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

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Abstract

Background—Deficits in neuronal plasticity underlie many neurobehavioral and cognitive problems presented in Fetal Alcohol Spectrum Disorders (FASD). Our lab has developed a ferret model showing that early alcohol exposure leads to a persistent disruption in ocular dominance (OD) plasticity. For instance, a few days of monocular deprivation results in a robust reduction of visual cortex neurons' responsiveness to stimulation of the deprived eye in normal animals, but not in ferrets with early alcohol exposure. Previously our lab demonstrated that overexpression of serum response factor (SRF) exclusively in astrocytes can improve neuronal plasticity in FASD. Here we test whether neuronal overexpression of SRF can achieve similar effects.

Methods—Ferrets received 3.5 g/kg alcohol i.p. (25% in saline) or saline as control every other day between postnatal day (P) 10 to 30, which is roughly equivalent to the third trimester of human gestation. Animals were given intracortical injections of a Herpes viral vector to express either GFP or a constitutively active form of SRF in infected neurons. They were then monocularly deprived by eyelid suture for 4–5 d after which single-unit recordings were conducted to determine if changes in ocular dominance had occurred.

Results—Overexpression of a constitutively active form of SRF by neurons restored OD plasticity in alcohol-treated animals. This effect was observed only in areas near the site of viral infection.

Conclusions—Overexpression of SRF in neurons can restore plasticity in the ferret model of FASD, but only in areas near the site of infection. This contrasts with SRF overexpression in astrocytes which restored plasticity throughout the visual cortex.

Keywords

Ocular dominance; fetal alcohol syndrome; visual development; serum response factor; monocular deprivation

INTRODUCTION

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term describing a range of effects that can occur in an individual whose mother imbibed alcohol during pregnancy. This

condition 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 with altered somatosensory, auditory and visual processing (Mattson et al., 1996; Uecker and Nadel, 1996; Coles et al., 2002; Rasmussen et al., 2006), and often autistic behavior (Harris et al., 1995). 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). However, the mechanisms that underlie such effects remain to be elucidated. We developed a unique ferret model of FASD that provides a novel approach to test mechanistic hypotheses of how early alcohol exposure can impair sensory cortex function.

In the primary visual cortex of higher mammals such as ferrets, alternating columns of neurons process information sent from either the left or the right eye. Unilateral eyelid closure for a few days (monocular deprivation; MD) during an early period in development leads to a dramatic loss of function of cortical cells wired to the deprived eye and an increase of function in cells wired to the experienced (open) eye. The ocular dominance columns serving the deprived eye shrink, and the columns representing the experienced (open) eye expand (Hubel and Wiesel, 1970; Hubel et al., 1977). This type of plasticity, known as ocular dominance plasticity (ODP), has been widely used to study the mechanisms that underlie neural plasticity. Moreover, it shares common mechanisms with learning and memory such as dependence on the NMDA receptor (Bear et al., 1990; Roberts et al., 1998) and the transcription factor cAMP/Ca⁺⁺ dependent response element binding protein (CREB) function (Mower et al., 2002). We previously showed that exposure of ferrets to alcohol from postnatal day 10 (P10) to P30, a period of development that for the visual system is roughly a human third trimester equivalent, leads to a long lasting reduction of ODP (Medina et al., 2003; Medina and Ramoa, 2005; Medina et al., 2006; Paul et al., 2010).

Early alcohol exposure has been shown to affect many signal transduction cascades related to the activation of serum response factor (SRF) (Davis et al., 1999; Luo and Miller, 1999; Kalluri and Ticku, 2003; Tsuji et al., 2003; Tang et al., 2006), and many of its target genes are associated with synaptic plasticity (Ramanan et al., 2005; Kuzniewska et al., 2015). 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 the serum response element (SRE) 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; Etkin et al., 2006).

Recently we have shown that overexpression of SRF by sindbis virus (which in ferrets infects mostly astrocytes), long after the period of alcohol exposure, rescues ODP (Paul et al., 2010). Moreover, plasticity restoration was observed in almost all V1 rather than being restricted to the area within the infection site. This finding suggests that released SRF products may diffuse through the neuropil, or are transported by the blood or CSF. These results were confirmed in a second study where we implanted SRF overexpressing astrocytes

instead of directly injecting the viral vector. This manipulation showed a similar restoration of ODP (Paul and Medina, 2012). Taken together these findings show that SRF overexpression in astrocytes is sufficient to rescue ODP. Here we asked whether expression of SRF specifically in neurons can achieve similar results. To address this, we used a similar constitutively active form of SRF construct packed into a herpes viral vector (HSV), which in ferrets is highly specific for neurons (Mower et al., 2002; Liao et al., 2004).

METHODS

All procedures described in this paper were approved by the Institutional Animal Care and Use Committee at the University of Maryland. 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). Near the peak of critical period of the OD plasticity (P39–P50), animals were injected in V1 with a Herpes simplex virus (HSV) carrying a constitutively active form of SRF or a control GFP. Animals were then monocularly deprived by eyelid suture for 4–5 d. After the period of monocular deprivation (MD), the animals were anesthetized, the deprived eye opened and a single-unit recording experiment was conducted. Nine females and eight males were used in these experiments, distributed approximately evenly across experimental groups.

Viral vectors

SRF constitutively active construct was made by fusing the dimerization and DNA binding domains of SRF (amino acids 90–222) with the transactivation domain of HSV protein VP16 (amino acids 363–490). This is the same construct used in our previous studies (Paul et al., 2010; Paul and Medina, 2012). However, for the current study the construct was packed into HSV viral vectors by Dr Rachael Neve at Massachusetts Institute of Technology viral core facility. The viral vector carrying the construct will be referred here as HSVSRF. As a control we use a HSV carrying green fluorescent protein (HSVGFPP)

Injection of viruses

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 secretions, anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg), and placed in a stereotaxic frame. No surgical procedures were initiated until animals were sufficiently anesthetized, as ascertained by the loss of withdrawal and corneal-blink reflexes. A small hole was drilled in the skull and the tip of a 31 gauge Hamilton Syringe (Hamilton Reno, Nevada) containing the HSV virus (titer = 4.0×10^7 infectious units/ml) was stereotaxically positioned at an angle of 15–22° from the midline and lowered 1 mm into the binocular region of the left primary visual cortex, as previously described (Paul et al., 2010). A volume of 2 μ l was injected at a rate of 0.1 μ l every 30 s. Each animal received two to three injections ~1 mm apart. The eyelid contralateral to the hemisphere in which virus was implanted was then sutured closed.

In vivo electrophysiology

Animals were premedicated by subcutaneous injection of acepromazine (1 mg/kg) and methyl atropine bromide (0.2 mg/kg), anesthetized with intraperitoneal sodium pentobarbital (35 mg/kg), and placed in a stereotaxic frame. No surgical procedures were initiated until the animals were 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. Expired $p\text{CO}_2$ and SpO_2 were monitored continuously and maintained at $\sim 4.0\%$ and above 90%, respectively. Body temperature was maintained at 38°C using a homeostatic blanket. Withdrawal reflexes and heart rate were monitored during the experiment. A craniotomy (3–5 mm in diameter) was performed over the binocular region of the primary visual cortex within which recordings were conducted. Supplemental doses of pentobarbital and acepromazine were given along with subcutaneous injections of lactated Ringer's solution every hour throughout the experiment or when heart rate or expired $p\text{CO}_2$ increased. The eyelids were opened, nictitating membranes were retracted using pseudoephedrine (2.5%), the pupils were dilated with atropine sulfate (1%), and contact lenses were placed on the corneas.

A 16-channel silicon probe, with $177\mu\text{m}^2$ recording sites separated vertically by $100\mu\text{m}$ (A1 $\times$ 16-5mm-100-177-A16; impedance $\sim 1\text{ M}\Omega$ at 1kHz; NeuroNexus Technologies, Ann Arbor, MI) was positioned over the visual cortex, and coated with DiI (to allow for later visualization of recording sites). The probe was advanced into the cortex to a depth of about $1500\mu\text{m}$ and allowed to stabilize for 30 minutes. For each recording site, 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. To assess OD, the moving bar of light was presented to each eye separately (20 stimulus presentations) at the optimal orientation. 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. Neuronal activity was recorded and digitized using an isolated preamplifier and RZ2 BioAmp Processor (Tucker Davis Technologies Alhula, FL) and stored for offline analysis. Offline, waveforms were sorted into individual units using principal component analysis followed by Bayesian classification with OpenSorter software (Tucker Davis Technologies Alhula, FL). Ocular dominance, spontaneous activity, and number of spikes per stimulus were then quantitatively determined for each cell. Spontaneous activity was determined by recording activity in the absence of stimulation. At the conclusion of the experiment, ferrets were killed with Euthasol (0.6 ml/kg, i.p.; Delmarva).

To quantify cortical binocularity, an ocular dominance index was calculated for each cell using the following equation: $(\text{LE})/(\text{LE} + \text{RE})$, where LE indicates the response to stimulation of the left eye, and RE indicates the response to stimulation for the right eye. The results were analyzed statistically using a Kruskal Wallis one-way analysis of variance followed by post-hoc Kolmogorov-Smirnov tests to determine where differences existed between individual pairs of experimental groups.

Tissue from recorded animals was sectioned and mounted on slides. Photographs of the injection sites (visible due to green fluorescence) and electrode recording sites (visible as red fluorescence from DiI) were taken using fluorescence microscopy. Recording locations were

separated into those that were close to virus injection sites, and those that were far away. This was used to subsequently analyze the local versus widespread effects of virus injection on neuronal responses.

RESULTS

We investigated whether neuronal overexpression of SRF by Herpes Simplex 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 et al., 2005). After an alcohol-free period, animals were injected with HSVSRF or HSVGFP in V1 (Fig. 1). We used single unit electrophysiology to assess changes in OD after monocular deprivation. The virus injection sites were easily identified in each animal and were used as a landmark to place the electrode, which was coated with DiI. Successful expression of the virus was confirmed by fluorescence microscopy after the experiment (Fig. 1A and B). The location of electrode tracks relative to virus injection sites was similarly confirmed (Fig. 1C). The spread of the viral infection as analyzed in coronal sections varied from about 1 to 2 mm at its greatest width (Fig. 1A). For analyses of recordings close to, and far from the virus infection sites, the following criteria were used. Sections which contained electrode penetrations which clearly passed through sites of infection (Fig. 1C) were classified as recordings close to the virus infection site. Electrode penetrations which did not clearly pass through infected areas and were more than 2.5 mm away from the center of a viral injection site were classified as being far away from the site of infection. Any penetrations which did not clearly pass through infected areas but were not more than 2.5 mm away from the center of a virus injection were excluded from analyses comparing recordings from sites near versus sites far from virus infection.

We created an ocular dominance index (ODI) 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 ODI of 1.0 indicates a neuron that is responsive only to the left eye whereas an ODI of 0.0 indicates responses only to the right eye. Figure 2 shows the average OD of left visual cortex from each group in which recordings were performed. Figure 2A presents the typical contralateral bias observed in naïve ferrets with no monocular deprivation (Issa et al., 1999; White et al., 1999; Medina et al., 2003). As expected, 4–5 days of MD in naïve and saline-treated animals led to a striking shift in ocular dominance toward the experienced (left) eye (Fig. 2B–C). Alcohol-treated animals that received HSVGFP presented a mild change in OD (Fig. 2D), which confirms our previous findings that early alcohol exposure impairs OD plasticity (Medina et al., 2003; Medina et al., 2005). Importantly, after the same period of MD, alcohol-treated animals that received the HSVSRF virus displayed restored OD plasticity at recording sites near the virus injection (Fig. 2F), but not far away from the injection (Fig. 2E).

Figure 3 shows the cumulative distributions of ocular dominance indices for the five groups in which recordings were performed. Note that the curve of ethanol-treated HSVSRF animals is completely skewed to the right at sites close to the virus injection when compared with sites far from the injection as well as GFP controls. Normal untreated animals with no monocular deprivation showed ODIs typical for juvenile ferrets, as previously reported (Paul

et al., 2010; Paul and Medina, 2012; Medina et al., 2003 and 2006). A Kruskal Wallis test showed a statistically significant difference between the ODIs of the different groups that received monocular deprivation ($\chi^2 = 39.62$, $df = 4$, $p < 0.001$). *Post hoc* analysis showed that alcohol-treated animals that received HSVSRF had significantly greater ODIs at sites near virus injection than at sites far away (K-S; $Z = 2.06$, $p < 0.001$). Near the virus injection sites, alcohol-treated, HSVSRF animals had ODIs similar to that of naïve (K-S; $Z = 0.71$, $p = 0.688$) and saline-treated (K-S; $Z = 0.68$, $p = 0.738$) animals, indicating that ocular dominance plasticity was restored at sites near the virus injection. At sites far from the virus injection, alcohol-treated HSVSRF animals had ODIs similar to that of alcohol-treated HSVGFP animals (K-S; $Z = 1.32$, $p = 0.062$) showing that ocular dominance plasticity was not restored at sites far from the virus injection.

DISCUSSION

Here we have shown that overexpression of SRF in neurons, via HSV, can rescue deficits in ODP in a ferret model of FASD. This effect was localized to areas close to the infection site, with distant sites displaying a profile similar to that of control alcohol exposed animals receiving injections of HSVGFP. This result differs from previous findings by our group, wherein SRF overexpression by astrocytes rescued ODP throughout primary visual cortex (Paul et al., 2010; Paul and Medina, 2012).

SRF is a transcription factor that binds to the Serum Response Element (SRE) in gene promoters. The SRE is an A/T rich core flanked by an inverted repeat (CCATATTAGG; also called CARG box). The SRF protein contains 508 aminoacids and contains a SRE binding domain, a transactivation domain and several phosphorylation sites (Chai and Tarnawski, 2002). The transcription activation domain is located in the C-terminal region of the SRF protein, within aminoacids 339 to 508 (Johansen and Prywes, 1993). SRF mediated transcription may require binding of ternary complex factors (TCFs) to the SRE. Examples of these TCFs are ELK-1, SAP-1 and SAP-2. While activation of SRF by Rho GTPases have been known for several years just recently this action has become better understood (Miralles et al., 2003; Vartiainen et al., 2007). Activation of Rho leads to the conversion of G-actin to F-actin through polymerization. The depletion of G-actin leads to the translocation of the megakaryocytic acute leukemia protein (MAL) from the cytoplasm to the nucleus, where it dimerizes with SRF and induces transcription (Miralles et al., 2003; Vartiainen et al., 2007).

Many of the target genes of SRF are associated with synaptic plasticity (Ramanan et al., 2005; Kuzniewska et al., 2015) and disruption of SRF function has revealed an important functional role in plasticity related processes. During development of the nervous system, SRF plays a major role in neuronal migration, axon guidance and myelination (Chai and Tarnawski, 2002; Alberti et al., 2005; Knoll et al., 2006; Wickramasinghe et al., 2008; Li et al., 2014). Also, several studies have shown that SRF can mediate neuronal plasticity through the expression of neurotrophins, immediate early genes, cytoskeletal proteins and thrombospondins (Morris et al., 1999; Chai and Tarnawski, 2002; Schrott et al., 2002; Christopherson et al., 2005; Ramanan et al., 2005; Knoll et al., 2006; Stritt et al., 2009). SRF has been shown to be important in the processes of both long-term potentiation (Ramanan et

al., 2005) and long-term depression (Etkin et al., 2006). Activation of SRF can also result in histone acetylation (Qiu and Li, 2002; Cao et al., 2005; Ebisuya et al., 2008), which indicates that this transcription factor may play a major role in epigenetic regulation (Grunstein, 1997; Jenuwein and Allis, 2001; Shukla et al., 2008).

It is known that early alcohol exposure affects many signal transduction pathways related to SRF activation (Davis et al., 1999; Luo and Miller, 1999; Kalluri and Ticku, 2003; Tsuji et al., 2003; Tang et al., 2006). Thus, 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 brings these molecules back to normal levels. Alternatively, the restoration of ocular dominance may be a result of a compensatory boost of plasticity caused by the release of plasticity-related molecules caused by SRF overexpression.

The restoration of ODP caused by neuronal overexpression of SRF observed in the present study, was localized to areas around the infection site. This is in contrast to previous findings by our group, which showed that astrocytic overexpression of SRF rescued plasticity throughout the primary visual cortex, even at areas far from the site of infection (Paul & Medina, 2012; Paul et al., 2010). Astrocytes may be particularly well-suited for widespread release of neuroactive substances, due to their connectivity with vasculature or the widespread gap-junction mediated interconnectivity of astrocytic networks. Indeed, it has been demonstrated that second messengers, amino acids, peptides and RNA can permeate through the gap-junctions of connected astrocytes (Giaume et al., 2010). Further, other groups have demonstrated the capacity of implanted astrocytes to produce effects in extensive areas. Implantation of astrocytes that were made to constitutively overexpress glial cell line-derived neurotrophic factor (GDNF) caused GDNF levels to be increased in brain regions both near and far from the implantation site, and even in the contralateral hemisphere, even though astrocyte migration away from the site did not occur (Cunningham and Su, 2002; Ericson et al., 2005).

In conclusion, we have shown that overexpression of SRF in neurons can restore deficits in ocular dominance plasticity in a ferret model of fetal alcohol spectrum disorder. This restoration is confined to areas close to the site of infection, in contrast to previous results obtained from overexpression of SRF in astrocytes which restored plasticity throughout primary visual cortex.

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Figure 1.

A) Representative coronal section through the visual cortex illustrates the spread of the infection. In this example, the area of greatest spread is roughly 1.7 mm. The morphology of infected cells (B) confirms that the virus infects neurons, as was expected. C) Electrode penetrations (DiI; blue arrow) were separated into sites near the infection site (GFP; yellow arrow) and those far away. Scale bars = 100 μ m.

Figure 2.

A) Ocular dominance profiles for neurons in the primary visual cortex of normal untreated ferrets (n=2). The primary visual cortex of these animals was characterized by a large number of binocular cells, with most neurons showing responses dominated by the contralateral eye. In contrast, after 4–5 days of monocular deprivation, histograms for naïve (n=4) (B) or saline-treated animals receiving intracortical injections of HSV-GFP (n=3) (C) indicate a shifted distribution of ocular dominance towards the non-deprived ipsilateral eye. Animals exposed to alcohol from P10–P30 display reduced ocular dominance if injected intracortically with a HSV virus encoding for GFP (n=4) (D). Ethanol exposed animals that received intracortical injections of HSV virus encoding for a constitutively active form of Serum Response Factor (SRF) (n=4) also showed reduced plasticity at electrode recording sites that were far away from the virus injection (E), but not from recordings performed near the location of virus injection (F). Data displayed as mean with error bars indicating standard error of mean.

Figure 3.

The Cumulative number of cells is (as percentage) plotted as a function of the OD index. Animals that did not receive a monocular deprivation are indicated with the shape that is the most to the left (n=145 cells). Recordings from naïve (n=222 cells) and saline (n=142 cells) treated animals, indicate a curve that is skewed to the right, indicating an OD shift (most cells respond better to ipsilateral eye stimulation). Animals receiving early ethanol exposure and treated with a HSV virus encoding for GFP (n=85 cells), displayed some ocular dominance plasticity, but not as much as naïve and saline-treated animals. Recordings from sites far away from intracortical injections of a HSV construct expressing a constitutively active form of SRF in ethanol animals (n=90 cells), displayed a similarly reduced OD shift. In contrast, recordings from these same animals, but near virus injection sites (n=93 cells) showed a restored ocular dominance shift that is similar to what is seen in naïve and saline-treated animals. In the figure legend black lines show several post-hoc comparisons (***, $p < 0.001$; NS, not significant).