of our construct indeed increases SRF expression, and that VP16 could be a good marker. **I**, VP16; **J**, SRF; **K,** merge.

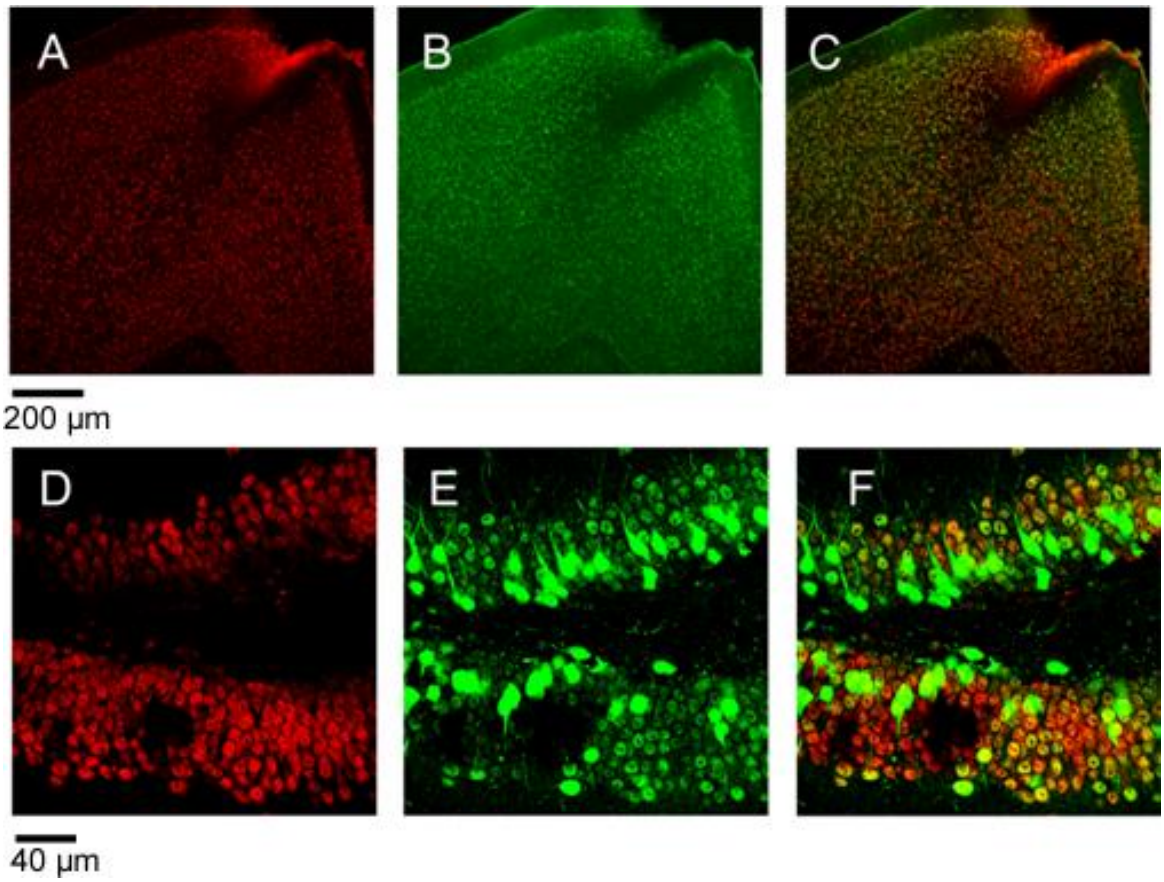

Figure 10. **Cell preference of the Sindbis virus is species dependent**. **A**-**C**, Intraventricular injection of 3µl of the sindbis virus resulted in a massive infection of visual cortex neurons in the mouse. **A**, NeuN; **B**, SRF; **C**, merge. **D**-**F**, Hippocampal injection of 1.5µl of the Sindbis virus resulted in a massive infection of neurons in the mouse hippocampus. **D**, NeuN; **E**, SRF; **F**, merge.

Discussion

Here we showed that overexpression of SRF by Sindbis virus, long after the period of alcohol exposure, rescues OD plasticity in the ferret model of FASD. In addition to this exciting result, we obtained two intriguing findings: First, in ferrets we did not see the Sindbis preference for neurons that was described for rodents (Ehrengruber, 2002). In fact our results showed that most of the cells infected were astrocytes rather than neurons. The preference for astrocytes seen here is probably due to species differences, since in mice the virus infected mostly neurons (Fig. 10). Second, plasticity restoration was observed in almost all V1 rather than being restricted to the area within the infection site. A possible interpretation is that released SRF products may diffuse through the neuropil, or are transported by the blood or CSF. While surprising, the capacity of brain cells to release signaling molecules and affect a large area had been demonstrated before. It was shown by independent groups that implantation of astrocytes overexpressing glial cell line-derived neurotrophic factor (GDNF) provide extensive neuroprotection to dopaminergic neurons (Cunningham and Su, 2002; Ericson et al., 2005). Moreover, the effects observed in these studies were not restricted to the injected site and increased levels of GDNF were observed even in the contralateral hemisphere (Cunningham and Su, 2002; Ericson et al., 2005). Recently, Stritt and colleagues provided evidence that activation of SRF can mediate the release of signaling molecules. They showed that lentiviral-mediated transfer of a constitutively active SRF construct in neurons dramatically affects maturation of oligodendrocytes (Stritt et al., 2009). In our present study, while most of the cells

infected by the Sindbis-SRF construct were astrocytes, we observed an effect on neuronal plasticity. This finding suggests the intriguing possibility that activation of SRF in astrocytes may result in the production and release of plasticity-related factors, boosting neuronal plasticity in an extensive cortical area. However, we cannot discard the possibility that the few infected neurons are actually either the major players or significantly contribute to the SRF-mediated restoration of ocular dominance plasticity in alcohol-treated animals.

Our understanding of astrocyte function and its relation to neurons has dramatically changed in the last 20 years. Modern neuroscience considers astrocytes major players in brain development and plasticity rather than mere supportive cells, see for review (Fellin, 2009; Stevens, 2008). In addition to producing an intricate pattern of spontaneous and neuronal evoked calcium oscillations, astrocytes can secrete glutamate (Parpura et al., 1994), D-serine (which potentiates the AMPA receptor) (Schell et al., 1995) and a constellation of plasticity related factors. Recently, studies from Ben Barres' lab provided convincing evidence that astrocytes can secrete molecules that can dramatically affect the establishment of synapses. For instance, this group showed that immature astrocytes can promote synaptogenesis through release of thrombospondins (Christopherson et al., 2005) and refinement of retinogeniculate synapses through secretion of C1q protein (Stevens et al., 2007). Astrocytes may also play an important role in synaptic plasticity. Recently, it was demonstrated that glial release of tumor necrosis factor alpha (TNFα) mediate the compensatory changes in synaptic strength that occur after an increase or decrease in global activity (homeostatic

plasticity) (Beattie et al., 2002). In addition, TNFα KO mice have altered OD plasticity (Kaneko et al., 2008).

Astrocytes have also been linked to the establishment of the critical period of ocular dominance plasticity. Christian Muller described that the time of the maturation of astrocytes is coincident with the closure of the critical period. In addition, implantation of immature astrocytes in the primary visual cortex of adult cats reopen the critical period of ocular dominance plasticity (Muller and Best, 1989). While these findings strongly indicated a role for astrocytes in the regulation of critical period, the mechanisms that underlie this process were never elucidated. Our findings here suggest that astrocytes may also contribute to the process of ocular dominance plasticity, perhaps through the release of plasticity-related factors, which could boost plasticity in neurons.

Since early alcohol exposure affects many cascades related to SRF activation (Davis et al., 1999; Luo and Miller, 1999; Kalluri and Ticku, 2003; Tsuji et al., 2003; Tang et al., 2006), as well as the secretory capacity of astrocytes (Tomas et al., 2005), it is plausible that a decrease of astrocytic-released molecules may contribute to the plasticity deficits seen in models of FASD. One possibility is that alcohol causes a reduction in the production and/or release of SRF-mediated molecules and the overexpression of this transcription factor bring these molecules to normal levels. This idea is supported by findings that show that early alcohol exposure can lead to an alteration in pathways that are known to activate SRF such as MAPK (Davis et al., 1999; Luo and Miller, 1999; Kalluri and Ticku, 2003; Tsuji et al., 2003; Tang et al., 2006) and RhoA (Joshi et al., 2006) as well as decrease the secretory capacity of astrocytes (Tomas et al., 2005). Alternatively, SRF function in astrocytes from alcohol-treated

animals might be normal, and the beneficial effects of SRF overexpression may be a result of a compensatory boost of plasticity caused by the release of many plasticity-related molecules.

In conclusion we show here that overexpression of SRF leads to a widespread plasticity enhancement in the visual cortex of ferrets exposed early to ethanol. Importantly, our results suggest the intriguing possibility that astrocytes are actually the major players in this restorative effect. Our findings contribute to our understanding of how early alcohol exposure affects astrocyte-to-neuron signaling and its effects on cortical plasticity. This information may be highly relevant to devise therapeutic interventions that will prevent or alleviate morbidity in FAS.

Chapter 3 - Overexpression of Serum Response Factor in Astrocytes Improves Neuronal Plasticity in a Model of Fetal Alcohol Spectrum Disorders

[Manuscript]

Arco P. Paul and Alexandre E. Medina

Department of Anatomy and Neurobiology, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298-0709

Key words: Astrocyte; Serum response factor; Sindbis virus; ocular dominance plasticity; visual development; ferret; monocular deprivation; optical imaging; neocortex development; fetal alcohol syndrome.

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Abstract

Neuronal plasticity deficits underlie many of the cognitive problems seen in Fetal Alcohol Spectrum Disorders (FASD). We have developed a ferret model showing that early alcohol exposure leads to a persistent disruption in ocular dominance (OD) plasticity. Recently, we showed that this deficit could be reversed by overexpression of serum response factor (SRF) in the primary visual cortex during the period of monocular deprivation (MD). Surprisingly, this restoration was observed throughout the extent of visual cortex and most of the cells transfected by the virus were positive for the astrocytic marker GFAP rather than the neuronal marker NeuN. Here we test whether overexpression of SRF exclusively in astrocytes is sufficient to restore OD plasticity in alcohol-exposed ferrets. To accomplish that, first we exposed cultured astrocytes to Sindbis viruses carrying either a constitutively active form of SRF (SRF+), a dominant negative (SRF-) or control GFP. After 24h, these astrocytes were implanted in the visual cortex of alcohol-exposed animals or saline controls one day before MD. Optical imaging of intrinsic signals showed that alcohol-exposed animals that were implanted with astrocytes expressing SRF, but not SRF- or GFP, showed robust restoration of OD plasticity in all visual cortex. These findings suggest that overexpression of SRF exclusively in astrocytes can improve neuronal plasticity in FASD.

Introduction

Alcohol consumption during pregnancy can lead to fetal alcohol spectrum disorders (FASD). This condition is characterized by a constellation of morphological, cognitive and behavioral problems with possible lifelong implications (Jones and Smith, 1973). In FASD, the sensory systems are particularly affected and learning and memory problems, as well as visual, somatosensory and auditory deficits are frequently observed (Mattson and Riley, 1998; Franklin et al., 2008; Manji et al., 2009; Coles et al., 2002).

The timing of alcohol exposure can be a determinant factor for the signs and symptoms observed in FASD. During the first-trimester equivalent of human gestation, alcohol exposure can alter normal development of the neural tube and crest, leading to microcephaly (Miller, 1996), hydrocephaly (Goez et al., 2011; Ronen and Andrews, 1991), ocular malformations (Stromland, 2004; Cook et al., 1987), and the facial dysmorphology that characterizes fetal alcohol syndrome (Sulik et al., 1981), a classical type of FASD. In the second trimester, alcohol exposure strongly affects the proliferation of neuronal precursors and the formation of the radial glia leading to abnormal neuronal migration (Vangipuram and Lyman, 2010; Tateno and Saito, 2008; Aronne et al., 2011; Miller and Robertson, 1993; Siegenthaler and Miller, 2004). Finally, during the third-trimester, alcohol can increase cell death, disrupt synaptogenesis and lead to persistent deficits on neuronal plasticity (Rema and Ebner, 1999; Medina et al., 2003; Climent et al., 2002; Moulder et al., 2002; Hoff, 1988; Isayama et al., 2009).

During the last decade, numerous studies have provided evidence that activity-dependent neuronal plasticity, a process crucial for the development of sensory cortices, is disrupted in animal models of FASD. For instance, early alcohol exposure has been shown to disrupt long term potentiation (Puglia and Valenzuela, 2010), eye-blink conditioning (Johnson et al., 2008), barrel cortex plasticity (Rema and Ebner, 1999) and ocular dominance plasticity (ODP) (Medina et al., 2003).

The ODP paradigm is based on the functional and anatomical changes that occur in the visual cortex after closing one eye by eyelid suturing (monocular deprivation, MD). In this paradigm, MD leads to shrinkage and expansion of OD columns receiving responses from the deprived (closed) eye and experienced (open) eye, respectively (Hubel et al., 1977; Issa et al., 1999; Wiesel And Hubel, 1963). As in the case of other types of activity-dependent plasticity, ODP relies on the integrity of the transmission of synaptic signals to the nucleus, where plasticity-related genes are regulated by transcription factors (Roberts et al., 1998; Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002; Mower et al., 2002).

Early alcohol exposure has been shown to affect the activity of many molecules that are important for ODP such as the NMDA receptor and many activity-dependent kinases (Savage et al., 1992; Joshi et al., 2006; Naseer et al., 2011; Davis et al., 2000). Many of these molecules are part of cascades that converge towards two important transcription factors, namely, cAMP response element binding protein (CREB) and Serum Response Factor (SRF) (Chai and Tarnawski, 2002; Knoll et al., 2006; Whitmarsh et al., 1995). While the role of CREB on activity-dependent plasticity is quite established (Silva et al., 1998; Mower et al., 2002), much less is known about the

contribution of SRF. This is surprising since both CREB and SRF can be activated by neuronal activity and both can regulate the expression of plasticity-related genes (Knoll and Nordheim, 2009; Ramanan et al., 2005; Lamprecht, 1999; Finkbeiner et al., 1997).

The transcription factor SRF has three functional domains: the dimerization and DNA binding domain, the transactivation domain and multiple phosphorylation sites (Chai and Tarnawski, 2002). It binds to its serum response element (SRE), which is present in the promoter regions of many genes. Some of the well known SRF target genes are IEGs (*c-fos, Egr-1* to *Egr-3, Junb,* etc.), actin cytoskeletal genes (*Actb, Actg1, Gelsolin, Arc,* etc.), axonal guidance genes (*Sema3a, Epha4, Epha7,* etc.), neurotrophin genes (*Bdnf, Ngf*) and genes involved in synaptic plasticity (Arc, Psd95) (Knoll and Nordheim, 2009; Etkin et al., 2006; Knoll et al., 2006). SRF has already been demonstrated to be essential for various forms of neuronal plasticity, including LTP (Ramanan et al., 2005), LTD (Etkin et al., 2006) and learning and memory (Etkin et al., 2006; Dash et al., 2005).

Recently, we used viral mediated gene transfer to test whether overexpression of SRF would reverse the disruption of ODP caused by early alcohol exposure in ferrets. We observed that during the period of MD, overexpression of a constitutively active form of SRF, but neither its dominant negative form nor GFP, restored OD plasticity in alcohol-exposed animals. Surprisingly, this restoration was observed throughout the extent of the visual cortex instead of being limited to the area of the virus transfection. Moreover, an analysis of the virus expression showed that while only 7% of the SRF-transfected cells expressed the neuronal marker NeuN, more than 60% cells showed expression of the astrocytic marker GFAP. Since glial cells can secrete

plasticity-related molecules, and because we observed that most of the transfected cells were GFAP positive, we hypothesized that overexpression of SRF in astrocytes can affect neuronal plasticity. However, we could not discard a major contribution of the few SRF-transfected neurons or the unidentified cells. Here we investigate whether overexpression of SRF in astrocytes is sufficient for the restoration of ODP in alcohol-exposed animals. To accomplish this, we overexpressed the constitutively active form of SRF in astrocyte cultures and implanted these modified cells in the visual cortex of ferrets exposed to alcohol during the third trimester equivalent of human gestation.

Materials and Methods

All procedures that are described in this article were approved by the Institutional Animal Care and use Committee at Virginia Commonwealth University. The protocols for some of the general experimental methods are the same as described in earlier papers (Medina et al., 2003; Medina et al., 2005; Paul et al., 2010). Please refer to Figure 11 for a summary of our experimental design. Briefly, ferrets were treated with 3.5 g/kg ethanol (25% in saline, i.p.) or saline as control every other day from postnatal day (P) 10 to P30. This alcohol treatment leads to a blood alcohol level of ~250 mg/dl for 1–5 h after injection (Medina et al., 2003). The animals were then left alcohol-free until the peak of the critical period of OD plasticity, which in ferrets is around P40 (Issa et al., 1999). Additional ferrets were sacrificed at P9/P10 and caudal portions of their brains containing the visual area were dissected to make pure astrocyte cultures. The cultures were transfected by Sindbis viruses carrying one of the following, a

constitutively active form of SRF (SRF+), a dominant negative form (SRF-) or control GFP.

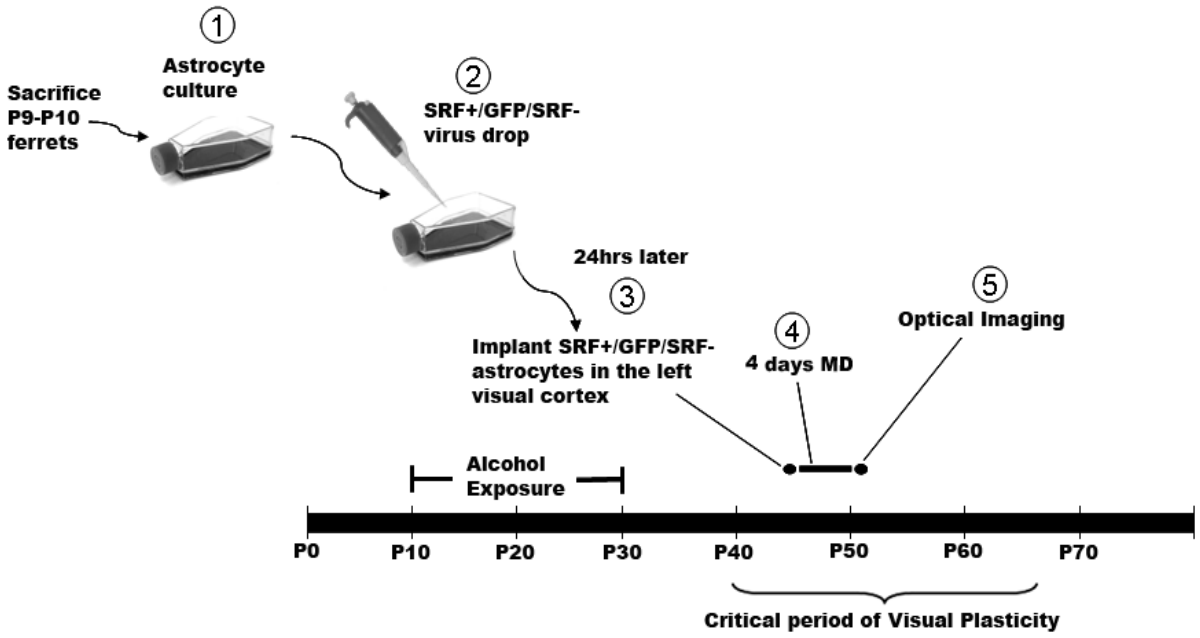

Figure 11. Experimental design. Pure astrocyte cultures are made from the occipital regions of ferret neonates (1). The astrocytes are then treated with the Sindbis SRF+, GFP or SRF- viruses for 24 hrs (2). These cells are implanted into the left visual cortex of animals exposed to alcohol during the third trimester-equivalent of human gestation (P10 – P30 in ferrets (Clancy et al., 2001). (3). Monocular deprivation (MD) is performed the following day by the suturing the right eyelids (4). After 4 days of MD, eyelids are opened and Optical Imaging of intrinsic signals are performed to assess Ocular Dominance plasticity (5).

After 24 hours of transfection, the astrocytes were implanted into the left visual cortex of the alcohol-exposed animals. One day after the implantation, the right eye of the animal was monocularly deprived (MD) by eyelid suture for 4 days. Following MD, the animal

was anesthetized, the deprived eye was opened and optical imaging of intrinsic signals was conducted on the left primary visual area.

Preparation of astrocyte cultures

The culture procedure is modified from the method established by McCarthy and de Vellis (McCarthy and de Vellis, 1980). Ferrets at P9 or P10 are sacrificed and the caudal portion of its brain is immediately cut out, cleaned from its meninges and put in a petri dish containing Hank's Balanced Salt Solution (HBSS) in sterile conditions inside a biosafety hood. The tissue block is minced to smallest possible pieces using a scalpel blade. The minced tissue is collected in a 15ml falcon tube with 3mls of 0.5% Trypsin-EDTA. The tube is gently agitated for 5-7 minutes. The tissue pieces are then mechanically triturated using a pipette to finer particles. Then serum media (DMEM/F12+10% fetal calf serum) is added to inactivate trypsin. The cell suspension is centrifuged at 3000 rpm for 3mins, resuspended in fresh serum media and plated in T-25 culture flasks (Falcon, BD). The flasks are kept in an incubator at 37^0C and 5% CO_2. The cells become confluent in 8-10 days. At that time, the flasks are agitated on an orbital shaker (250rpm, 37^0C, 12-15 hrs) in order to remove the oligodendrocytes and microglia. The supernatant is removed and an astrocyte purity of ~ 98% is achieved (McCarthy and de Vellis, 1980).

Ex-vivo gene delivery procedure

The Sindbis viral vectors were made and kindly provided by Drs. Amit Etkin, Angel Barco and Eric Kandel (Columbia University, New York, NY). The SRF+ construct

was made by fusing the dimerization and DNA binding domains of SRF (aa 90–222) with the transactivation domain of HSV protein VP16 (aa 363–490) (Paul et al., 2010). This construct was then cloned into the pSinRep5 vector (Invitrogen, Carlsbad, CA). The SRF- was made by fusing a FLAG epitope sequence to the N terminus of SRF (aa 1- 272), which was been found to effectively inhibit SRF activity (Etkin et al., 2006). The control GFP construct was made by cloning an EGFP cDNA into the vector.

The astrocyte media is replaced by serum-free DMEM and 10µls of a solution of the Sindbis virus carrying one of the constructs (SRF+, SRF- or GFP) (viral titer = 4.0 x 10^7 infectious units/ml). The virus is allowed to transfect the cells for 24 hours inside the incubator. After this period, the media is discarded and the cells are washed multiple times to remove any free viral particles. The cells are then dissociated from each other using 0.5% trypsin-EDTA and lifted from the floor of the flask using a sterile cell scraper (Falcon, BD). Trypsin is deactivated by adding serum media to the flask. The cell suspension is transferred to a 15ml falcon tube. The tube is centrifuged at 3000 rpm for 3 min and supernatant discarded. The cells are washed again with PBS. Finally, the cells are suspended in PBS in a 1ml microcentrifuge tube at a concentration of ~ 10,000-15,000 cells/µl.

These virus-transfected astrocytes are then immediately implanted into the left visual cortex of an alcohol or a saline-treated animal. For implantation, a small hole is drilled in the caudal end of the skull of an anesthetized animal. The tip of a 26 gauge syringe (Hamilton, Reno, NV)) containing the astrocyte suspension is stereotaxically positioned at an angle of 15–22° from the midline and lowered 1mm into the primary

visual cortex (V1). Two injections of 4µl each ~1mm apart are administered at a rate of 10 nl/s using a hydraulic microsyringe pump controller (Micro 4, WPI, Sarasota, FL).

Optical Imaging of intrinsic signals

Optical imaging of intrinsic signals is performed with Imager 3001 VSD+ (Optical Imaging Ltd., Rehovot, Israel) by using imaging methods similar to those described previously (Medina et al., 2003; Medina et al., 2006; Paul et al., 2010). Briefly, animals are premedicated with subcutaneous injections of a tranquilizer (acepromazine, 2mg/kg), a muscarinic antagonist (methyl atropine bromide, 0.2mg/kg) to reduce bronchial secretion, and dexamethasone sodium phosphate (0.5mg/kg) to reduce inflammation. Animals are then anesthetized using sodium pentobarbital (35mg/kg, i.p., Abbott Laboratories) and placed in a stereotaxic frame. No procedures are performed until the animal is sufficiently anesthetized, as determined by the loss of withdrawal and corneal-blink reflexes. A tracheal cannulation is performed, and the animal is placed on a ventilator. Heart rate, expired CO_2 and arterial blood oxygen saturation (SpO_2) are monitored continuously and maintained at ~4% and above 90%, respectively. Body temperature is maintained at 38^{o}C using a homeostatic blanket. The eyelids are opened, nictitating membranes are retracted using phenylephrine hydrochloride (2.5%), the pupils are dilated with atropine sulfate (1%), and contact lenses are placed on the corneas. A bigger craniotomy (3-4 mm) is performed around the hole already made for the astrocyte implantation to expose the dorsal area of the caudal end of occipital cortex. The duramater is reflected to expose the primary visual cortex, the opening is filled with agar (2.5% in saline) and covered with a glass coverslip. First, an image of

the vascular pattern is obtained by illuminating the cortical surface with a green filter (~550 nm) using a tungsten-halogen light source. Next, images of intrinsic signals are obtained using a red filter (~700nm). Visual stimulation consisting of high-contrast square wave gratings (8.75° dark phase/1.25° light phase) is generated by a 21-inch monitor (Sony Trinitron) using SGT+ graphics board and STIM software. Gratings are presented to each eye at an angle of 0°, 45°, 90°, or 135° and drifted (22.5°/s) in both directions along the axis orthogonal to the orientation of the grating. A single trial consists of these four gratings and a blank screen presented to each eye for 9 s in a pseudorandom sequence, with data acquisition during the last 8 s. Eye shutters (Optical Imaging Ltd.) are used to stimulate each eye separately. A total of 20 trials are performed for both eyes. For each eye, a monocular response/magnitude map is obtained by summing the different orientation images and subtracting that from the responses to the blank screen. In these images, dark areas correspond to regions that are visually responsive. The ocular dominance/differential maps are obtained by subtracting the summed left eye monocular maps from the summed right eye maps. In these images, dark and light regions are areas that respond predominantly to right and left eye stimulation, respectively.

To quantify the OD profile of each animal from the OD map, a region of interest (ROI) outlining V1 is drawn manually using the ImageJ software (NIH) by an investigator who is blind to the animal's treatment. The contralateral eye band, which marks the anterior border of V1 and lies caudal to the big V2 ipsilateral modules (White et al., 1999), is used as the rostral reference to draw the ROI. The caudal reference is the caudal pole. Next, each pixel in the ROI is assigned a gray value on a grayscale of 0 –

255. In this scale, the darkest pixel has a value of 0 and it responds only to the right eye, whereas, the lightest pixel has a value of 255 and it responds only to the left eye. An ocular dominance histogram is created by dividing the pixels into 5 class intervals of gray values, namely, 0-51, 52-102, 103-153, 154-204, 205-255.

Immunohistochemistry and confocal microscopy

Ferrets are perfused with cold saline followed by cold 4% paraformaldehyde solution. After dissection of the occipital cortex, 50 µm coronal sections are obtained using a vibratome. For immunohistochemistry, the following primary antibodies are used: GFAP (1:500, mature astrocytic marker, rabbit; Dako); Vimentin (1:500, Immature astrocytic marker, mouse-Dako, rabbit-Abcam), VP16 (1:200, Sindbis/SRF+ expression marker, mouse; Santa Cruz Biotechnology), FLAG (1:200, Sindbis/SRF- expression marker, mouse; Sigma). The secondary antibodies are Alexa Fluor 594 (1:400, anti-rabbit, Invitrogen) and Alexa Fluor 488 (1:200, anti-mouse, Invitrogen). Tissue sections are alternately double-stained for VP16/GFAP and VP16/Vimentin in SRF+ astrocyte implanted animals, for GFAP and Vimentin in GFP astrocyte implanted animals and for Flag/GFAP in SRF- astrocyte implanted animals. Sections are visualized in a Leica TCS-SP2 AOBS confocal laser scanning microscope for colocalization of VP16/GFP/Flag with GFAP/Vimentin.

Results

We confirmed the purity of our astrocyte cultures by observing expression of vimentin (Dako) and GFAP (Dako) in almost all of the cells (Figure 12). Quantification showed that ~98% of the cells could be identified as astrocytes, Similar purity of astrocytes were obtained by different groups using the same protocol (Coco et al., 2003; Guizzetti et al., 1996). We also found that the Sindbis virus robustly transfects astrocytes in culture. We used an antibody against VP16 (Santacruz) to identify the expression of the SRF-VP16 transgene (Paul et al., 2010) (Figure 13).

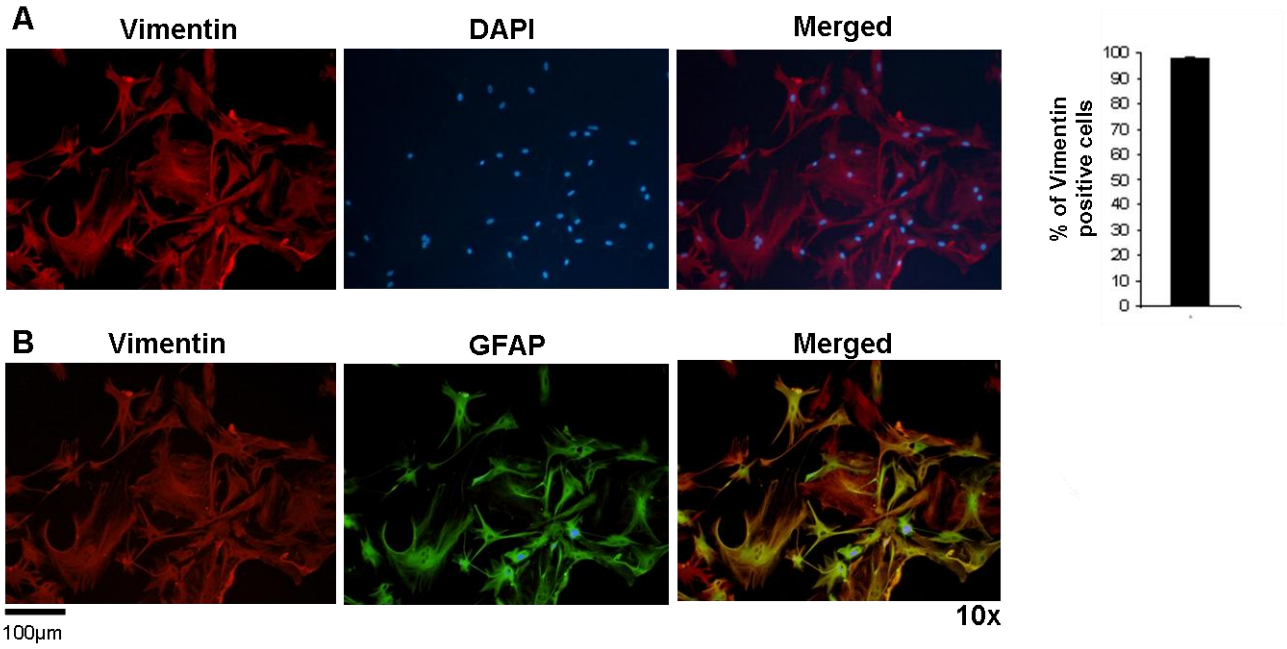

Figure 12. Immunohistochemistry for vimentin (red) and GFAP (green) confirms that the astrocyte cultures are pure. In **A**, the cultures are stained for Vimentin and DAPI. Quantification was done using 9 images from 3 different culture wells, which showed that 98% of the cells are vimentin-positive. In **B**, the same astrocytes also express GFAP (marker for mature astrocytes).

After transfecting the cultured astrocytes by SRF+, SRF- or GFP, cells were implanted in the left visual cortex of the alcohol-exposed animals. One day after the astrocyte implantation, the right eye of the animal was monocularly deprived for 4 days. After the period of MD, the deprived eye was opened and optical imaging of intrinsic signals was conducted on the left primary visual cortex.

Figure 13. VP16 immunoreactivity reveals that astrocytes show robust expression of SRF by the Sindbis virus particles after 24 hours of transfection.

Figure 14A shows the left visual cortex of representative cases from each group of animals. Dark and light areas in OD maps represent regions that responded preferentially to the stimulation of the right and the left eye, respectively. In the magnitude maps, dark regions represent the strength with which each area responded to visual stimulation of each eye. As expected, MD in saline-treated control animals showed a clear shift in ocular dominance towards the left experienced eye, as evident by the predominantly light regions in the V1 area of the OD map. Notably, we saw a similarly prominent OD shift following MD in alcohol-exposed animals that were implanted by astrocytes expressing SRF+ (Figure 14A), indicating that plasticity was

restored. In contrast, alcohol-exposed animals that were implanted with astrocytes expressing SRF- or GFP continued to show responses from both eyes following MD, as evident from the presence of comparable amounts of light and dark areas in V1. This also confirmed our previous findings that early alcohol exposure impairs OD plasticity (Medina et al., 2003; Medina and Ramoa, 2005). To quantify the OD profile of each animal from the OD map, we obtained a histogram of intensity distribution of the pixels in the V1 area in a 0-255 grayscale.

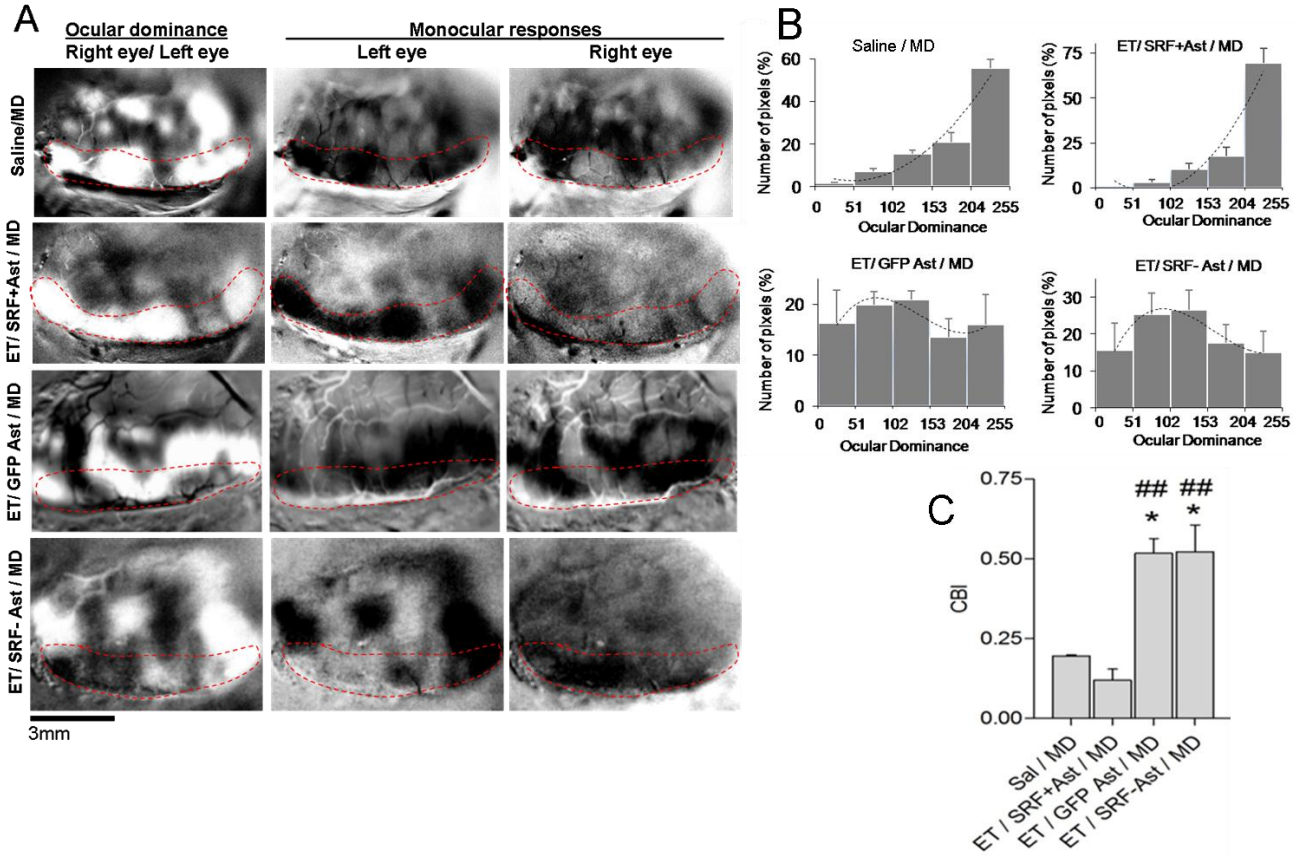

Figure 14. Optical imaging of intrinsic signals. It shows that implantation of astrocytes that overexpress SRF restores ocular dominance plasticity in alcohol-exposed animals. **A,** The first column shows ocular dominance maps of representative cases of each experimental group. Dark and light areas represent regions dominated by the right and left eye respectively. In animals implanted by the SRF-

overexpressing astrocytes (SRF+), monocular deprivation of the right eye resulted in a shift in dominance towards the left eye. In contrast, in animals implanted by astrocytes that overexpressed either GFP or the dominant-negative form of SRF (SRF−), the deprived eye remained responsive. **B**, Histograms display the percentage of pixels fitting in a grayscale (0-Black, 255-white). Note the predominance of lighter pixels in animals implanted by the SRF+ astrocytes, which indicates left (experienced) eye dominance. In contrast, note the predominance of black and gray pixels in animals implanted by astrocytes that were overexpressing either GFP or a dominant negative form of SRF, which indicates that the right (deprived) eye is still responsive. Saline/MD, n = 2; ET/SRF+Ast/MD, n = 4; ET/GFP Ast/MD, n = 4; ET/SRF−Ast/MD, n=4. **C**, Mean contralateral bias indexes for all groups. ET/SRF+Ast/MD animals presented significantly lower CBIs than ET/GFP Ast/MD or ET/SRF−Ast/MD. The statistical significance was established using one-way ANOVA followed by Bonferroni post-hoc analysis (*,$p< 0.05$ versus Sal/MD. ##, $p<0.01$ versus ET/SRF+Ast/MD). These findings indicate that overexpression of SRF in astrocytes can rescue plasticity in alcohol-exposed animals.

We then divided this grayscale into 5 class intervals, where 0-51 and 205-255 are classes containing the darkest and lightest pixels, respectively. An average histogram distribution was calculated by combining the values of all the animals in each group (Figure 14B). Accordingly, after 4 days of MD of the right eye in the saline-treated animals, the OD histogram was skewed towards the right, indicating the dominance of responses from the left experienced eye. The alcohol-exposed animals that were implanted by the SRF+ astrocytes also presented an OD histogram skewed similarly towards the right following same period of MD of the right eye, indicating restoration of normal OD plasticity. In contrast, alcohol-exposed animals that received astrocytes expressing either GFP or SRF- did not show this shift, indicating that they continued to

show good responses from the deprived eye. In order to further quantify these differences, we created a contralateral bias index (CBI), which is calculated as follows: $[(P_{0\text{-}51} - P_{205\text{-}255}) + \{(P_{52\text{-}102} - P_{154\text{-}204})/2\} + 100]/200$, where $P_{A\text{-}B}$ denotes the percentage of pixels with gray values between A and B. A CBI has values between 0 and 1. Lower CBI values indicate prevalence of lighter pixels and left eye dominance. Higher CBI values indicate prevalence of darker pixels and right eye dominance. A one-way ANOVA followed by Bonferroni *post-hoc* analysis showed that alcohol-exposed animals that were implanted by the SRF+ astrocytes had significantly smaller CBI values than the alcohol-exposed animals that received astrocytes expressing either GFP or the SRF- ($p<0.01$) (Figure 14C).

To confirm the successful implantation of the astrocytes in the visual cortex, we perfused the animals after optical imaging experiments, sectioned the visual cortex coronally and then identified the implanted astrocytes by using immunohistochemistry and confocal microscopy. Implanted cells could still be observed after 5 days of implantation (Figures 15A and B). Figure15C shows a highly magnified image of an implanted astrocyte expressing SRF. To further investigate the initial spread of implanted cells, an additional animal was perfused after 24h of implantation. Figure 16 shows the track of two implantation injections in a coronal section of the visual cortex as seen by the expression of the SRF-VP16 transgene. Figures 17A and B show the spread of implanted astrocytes in an adjacent section. Astrocytes were observed in all cortical layers and in the white matter. The reduced number of cells observed after 5 days of implantation compared to 24h (compare Figs. 15A with 17A), suggests that implanted cells are labile.

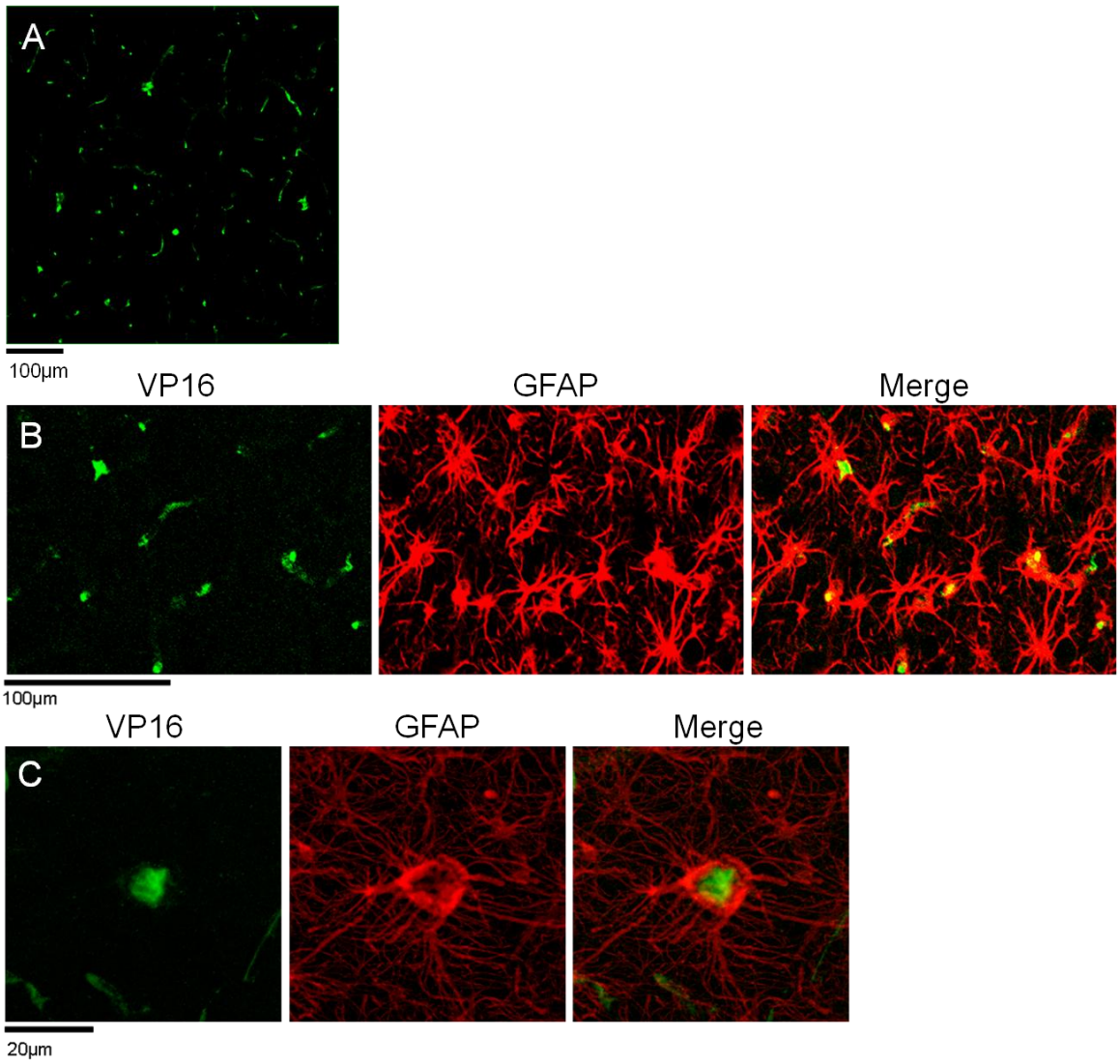

Figure 15. **Spread of implanted astrocytes after 5 days**, as shown by **A**, the expression of SRF-VP16. **B**, A higher magnification showing the cells express GFAP. **C**, An implanted astrocyte expressing SRF.

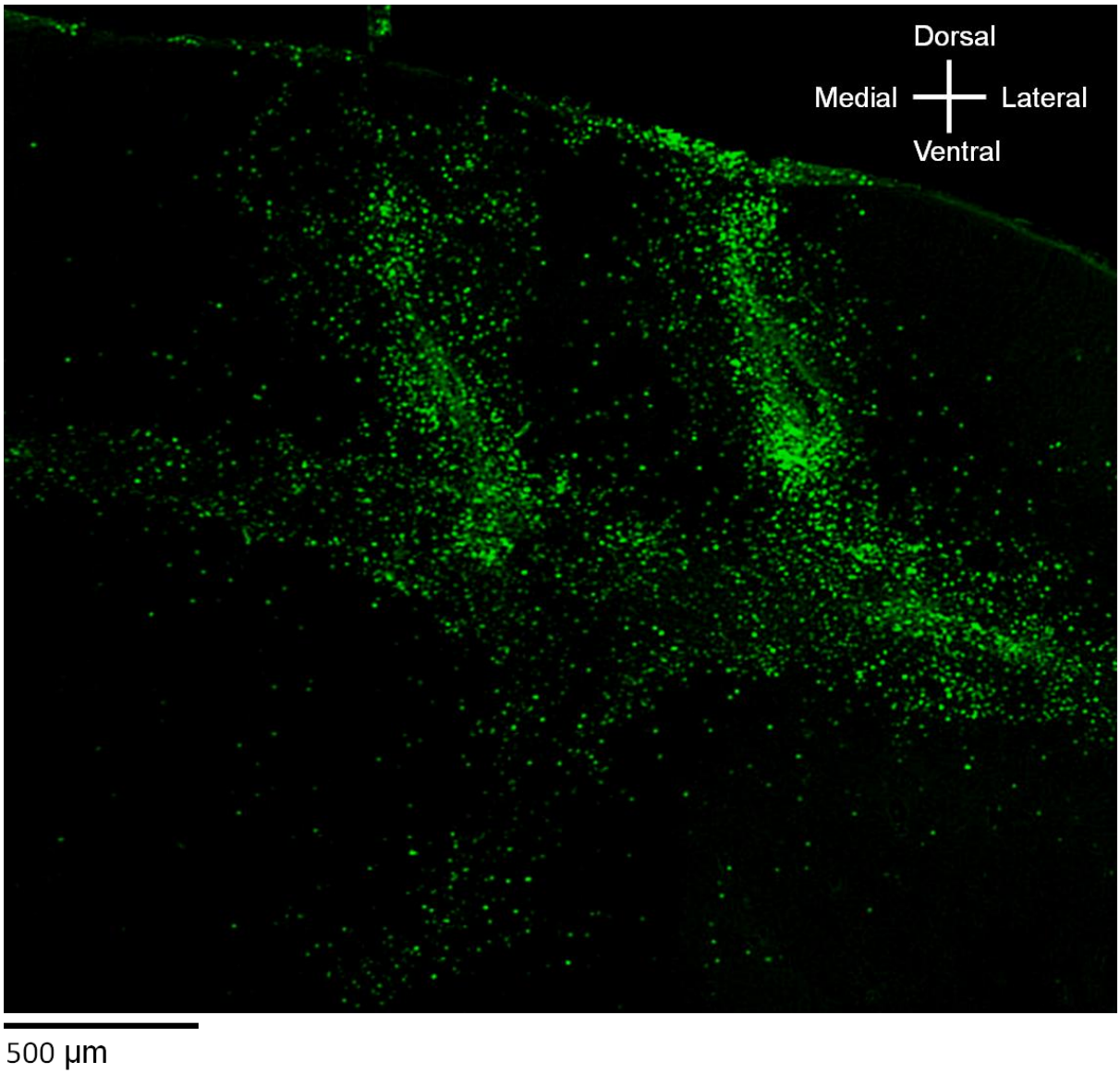

Figure 16. **Spread of implanted astrocytes after 24 h**. A coronal section of the visual cortex showing the track of the two injections 24h after implantation. The two injections were administered from the dorsal surface. It shows the spread of the implanted cells as seen by the expression of the SRF-VP16.

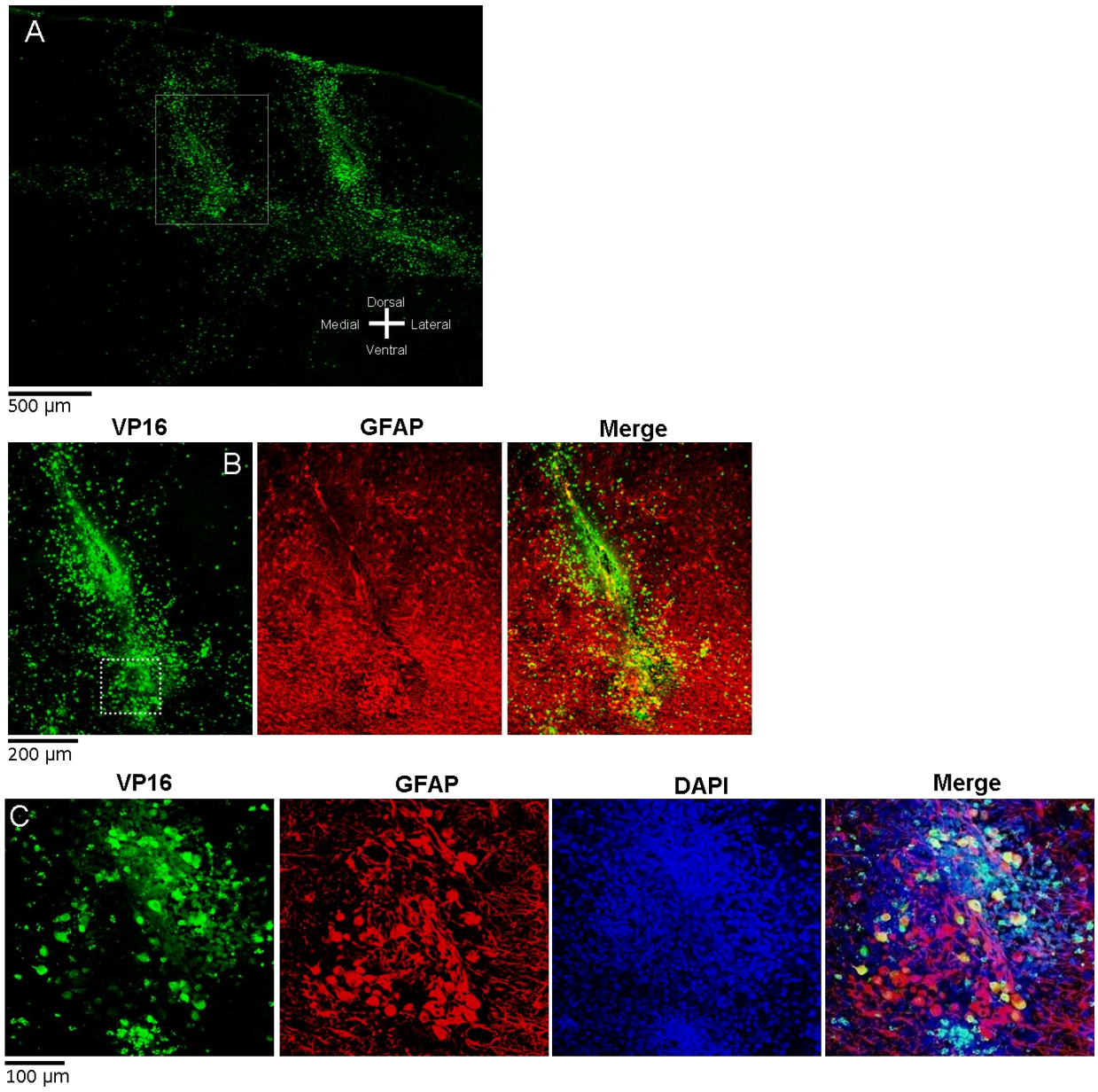

Figure 17. Many of the implanted cells show good colocalization with GFAP. A, Spread pattern of the implanted astrocytes after 24h, expressing SRF-VP16 (same image as figure 16). **B**, A higher magnification of one of the injection sites in the boxed area of **A**. **C**, Many of the VP16 positive implanted cells can be identified as astrocytes by their colocalization with GFAP, as shown by magnifying the boxed area in **B**.

Discussion

In this study, we showed that implantation of astrocytes that overexpressed SRF led to restoration of OD plasticity in alcohol-exposed ferrets. These findings support our previous study (Paul et al., 2010) and suggest that the expression of the transcription factor in astrocytes is sufficient to reverse the detrimental effects of early alcohol exposure in the visual cortex plasticity.

Confirming our previous findings, the optical imaging showed that the plasticity was restored in an extended visual area (Paul et al., 2010). Since the implanted astrocytes were restricted around the injected sites, it strongly suggests that astrocytes might be releasing neuroactive products over a wide cortical area upon activation by SRF. The capacity of astrocytes to produce effects in extensive areas has been previously demonstrated. Implantation of astrocytes that were made to constitutively overexpress glial cell line-derived neurotrophic factor (GDNF) using retroviral plasmids or lentiviral vectors, provided widespread protection of dopaminergic nigrostriatal neurons in mouse or rat models of Parkinson's disease (Cunningham and Su, 2002; Ericson et al., 2005). Interestingly, GDNF levels were found to be increased in brain regions both proximal and distal to the implantation site and even in the contralateral hemisphere, even though astrocyte migration away from the site did not occur.

While astrocytes cannot fire action potentials, they are responsive to neuronal activity and can influence neurotransmission. In-vivo studies have shown that astrocytes in the olfactory, somatosensory and visual cortex respond to sensory activity by transient Ca^{2+} increases (Schummers et al., 2008; Petzold et al., 2008; Wang et al.,

2006). Moreover, astrocytes express a wide variety of neurotransmitter receptors, which upon stimulation activate phospholipase C that in turn produce inositol triphosphate (IP_3). IP_3 induces the release of Ca^{2+} from the intracellular stores (Fellin, 2009; Araque et al., 2002). The Ca^{2+} signal can propagate to neighboring astrocytes as intercellular Ca^{2+} waves via gap junctions involving multiple cells in an extensive area (Cornell-Bell et al., 1990; Scemes and Giaume, 2006; Agulhon et al., 2008). Besides being connected to each other, astrocytes also directly contact nearby neurons at thousands of synapses (Bushong et al., 2002) to process synaptic transmission and plasticity (Perea et al., 2009; Stevens, 2008). Intracellular calcium elevations can also stimulate the release of neurotransmitters from astrocytes, as has been described for glutamate (Reyes and Parpura, 2009), ATP (Coco et al., 2003) and D-serine (Mothet et al., 2005). Glutamate and ATP released from astrocytes has been shown to signal to other unconnected synapses by diffusing through the neuropil (Navarrete and Araque, 2010; Serrano et al., 2006).

Astrocyte-released molecules have been shown to be important for various forms of neuronal plasticity. A prototypic form of plasticity, LTP, has also been shown to be influenced by the release of D-serine from astrocytes (Henneberger et al., 2010). Moreover, astrocyte-secreted thrombospondins, which is a product of SRF activation (Framson and Bornstein, 1993), has been found to be responsible for synaptic plasticity and functional recovery after stroke (Liauw et al., 2008a). Another study demonstrated that the release of TNFα from astrocytes contributes to homeostatic plasticity by synaptic scaling (Stellwagen and Malenka, 2006). Mostly importantly, a report showed

that the TNFα-mediated synaptic scaling is involved in OD plasticity (Kaneko et al., 2008).

It is quite established that astrocytes can produce several proteins that are regulated by SRF. However, it is puzzling that the basal levels of this transcription factor are very low in astrocytes *in vivo* (Knoll and Nordheim, 2009; Paul et al., 2010). One possibility is that SRF is expressed in astrocytes only when a particular set of proteins need to be produced. This possibility is supported by the fact that SRF can regulate its own transcription, which could lead to a rapid increase of the levels of this transcription factor in a positive feedback manner (Philippar et al., 2004). Another more speculative possibility is that astrocytes have a variant of SRF that cannot be identified by the commercially available antibodies.

In conclusion, our study shows that enhancement of SRF function in astrocytes can rescue neuronal plasticity that is impaired by early alcohol exposure. It supports the prevailing theory that astrocytes actively process and modulate neuronal functions by releasing various kinds of neuroactive substances. Future studies investigating what molecules and what mechanisms underlie the restorative effect seen here may contribute to the development of new interventions to improve neuronal plasticity in FASD.

Chapter 4 - Conclusion and future directions

In the first part of this project, we used a Sindbis viral vector to overexpress a constitutively active form of the transcription factor SRF in ferrets exposed to early alcohol exposure. Using optical imaging of intrinsic signals in tandem with extracellular electrophysiology (single-unit recordings) we showed that overexpressing SRF restored OD plasticity in a large area of the visual cortex in the experimental animals. Importantly, OD plasticity was still impaired in alcohol-treated animals that received the dominant negative form of SRF or the control GFP. Interestingly, we found that the Sindbis virus transfected mostly astrocytes and only a few transfected cells could be identified as neurons. This suggested a role of astrocytes in the SRF-mediated rescue of plasticity. But we could not discard the possibility of a role of the few transfected neurons in this restorative effect.

In the second part, we investigated whether the enhancement of SRF function exclusively in astrocytes is sufficient to rescue the impairment in visual plasticity. Here we first transfected cultured astrocytes with the Sindbis SRF+ virus *in vitro*. Then we implanted these astrocytes into the visual cortex of ferrets that had been exposed to alcohol during the third trimester- equivalent of human gestation. Using optical imaging of intrinsic signals we found that implantation of 'SRF overexpressing' astrocytes

restored the plasticity deficit in our FASD model. Again, even though the implanted astrocytes were restricted to the injection sites, the plasticity was restored in an extensive visual area.

General Hypothesis

The overall finding from the two parts of the project can be summarized as - overexpression of SRF in astrocytes results in a widespread restoration of OD plasticity in ferrets subjected to early alcohol exposure. This has led us to the following two working hypotheses regarding why plasticity was restored and why this effect was observed in an extensive area. In the first, we hypothesize that the SRF dependent restoration of plasticity is due to a cell-to-cell signaling. In the second, we hypothesize that this restoration is due to the production and release of SRF dependent molecules in the neuropil.

Upon stimulation by SRF, astrocytes may activate some plasticity-enhancing factors which spread from one to cell another through physical connections, thus having a cascading effect on astrocytes and neurons of a large area. This possibility is supported by the fact that astrocytes contact other astrocytes and neurons by gap junctions (Fellin, 2009; Rouach et al., 2004). Moreover, astrocytes form a highly interconnected network and propagate calcium signals over large distances (Fellin, 2009). Importantly, these calcium signals can be triggered by changes in sensory activity, including visual stimulation (Schummers et al., 2008; Wang et al., 2006).

Alternately, SRF activation may induce signaling pathways in astrocytes to produce and release neuroactive substances, which can diffuse through the neuropil and enhance plasticity in a wide area. The immense secretory capacity of astrocytes has been demonstrated previously. In one such experiment, 'GDNF-overexpressing' astrocytes were implanted in one cerebral hemisphere, and increased levels GDNF were detected even in the contralateral hemisphere in the absence of astrocyte migration (Cunningham and Su, 2002). Some of the plasticity-related factors that are known to be secreted by astrocytes include glutamate, GABA, ATP, D-serine, BDNF, NGF, thrombospondins, TNFalpha and components of extracellular matrix like matrix metalloproteinases (MMP) and chondroitinsulphate proteoglycans (CSPG) (Henneberger et al., 2010; Liauw et al., 2008a; Beattie et al., 2002; Reyes and Parpura, 2009; Lee et al., 2011; Jean et al., 2008; Zhang et al., 2003; Faissner et al., 2010; Ralay Ranaivo et al., 2011; Middlemiss et al., 1995). While many of these molecules affect plasticity by modulating synaptic physiology (Jourdain et al., 2007; Henneberger et al., 2010), some are involved in structural remodeling of synapses (Faissner et al., 2010). Among these, BDNF, NGF, thrombospondins and MMPs-2 and -9 are most probable candidates as they can be produced following activation of SRF (Chai and Tarnawski, 2002; Etkin et al., 2006; Middlemiss et al., 1995). In addition, alcohol exposure has been shown to perturb the secretory capacity of astrocytes (Tomas et al., 2005). It was demonstrated that ethanol exposure led to retention of proteins in various stages of the secretory pathway (RER-Golgi-plasma membrane), causing delay in their release. This impairment occurred because of alcohol-induced alteration of Golgi complex morphology, downregulation of the proteins required for vesicular trafficking and

disruption of microtubule assembly. In this case, the 'SRF overexpressing' implanted astrocytes might be compensating for the damaged endogenous astrocytes.

Both of these hypothetical processes might occur simultaneously, which could have a synergistic effect on the spread plasticity.

Future directions

Investigation of morphological and functional contacts between implanted and endogenous astrocytes

One way to refute the 'cell-to-cell' hypothesis would be to demonstrate that the implanted astrocytes are not incorporated into the astrocytic network. Since the implanted astrocytes remained in the brain for only five days before we assessed OD plasticity, it is conceivable that they never grew functional processes and never formed gap junctions with adjacent cells. If this is the case, the restorative effect caused by SRF may not be caused by a cell-to-cell transmission, favoring our alternate hypothesis of the release of plasticity-related molecules in the neuropil. A possible experiment would be to make cultured slices from animals that were implanted with GFP-transfected astrocytes. After locating an implanted cell, we can inject a dye that is small enough to pass through gap junctions (such as Fluorescein, Lucifer yellow or biocytin (Schools et al., 2006)) and observe the extent to which the dye reached the other endogenous cells. If we do not find any dye in the neighboring cells, this should indicate that the implanted cells were not incorporated in the network and the cell-to-cell

mechanism hypothesis would be unlikely. If we find the dye to spread out to other cells, it can provide a structural substrate for the spread of plasticity.

In order to show functional connectivity between the implanted and endogenous astrocytes, we can also implant GFP-transfected astrocytes into the visual cortex and make slice cultures as mentioned before. Then we can load the slices with calcium indicator dyes, like Fluo-3 or Oregon Green Bapta (Schummers et al., 2008; Charles et al., 1991b). We can then stimulate the implanted cell electrically or mechanically to activate it. Electrical or mechanical stimulation of astrocytes by using micropipettes have been used previously to produce calcium waves in neighboring astrocytes (Charles et al., 1991a; Rao and Sikdar, 2007; Araque et al., 1998). We can visualize the extent of increase in calcium activity in the neighboring astrocytes by using two-photon microscopy.

It is important to emphasize that even if we observe functional connectivity between implanted and endogenous cells, we cannot discard the alternate hypothesis of secretion of diffusible proteins from astrocytes.

Investigation of release of molecules from implanted astrocytes

In order to probe the possibility of secretion of SRF dependent molecules into the neuropil, we can implant astrocytes transfected by Sindbis SRF+ virus and loaded with radioactive amino acids like 35S-methionine or 3H-leucine (Barbin et al., 1988; Mellon et al., 1989). We can then implant these cells in the visual cortex. The radioactive amino acids would be incorporated in the proteins made by these astrocytes. If these proteins are released into the neuropil, we would be able to visualize the extent of their spread

using autoradiography (Plunkett et al., 1989). We can also counter-stain the slices with markers for astrocytes or neurons to detect if these proteins are taken up by neighboring cells. We can compare the extent of the spread of radioactivity by implantation of 'SRF expressing' astrocytes versus the control astrocytes.

Another way to show that implanted astrocytes are releasing plasticity factors is by infusing conditioned media from the cultured SRF transfected astrocytes into the visual cortex of alcohol-exposed animals during the period of MD. This could be accomplished by an intracortical infusion using an osmotic minipump. A possible pitfall of this experiment is that the SRF dependent plasticity-related molecules might be found in the media in very low concentrations. We can circumvent that possibility by using filtration devices to produce highly concentrated media.

Investigation of candidate plasticity factors released by astrocytes

As mentioned before, some of the different class of plasticity-related factors that are known to be released by astrocytes are neurotransmitters (Lee et al., 2011; Montana et al., 2006; Panatier et al., 2006), neurotrophic factors (Jean et al., 2008), cytokines (Beattie et al., 2002) and other kinds of secreted extracellular proteins (Christopherson et al., 2005). Indeed, SRF activation has been shown to be involved in the production of some of these plasticity-enhancing molecules (Chai and Tarnawski, 2002; Etkin et al., 2006; Middlemiss et al., 1995).

In order to identify plasticity-related factors that are being released by astrocytes upon activation by SRF, we can collect the media from the astrocyte cultures that have been transfected by the SRF+ virus or the control GFP virus. We can then perform a

multiple antibody ELISA on the media by using a set of antibodies against the most probable proteins that might be released from astrocytes. An ideal set of antibodies would consist of a mix of neurotransmitters, neurotrophic factors, cytokines, matrix metalloproteinases and CSPG proteins, which are known to be released by astrocytes. We would compare the results of the SRF media and the control media (both SRF- and GFP) to determine candidate molecules. Multiple antibody ELISA kits are available commercially (www.raybiotech.com) and they can be custom made to detect different class of proteins.

Investigation of the requirement of neurons in restoration of OD plasticity

Finally, in order to prove that astrocytes are necessary for the SRF mediated restoration of plasticity, we have to show that 'SRF overexpressing' neurons cannot restore OD plasticity in alcohol-exposed ferrets. In order to accomplish that we have acquired a new HSV virus that overexpress the same constitutively active form of SRF (HSV-SRF+) from Rachael Neve of MIT. This virus transfects mostly neurons in the ferret visual cortex (Figure 18). If overexpression of SRF in neurons is unable to rescue OD plasticity, it would mean that astrocytes are necessary for the restoration. It is possible that astrocytes have different cofactors that together with SRF lead to expression of specific proteins. If, on the other hand, the 'SRF overexpressing' neurons restore OD plasticity, we can say that both astrocytes and neurons are capable of restoring plasticity by themselves. Although this conclusion might attenuate the cruciality of astrocytes, our previous findings still show a role of astrocyte-to-neuron communication in process of neuronal plasticity

In conclusion, successful identification of the factors involved in the SRF mediated plasticity restoration would unravel a previously unknown role of astrocytes in neuronal plasticity. It might also bring us one step closer to finding potential therapeutic avenues for curing neurobehavioral deficits observed in FASD.

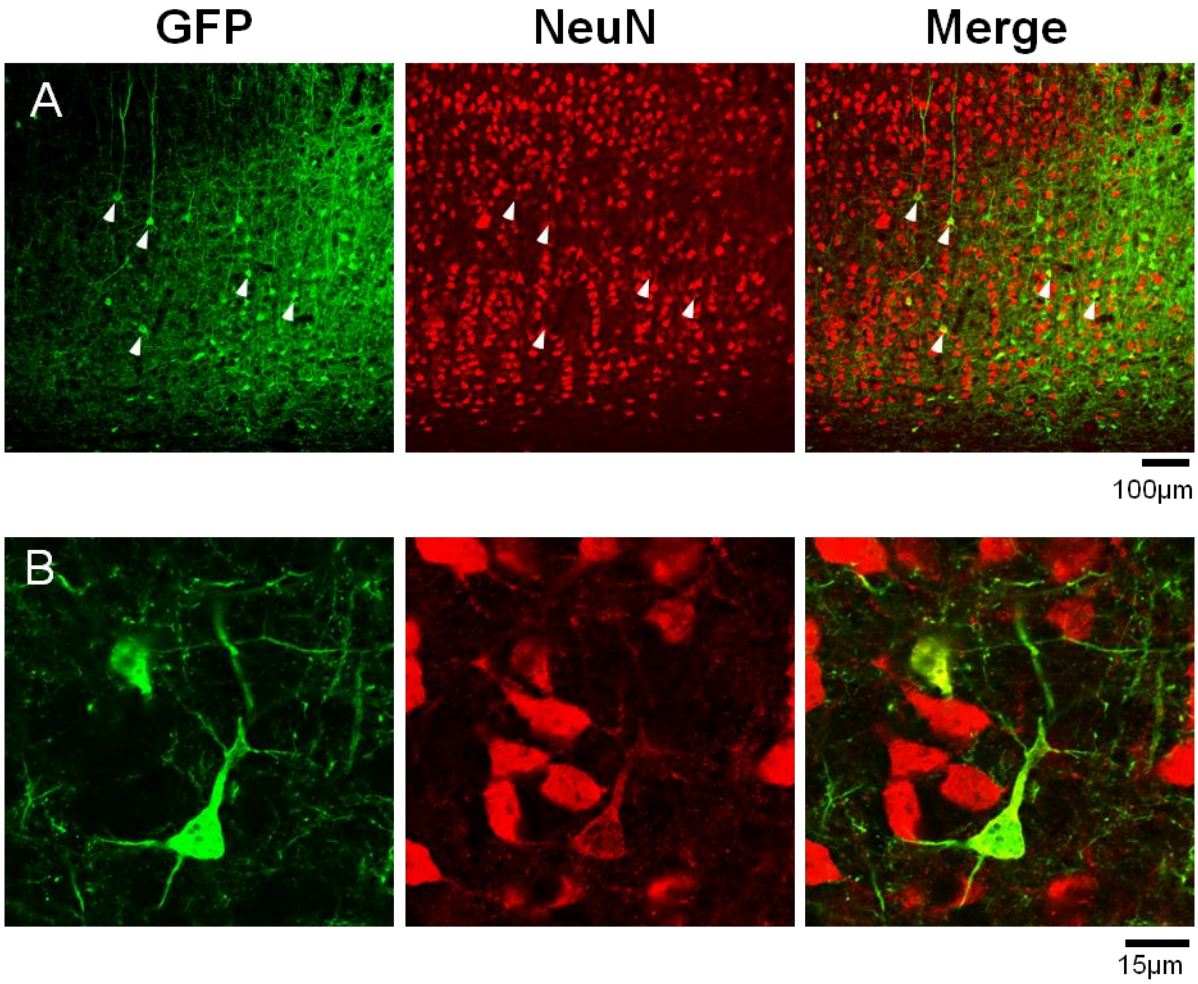

Figure 18. A, **The HSV-SRF+ virus transfects neurons in the visual cortex of ferrets**, as shown the expression of GFP tagged to the SRF-VP16 sequence. **B**, A highly magnified image showing a pyramidal neuron transfected by the HSV virus. Note the colocalization between GFP and NeuN.

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Vita

Arco Prabha Paul was born on January 11, 1977 in Jamshedpur, India, and is an Indian citizen. He completed high school from Kendriya Vidyalaya, Ishapore in 1994. He graduated from The National Institute for the Orthopaedically Handicapped where he received his Bachelor of Physiotherapy in September 1999. Then he received a Post-Graduate Diploma in Management from Goa Institute of Management in March 2003.