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OVEREXPRESSION OF SERUM RESPONSE FACTOR IN ASTROCYTES IMPROVES NEURONAL PLASTICITY IN A MODEL OF EARLY ALCOHOL EXPOSURE

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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.

Keywords

serum response factor; astrocyte; ocular dominance plasticity; visual development; ferret; fetal alcohol spectrum disorders

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; Coles et al., 2002; Manji et al., 2009; Franklin et al., 2008).

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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 (Climent et al., 2002; Moulder et al., 2002; Hoff, 1988; Isayama et al., 2009; Rema and Ebner, 1999; Medina et al., 2003).

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 (Wiesel And Hubel, 1963; Hubel et al., 1977; Issa et al., 1999). 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 protein kinases (Savage et al., 1992; Naseer et al., 2011; Davis et al., 2000; Joshi et al., 2006). 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) (Knoll et al., 2006; Chai and Tarnawski, 2002; Whitmarsh et al., 1995). While the role of CREB on activity-dependent plasticity is quite established (Mower et al., 2002; Silva et al., 1998), 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; Finkbeiner et al., 1997; Lamprecht, 1999).

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 et al., 2006; Knoll and Nordheim, 2009; Etkin 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 (Paul et al., 2010). 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.

EXPERIMENTAL PROCEDURES

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 1 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.

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°C and 5% CO₂. The cells become confluent in 8–10 days. At that time, the flasks are agitated on an orbital shaker (250rpm, 37°C, 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 10 μ l of a solution of the Sindbis virus carrying one of the constructs (SRF+, SRF– or GFP) (viral titer = 4.0×10^7 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/ μ l.

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 stereotactically positioned at an angle of 15–22° from the midline and lowered 1mm into the primary visual cortex (V1). Two injections of 4 μ l each ~1mm apart are administered at a rate of 10 nl/s using a hydraulic microsyringe pump controller (Micro 4, WPI, Sarasota, FL). Regardless the construct (SRF+, SRF–, GFP) the virus infected almost all cells in culture. Therefore, we can assume that in each animal the total number of modified astrocytes injected were between 80,000–120,000.

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; Paul et al., 2010; Medina et al., 2006). 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 CO₂ and arterial blood oxygen saturation (SpO₂) are monitored continuously and maintained at ~4% and above 90%, respectively. Body temperature is maintained at 38°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 2). 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). To identify the expression of the SRF-VP16 transgene we used an antibody against VP16 (Santacruz) (Paul et al., 2010). Fig 3 shows that Sindbis virus robustly transfects cells

in culture and almost all astrocytes were positive for VP16. A similar pattern of infection was obtained with the SRF⁺ and GFP constructs (data not shown).

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 4A 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 4A), 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. 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 4B). 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:

$$[(P_{0-51} - P_{205-255}) + \{(P_{52-102} - P_{154-204})/2\} + 100]/200$$
, where P_{A-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 4C).

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 5A and B). Figure 5C 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 6 (upper row) 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. Implanted cells 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. 5 and 6), suggests that the implanted cells are labile.

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.

Astrocytes and neuronal plasticity

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 (Petzold et al., 2008; Wang et al., 2006; Schummers et al., 2008). 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 (Araque et al., 2002; Fellin, 2009). 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 (Stevens, 2008; Perea et al., 2009). 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., 2008). Another study demonstrated that the release of $\text{TNF}\alpha$ from astrocytes contributes to homeostatic plasticity by synaptic scaling (Stellwagen and Malenka, 2006). Mostly importantly, a report showed that the $\text{TNF}\alpha$ -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 (Philippart 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 the present study we observed an effect on neuronal function following activation of SRF in astrocytes. Such SRF-directed paracrine communication has also been observed between neurons and oligodendrocytes. For instance, SRF activity in neurons was found to regulate the differentiation of oligodendrocytes and myelination via the expression of connective tissue growth factor (CTGF) (Stritt et al., 2009).

The ferret model of FASD

One of the biggest challenges to translate developmental age across species is posed by the fact that distinct regions mature differently. In a seminal paper Clancy and collaborators created a “translation table” using numerous developmental events. According to this table, P10–P30 in the ferret (and P2–P10 in the mouse) is equivalent to mid second trimester to beginning of the third trimester in humans. However, we don’t think this is valid for the visual cortex, where retinal activity plays a major role in cortical development.

In humans, retinal waves, a spontaneous and periodic cellular activity which is crucial for the proper development of retinothalamic afferents occur between 22 and 30 weeks of gestation (Graven, 2004) while in ferrets these waves are observed between P0–P28 (Daw, 1998; Firth et al., 2005).

In ferrets eye opening occurs around P32 (~P12 in rodents). This important milestone, which is characterized by the presence of pattern-visual stimulation, should be equivalent to birth in humans. In fact, we can see some similarities in events that occur immediately after the eye opening in ferrets and after birth in humans. For instance the maturation of neuronal orientation selectivity in the visual cortex occurs at P38 in ferrets (Chapman and Stryker, 1993) and, at 6 weeks after birth in humans (Braddick et al. 1986). In addition, the critical period of ocular dominance plasticity which is between P35 to P65 in the ferret (Issa et al., 1999), in humans it occurs roughly during the first seven years of age (Hooks and Chen, 2007). Taking all these events together, we consider here P10–P30 in the ferret to be equivalent roughly to the third trimester of the human gestation.

The blood alcohol level obtained by our paradigm (~250 mg/dl), in humans, is considered a mild to heavy intoxication. Higher levels, such as 400 mg/Kg are usually associated with a comatose state. However, in chronic alcoholism this value can be reached without loss of conscience and patients can be oriented regarding self, place and time even with levels as high as 500 mg/dl (Minion et al. 1999). These levels can be particular harmful to the fetus since alcohol freely crosses the placenta and the blood alcohol levels in the mother are similar to those observed in the offspring (Burd et al., 2012).

CONCLUSION

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.

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Highlights

Early alcohol exposure leads to a long lasting impairment of neuronal plasticity
Implantation of cultured astrocytes overexpressing SRF restored neuronal plasticity
Restoration of neuronal plasticity occurred in all visual cortex
SRF-related molecules produced by astrocytes can improve plasticity in neurons.

Figure 1. 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).

Figure 2.

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).

Figure 3.

VP16 immunoreactivity (green) reveals that astrocytes show robust expression of SRF by the Sindbis virus particles after 24 hours of transfection. Panel A shows that almost all cells were positive for VP16. Panel B shows the nuclear expression of VP16 compared with cytoplasmic expression of the immature astrocyte marker vimentin (red).

Figure 4.

Optical imaging of intrinsic signals show 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 Saline/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.

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

Figure 6.

Coronal section of the visual cortex 24h after implantation. Note the track of two injections and the spread of the implanted cells as seen by the expression of the SRF-VP16. Note that many of the VP16 positive implanted cells can be identified as astrocytes by their colocalization with GFAP.