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Differential regulation of synapsin phosphorylation by monocular deprivation in juveniles and adults

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Abstract

The rodent visual cortex retains significant ocular dominance plasticity beyond the traditional postnatal critical period. However, the intracellular mechanisms that underlie the cortical response to monocular deprivation are predicted to be different in juveniles and adults. Here we show monocular deprivation in adults, but not juveniles, induced an increase in the phosphorylation of the prominent presynaptic effector protein synapsin at two key sites known to regulate synapsin function. Monocular deprivation in adults induced an increase in synapsin phosphorylation at the PKA consensus site (site 1) and the CaMKII consensus site (site 3) in the visual cortex ipsilateral to the deprived eye, which is dominated by non-deprived eye input. The increase in synapsin phosphorylation was observed in total cortical homogenate, but not synaptoneurosomes, suggesting that the pool of synapsin targeted by monocular deprivation in adults does not co-fractionate with excitatory synapses. Phosphorylation of sites 1 and 3 stimulates the release of synaptic vesicles from a reserve pool and increases in the probability of evoked neurotransmitter release, which may contribute to the strengthening of the non-deprived input characteristic of ocular dominance plasticity in adults.

INTRODUCTION

The shift in ocular dominance induced by brief monocular deprivation is a sensitive assay of the level of synaptic plasticity available to synapses in the binocular region of the primary visual cortex. Ocular dominance plasticity is robust in juveniles (Wiesel and Hubel, 1963) and was previously thought to be confined to an early postnatal critical period ending near puberty. However, accumulating evidence demonstrates that the rodent visual cortex retains significant ocular dominance plasticity beyond the traditional critical period. It remains to be determined if the intracellular signaling cascades linking monocular deprivation to a shift in ocular dominance are the same in the juvenile and adult cortex.

In juveniles, two temporally distinct changes in thalamocortical synaptic transmission are observed following monocular deprivation: a rapid decrease in the strength of synapses serving the deprived eye, followed by a slower increase in strength of synapses serving the non-deprived eye (Frenkel and Bear, 2004). The rapid decrease in deprived eye synaptic

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strength is similar to the long-term depression (LTD) of excitatory synaptic responses induced by low frequency stimulation (LFS). Depression of inputs serving the deprived eye and LFS-LTD are both activity-dependent and require NMDAR activation. In addition, monocular deprivation in juveniles occludes subsequent LFS-LTD in layer IV (Heynen et al., 2003). Over the course of development, the ability to express deprivation-induced depression of the thalamo-cortical projections decreases (Fox and Wong, 2005), with a time course similar to the developmental loss of LFS-LTD in layer IV (Dudek and Friedlander 1996; Jiang et al., 2007). The slower increase in the strength of synapses serving the non-deprived eye following prolonged MD in juveniles shares many characteristics with the long-term potentiation (LTP) of synaptic responses induced by high frequency stimulation (HFS). The potentiation of inputs serving the non-deprived eye and HFS-LTP are both activity-dependent and require activation of NMDARs (Sawtell et al., 2003).

The response to monocular deprivation in adults differs in many ways from the response in juveniles. In adult rodents, the primary response to monocular deprivation is an increase in the physiological response to stimulation of the non-deprived eye, an increase in the spatial acuity of the non-deprived eye and an expansion of cortical territory representing the non-deprived eye (Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Prusky et al., 2006; Fisher et al., 2007; Sato and Stryker, 2008; Lehmann and Lowel, 2008). The duration of monocular deprivation required to reveal a maximal ocular dominance shift in adults is longer than what is required in juveniles. Other factors influencing ocular dominance plasticity in adults include the age at initiation of monocular deprivation and the history of visual experience prior to the monocular deprivation (He et al., 2006; Hofer et al., 2006; He et al., 2007; Hofer et al., 2009).

Pharmacological and transgenic manipulations implicate the involvement of several second messenger pathways and subsequent activation of intracellular protein kinases as mediators of ocular dominance plasticity in juveniles. Of particular interest is the role of activity-dependent protein kinases, which can rapidly translate the asymmetry in visual experience to changes in synaptic function. Inhibition of the cAMP-dependent protein kinase PKA activity blocks the ocular dominance shift observed in response to monocular deprivation in juvenile rodents and cats (Beaver et al., 2001; Fischer 2004; Rao et al., 2004). In addition, activation of PKA promotes the strengthening of the non-deprived input following monocular deprivation in adult cats (Imamura et al., 1999). Similarly, transgenic mice expressing a mutant form of the calcium-calmodulin-dependent kinase CaMKII that renders it incapable of calcium-independent activity show a significant deficit in ocular dominance plasticity (Taha et al., 2002). Examination of the role of activity-dependent protein kinases in ocular dominance plasticity has focused primarily on postsynaptic targets, consistent with the significant contribution of the postsynaptic specialization to the maintenance of long-term changes in synaptic strength. For example dephosphorylation of a PKA-consensus site (serine 845) on the GluR1 subunit of the AMPAR is observed in the visual cortex contralateral to the deprived eye following brief monocular deprivation in juveniles, but not adults (Heynen et al., 2003). Phosphorylation of the PKA-consensus site (Serine 133) is observed following monocular deprivation in juveniles, but not adults (Pham et al., 2004).

However, one of the primary targets of activity-dependent protein kinases is the presynaptic vesicle-associated phosphoprotein synapsin (Leenders and Sheng, 2005). Activity-dependent regulation of synapsin phosphorylation/dephosphorylation has been implicated in synaptogenesis, synaptic plasticity, neurotransmitter release, and aging (Greengard et al., 1993; Hilfiker et al., 2005; Corradi et al., 2008). Three genes encode the family of synapsin proteins (synapsin I, II and III). The mRNA for synapsin I and II are alternatively spliced to produce two isoforms, synapsin Ia and b and synapsin IIa and b (Evergren et al., 2007). All synapsin isoforms share a consensus sequence for phosphorylation by PKA at the amino

terminus called site 1 (serine 9 in synapsin Ia/b, serine 10 in synapsin IIa/b). In addition, synapsin 1a and Ib share a consensus sequence for phosphorylation by CaMKII at the carboxy terminus called site 3 (serine 603). Phosphorylation at sites 1 and 3 reduces the affinity of synapsins for both actin and the lipid bilayer of synaptic vesicles (Hosaka et al., 1999; Stefani et al., 1997), and thereby initiates the disassociation of synapsin from synaptic vesicles. Activity-dependent phosphorylation of synapsins has been implicated in the plasticity of activity-dependent regulation of neurotransmitter release, including an increase in synaptic vesicle exocytosis in response to synaptic stimulation (Fiumara et al. 2007; Hilfiker et al., 2005; Menegnon et al., 2006). To characterize the role of synapsin and synapsin phosphorylation in ocular dominance plasticity, we determined the distribution of synapsin isoforms in the juvenile and adult visual cortex, as well as the regulation of synapsin phosphorylation following monocular deprivation in juvenile and adult rats.

EXPERIMENTAL METHODS

Male and female pigmented Long Evans rats were raised on site with 12 hours light: 12 hours dark per day, with food and water available *ad libitum*. Animal care and use conformed to the guidelines of the U.S. Department of Health and Human Services and have been approved by the University of Maryland Institutional Animal Care and Use Committee.

Monocular deprivation

Juveniles (P28) or adults (P70-90) were anesthetized with ketamine/xylazine (50mg/10mg/kg, i.p.). The margins of the upper and lower lids of one eye were trimmed and sutured together. The animals were then sacrificed immediately (sham operated controls) or returned to a normally lighted environment for 3 days of monocular deprivation. If any sign of suture opening or infection was observed, animals were excluded from further analysis.

Quantitative Immunoblots

Subjects were anesthetized with sodium pentobarbital (75 mg/kg, i.p.), and the binocular region of the primary visual cortex was dissected in dissection buffer (2.6 mM KCl, 1.23 mM NaH_2PO_4 , 26 mM NaCO_3 , 212.7 mM sucrose, 10 mM dextrose, 0.5 mM CaCl_2 , 1 mM MgCl_2 , 100 μM kynurenic acid, pH 7.2, saturated with O_2/CO_2). Synaptoneurosome were prepared from visual cortex as described (Hollingsworth et al., 1985; Quinlan et al., 1999; Heynen et al., 2000). Equal concentrations of synaptoneurosome or homogenate protein, determined using the BCA assay (Pierce), were resolved on 7.5% polyacrylamide gels, transferred to nitrocellulose and probed with anti-phospho-synapsin antibody (site 1, 1:700, rabbit polyclonal, Cell Signaling; site 3, 1:500 rabbit polyclonal, Santa Cruz Biotechnology) then stripped and reprobed with anti-synapsin antibody (1:500, rabbit polyclonal, Cell Signaling) in Tris buffered saline, pH 7.3 containing 1% milk and 0.1% Triton X-100, followed by incubation in appropriate secondary antibody coupled to horseradish peroxidase (1:3500, Amersham). Visualization of immunoreactive bands was induced by enhanced chemiluminescence (Amersham ECL) captured on autoradiography film (Amersham Hyperfilm). Each immunoblot was exposed to autoradiography film multiple times to insure we were operating within the linear range of the assay. Digital images, produced by densitometric scans of autoradiographs on a ScanJet 7400C (Hewlett Packard), were quantified using NIH Image 1.60 software. All values were normalized to a standard run on the same gel, to allow comparisons across gels. In figures 1 and 2, the data are normalized to actin as a gel-loading control. In figure 3-6, normalization to a gel-loading control was not necessary as the data are expressed as the ratio of sequential probes (phosphorylated synapsin/total synapsin) of the same gel. Quantification was performed blind to the experimental condition.

RESULTS

To ask if synapsin expression and phosphorylation was regulated over the course of cortical development, we compared the level of synapsins and phosphorylated synapsins in the visual cortex of juvenile (postnatal day 28) and adult (postnatal day 70-90) pigmented Long Evens rats. All synapsin isoforms share a consensus sequence for phosphorylation at the amino terminus called site 1 (serine 9 in synapsin Ia/b, serine 10 in synapsin IIa/b). Site 1 phosphorylation is targeted *in vivo* by protein kinase A PKA, which reduces the affinity of synapsin for synaptic vesicles and actin. Homogenate prepared from the primary visual cortex of normal-reared (12 hours light:12 dark/day) juveniles and adults were subjected to quantitative immunoblotting with an antibody that recognizes each isoform of synapsin only when phosphorylated on site 1, (phospho-synapsin; P-synapsin). Equal concentrations of homogenate proteins (12 ug) were separated by SDS-PAGE and the optical density of each immunoreactive band was normalized to the average of all of the juvenile samples run on the same gel. Synapsin Ia and Ib have similar electrophoretic mobilities, and were quantified together as synapsin Ia/b, as previously reported (Bogen et al., 2009). Synapsin IIa and IIb were quantified separately. Synapsin III levels are low in the postnatal mammalian cortex, and were not quantified in our experiments. We found no difference in the level of site 1 phosphorylation of any synapsin isoform in adult homogenate relative to juveniles (% of juvenile homogenate, average $\pm$ SEM: phospho-synapsin Ia/b = 106.13 ± 9.62 ; phospho-synapsin IIa = 112.37 ± 10.37 , phospho-synapsin IIb = 112.04 ± 11.67 , all $p > 0.05$, unpaired t-test versus juvenile, $n = 16$, Figure 1A). In contrast, the expression of total synapsin independent of phosphorylation state (total synapsin; T-synapsin) was developmentally regulated. We observed a significant increase in the expression of synapsin IIa and IIb, but not synapsin Ia/b, in adult homogenates relative to juveniles (% of juvenile homogenate, average $\pm$ SEM: total-synapsin Ia/b = 115.90 ± 16.51 ; total-synapsin IIa = 163.87 ± 21.26 ; total-synapsin IIb = 144.82 ± 13.11 ; both $p < 0.05$, unpaired t-test versus juvenile, $n = 16$, Figure 1B). However, when expressed as a ratio of phosphorylated synapsin/total synapsin, there was no significant difference in the percent of synapsin phosphorylated at site 1 in adult visual cortical homogenate relative to juvenile (% of juvenile homogenate, average $\pm$ SEM: phospho/total synapsin Ia/b = 126.79 ± 19.37 ; phospho/total synapsin IIa = 86.83 ± 12.91 ; phospho/total synapsin IIb = 83.18 ± 8.54 , all $p > 0.05$, unpaired t-test versus juvenile, $n = 16$, Figure 1C).

Two, anatomically distinct pools of immuno-gold labeled synapsin have been identified in the presynaptic terminals of young excitatory neurons in culture: a proximal pool that is 66 nm from the presynaptic membrane and distal pool that is 67 to 200 nm from the presynaptic membrane (Tao-Cheng, 2006). To ask if the distribution of synapsins is regulated over the course of postnatal development, we prepared synaptoneurosomes, a subcellular fraction that enriches for excitatory synaptic profiles (Hollingsworth et al., 1985). Previous work has demonstrated that synaptoneurosomes enrich for a subset of neuronal proteins that participates in excitatory synaptic transmission (Quinlan et al., 1999; Heynen et al., 2000; Whitlock et al., 2006). In the juvenile visual cortex, the level synapsins phosphorylated at site 1 is reduced in synaptoneurosomes relative to an equal concentration of homogenate (% of paired homogenate, average $\pm$ SEM: phospho-synapsin Ia/b = 76.6 ± 4.7 , $*p < 0.002$; phospho-synapsin IIa = 75.8 ± 13.7 , $*p < 0.05$, phospho-synapsin IIb = 72.8 ± 8.2 , $*p < 0.03$, paired t-test versus homogenate, $n = 12$, Figure 2A). In contrast, in the adult visual cortex, the level of synapsins phosphorylated at site 1 is not reduced in synaptoneurosomes relative homogenate (% of paired homogenate average $\pm$ SEM: phosphosynapsin Ia/b = 96.4 ± 4.1 ; phospho-synapsin IIa = 111.3 ± 15.4 ; phospho-synapsin IIb = 121.4 ± 12.1 ; $p > 0.05$ paired t-test versus homogenate, $n = 12$, Figure 2D). To ask if changes in synapsins phosphorylated at site 1 in synaptoneurosomes could be explained by changes in the total levels of synapsins, we quantified synapsin levels independent of phosphorylation state, (total

synapsin; T-synapsin). However, we observed no difference in the total level of synapsins in the synaptoneurosomes versus the homogenate in the juvenile visual cortex (% of paired homogenate, average $\pm$ SEM: total-synapsin 1 a/b = 97.1 ± 10.3 ; total-synapsin IIa = 111.6 ± 17.0 ; total-synapsin IIb = 106.5 ± 12.7 ; $p > 0.05$, paired t-test versus homogenate, $n = 12$, Figure 2B). Similarly, we observed no difference in the total level of synapsins in the synaptoneurosomes versus the homogenate in the adult visual cortex (% of homogenate, average $\pm$ SEM: synapsin Ia/b = 94.3 ± 5.9 ; synapsin IIa = 130.0 ± 23.4 ; synapsin IIb = 128.9 ± 19.1 , $n = 12$, $p > 0.05$ paired t-test versus homogenate, $n = 12$, Figure 2D). When expressed as a ratio of phosphorylated synapsin/total synapsin, we observe a significant decrease in the percent of phosphorylated synapsin in synaptoneurosomes prepared from juveniles (% of paired homogenate, average $\pm$ SEM: juvenile phospho/total-synapsin 1 a/b = 86.0 ± 8.1 , $*p < 0.05$; phospho/total-synapsin IIa = 77.1 ± 1.1 , $*p < 0.03$; phospho/total-synapsin IIb = 75.2 ± 7.6 , $*p < 0.005$, paired t-test versus homogenate, $n = 12$, Figure 2C) than adults (phospho/total-synapsin 1 a/b = 109.9 ± 5.6 ; phospho/total-synapsin IIa = 92.1 ± 5.5 ; phospho/total-synapsin IIb = 97.5 ± 4.6 , $p > 0.05$, paired t-test versus homogenate, $n = 12$, Figure 2E).

Strong depolarizing conditions can recruit synapsin to the synaptic membrane, demonstrating the concentration of synapsin present at the active zone can be regulated by activity (Chi et al., 2001; Bloom et al., 2003; Tao-Cheng, 2006). To ask if visual experience regulates the concentration of synapsin present in synaptoneurosomes, we performed brief (3 day) monocular deprivation to induce an asymmetry in activity across the two visual cortices. Synaptoneurosomes were prepared from each hemisphere to allow comparison of synapsin levels across visual cortical hemispheres. However, monocular deprivation did not regulate the levels of synapsin in the visual cortex of juveniles or adults. In juveniles, synapsin levels were maintained at low levels in synaptoneurosomes relative to homogenate following monocular deprivation (% of paired homogenate, average $\pm$ SEM: synapsin Ia/b contralateral to deprived eye = 85.2 ± 3.4 , ipsilateral = 94.7 ± 9.4 ; synapsin IIa contralateral = 73.3 ± 18.9 , ipsilateral = 72.4 ± 5.8 ; synapsin IIb contralateral = 78.6 ± 8.5 , ipsilateral = 82.9 ± 3 ; $p > 0.05$ unpaired t-test for each isoform versus control, $n = 6$, not shown). In adults, synapsin levels were maintained at control levels in synaptoneurosomes relative to homogenate following monocular deprivation (% of paired homogenate, average $\pm$ SEM: synapsin Ia/b contralateral to deprived eye = 95.7 ± 12.7 , ipsilateral = 95.5 ± 3.4 ; synapsin IIa contralateral = 92.7 ± 38.5 , ipsilateral = 96.8 ± 5.1 ; synapsin IIb contralateral = 127.5 ± 26.8 , ipsilateral = 136.9 ± 17.2 , $p > 0.05$ paired t-test for each isoform versus homogenate, $n = 6$, not shown).

To ask if monocular deprivation activates signaling pathways that target phosphorylation/dephosphorylation of synapsins, we tracked the proportion of synapsin phosphorylated at site 1. Surprisingly, we saw no change in the proportion of synapsin Ia/b, IIa or IIb phosphorylated on site 1 in total homogenate following monocular deprivation in juveniles (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral to deprived eye = 94.1 ± 14.5 , ipsilateral = 93.0 ± 16.8 ; phospho/total synapsin IIa contralateral = 108.9 ± 15.9 , ipsilateral = 105.6 ± 8.5 ; phospho/total synapsin IIb contralateral = 125.4 ± 11.3 , ipsilateral = 132.4 ± 19.6 , $p > 0.05$ unpaired t-test for each isoform versus control, $n = 7$, Figure 3A, 3B, 3C). Similarly, we saw no change in the proportion of synapsin Ia/b, IIa or IIb phosphorylated on site 1 in synaptoneurosomes following brief monocular deprivation in juveniles (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 118.9 ± 33.3 , ipsilateral = 119.7 ± 33.2 ; phospho/total synapsin IIa contralateral = 88.4 ± 16.6 , ipsilateral = 98.2 ± 23.1 ; phospho/total synapsin IIb contralateral = 111.5 ± 37.5 , ipsilateral = 125.9 ± 43.6 ; $p > 0.05$ unpaired t-test for each isoform versus control, $n = 9$, Figure 3D, 3E, 3F).

In contrast, monocular deprivation in adults induced a significant increase in synapsin phosphorylated at site 1 in homogenates prepared from visual cortex ipsilateral to the

deprived eye (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 84.4 ± 17.4 , ipsilateral = 126.2 ± 26.4 , $*=p<0.01$; phospho/total synapsin IIa contralateral = 106.4 ± 21.4 , ipsilateral = 158.8 ± 32.5 , $*=p<0.05$; phospho/total synapsin IIb: contralateral = 79.6 ± 13.6 , ipsilateral = 146.2 ± 23.8 , $*=p<0.005$ unpaired t-test for each isoform versus control, $n = 7$, Figure 4A₁, 4B₁, 4C₁). The increase in synapsin phosphorylation appears to be a specific consequence of monocular deprivation, because we observe no difference in synapsin phosphorylation in sham-operated adults (% of normal-reared controls, average $\pm$ SEM: synapsin Ia/b contralateral = 107.1 ± 18.2 , ipsilateral = 93.2 ± 15.6 , synapsin IIa contralateral = 104.3 ± 27.4 , ipsilateral = 95.4 ± 18.4 , synapsin IIb: contralateral = 107.6 ± 18.5 , ipsilateral = 93.2 ± 13.6 , all $p>0.05$ unpaired t-test for each isoform versus control, $n = 7$, Figure 4A₂, 4B₂, 4C₂).

We next asked if monocular deprivation in adults regulates the phosphorylation of the subset of synapsin enriched in the synaptoneurosomes preparation. However, we observed no significant difference in synapsin phosphorylation on site 1 in synaptoneurosomes prepared from the adult visual cortex contralateral or ipsilateral to the deprived eye (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 109.2 ± 24.7 , ipsilateral = 106.2 ± 28.5 ; phospho/total synapsin IIa contralateral = 93.2 ± 29.2 , ipsilateral = 83.9 ± 21.3 ; phospho/total synapsin IIb contralateral = 112.3 ± 20.8 , ipsilateral = 92.4 ± 25.4 ; $p>0.05$ unpaired t-test for each isoform versus control, $n = 9$, Figure 5A₁, 5B₁, 5C₁) or sham-operated adults (% of normal-reared control, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 101.4 ± 18.5 , ipsilateral = 98.5 ± 23.5 ; phospho/total synapsin IIa contralateral = 97.1 ± 28.5 , ipsilateral = 103.4 ± 29.6 ; phospho/total synapsin IIb contralateral = 99.3 ± 15.7 , ipsilateral = 100.1 ± 16.8 ; $p>0.05$ unpaired t-test for each isoform versus control, $n = 7$, Figure 5A₂, 5B₂, 5C₂).

The experience-dependent increase in synapsin phosphorylation at site 1 in the adult visual cortex ipsilateral to the deprived eye is present in total cortical homogenate, but not synaptoneurosomes fraction, suggesting that monocular deprivation in adults may target a population of synapsin not tethered to excitatory synapses. The non-synaptoneurosomes synapsin may reside at inhibitory synapses, which are not enriched in the synaptoneurosomes fraction, or may reside in excitatory axons but not sediment during the synaptoneurosomes isolation procedure. To distinguish between these two possibilities, we tracked experience-dependent regulation of synapsin phosphorylation at site 3, (serine 603), a phosphorylation site present at the carboxy terminus of synapsin Ia/b also regulates the binding of synapsin to synaptic vesicles and actin. Site 3 is exclusively phosphorylated by CaMKII, and therefore site 3 phosphorylation of synapsin is restricted to excitatory neurons.

Following monocular deprivation in adults, we observed a significant increase in site 3 phosphorylation in homogenate prepared from the visual cortex ipsilateral to the occlusion (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 100.9 ± 15.2 , ipsilateral = 191.1 ± 15.4 , $*=p<0.005$ unpaired t-test versus control, $n = 7$, Figure 6A). We observed no significant difference in synapsin phosphorylation on site 1 in synaptoneurosomes prepared following monocular deprivation in adults (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 100.7 ± 9.8 , ipsilateral = 99.31 ± 9.8 , $p>0.05$ unpaired t-test versus control, $n = 9$, Figure 6B).

Following monocular deprivation in juveniles, we observed no difference in the phosphorylation of synapsin at site 3 in homogenate (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 100.2 ± 6.2 , ipsilateral = 78.9 ± 7.3 ; $p>0.05$ unpaired t-test versus control, $n = 9$, Figure 6A) or synaptoneurosomes prepared from the visual cortex contralateral or ipsilateral to the occluded eye (% of normal-reared controls, average $\pm$ SEM: phospho/total synapsin Ia/b contralateral = 107.3 ± 5.8 , ipsilateral = 93.4

± 5.4 ; $p > 0.05$ unpaired t-test versus control, $n = 9$, Figure 6D). The regulation of synapsin phosphorylation at site 3 in homogenate but not synaptoneurosomes suggests the subset of synapsin regulated by monocular deprivation in adults is present in excitatory neurons.

DISCUSSION

A significant body of evidence now demonstrates that ocular dominance plasticity persists beyond the traditional postnatal critical period in rodents. However, ocular dominance plasticity in adults differs in many ways from ocular dominance plasticity in juveniles. In juveniles, the initial response to monocular deprivation is an activity-dependent weakening of thalamocortical synapses serving the deprived eye, while in adults, the response to monocular deprivation is dominated by an activity-dependent strengthening of synapses serving the non-deprived eye (Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Prusky et al., 2006; Fischer et al., 2007; Sato and Stryker, 2008). Not surprisingly, the intracellular signaling cascades recruited by monocular deprivation are predicted to be different in juveniles and adults (Majdan and Shatz, 2006; Heynen et al., 2003; Pham et al., 2004). However, to date, only a small number of signaling pathways activated by monocular deprivation have been identified at any age. Here we demonstrate that monocular deprivation regulates the phosphorylation of the synapsin family of presynaptic effector proteins in adults but not juveniles. In addition to suggesting that the regulation of presynaptic function may contribute to the response to monocular deprivation in adulthood, our work complements a growing body of data demonstrating different mechanisms underlying receptive field plasticity in juveniles versus adults (Fox and Wong, 2005).

The classical roles assigned to synapsin include the maintenance of a reserve pool of synaptic vesicles, and the activity-dependent release from the reserve pool upon increased synaptic stimulation. Accordingly, transgenic mice with targeted deletion of the genes for synapsin I and II have normal synapse number (Rosahl et al., 1995) but reduced number of synaptic vesicles at synapses (Bogen et al., 2006) and reduced potentiation in response to tetanic stimulation (Jensen et al., 2007). Several recent observations suggest additional roles for synapsin, including regulation of the readily releasable pool of synaptic vesicles and the regulation of synaptic vesicle exocytosis (Coleman 2009; Hilfiker et al., 2005; Baldelli et al., 2007). It remains to be determined if distinct pools of synapsin perform each of these different functions. However, immuno-gold electron microscopy identifies two anatomically distinct pools of synapsin in the presynaptic terminal of cultured hippocampal neurons, one that is proximal to the presynaptic membrane, and one that is more distal (Tao-Cheng, 2006). This is consistent with recent reports that synapsin distribution in the presynaptic terminal is broader than the synaptic vesicle protein synaptophysin or the active zone proteins bassoon and cask (Siksou et al., 2007).

Here we show that the synaptoneurosomes preparation separates synapsin into two pools that are independently regulated by visual experience in adulthood. The level of synapsin in the synaptoneurosomes fraction does not increase from postnatal day 28 to adulthood, consistent with recent observation that adult synapsin expression plateaus during the third postnatal week (Bogen et al., 2009). Similarly, the level of synapsin enriched in the synaptoneurosomes fraction is stable following monocular deprivation in juveniles or adults. The synaptoneurosomes complement of synapsin may represent a pool bound to the presynaptic active zone of excitatory synapses (Phillips et al., 2001), which is predicted to sediment with the excitatory synapses during synaptoneurosomes preparation, and may participate in the organization of the presynaptic actin-rich cytomatrix of the endocytic zone (Bloom et al., 2003). We observed an increase in phosphorylation of all synapsin isoforms at site 1 in synaptoneurosomes prepared from adult compared to juvenile visual cortex, similar to an increase in site 1 phosphorylation observed over the course of postnatal development of the

rat dentate gyrus (Parfitt et al., 1991). The developmental increase in synaptoneurosome synapsin phosphorylation may be due to a developmental increase in presynaptic PKA activity, a developmental decrease in presynaptic PP2a activity, or a developmental decrease in presynaptic metabotropic receptors coupled to G_i. However, we observed no change in the phosphorylation of synapsin in synaptoneurosomes prepared from juvenile or adult visual cortex following monocular deprivation, demonstrating the pool of synapsins enriched in synaptoneurosomes is not a target of signaling pathways activated by MD.

In contrast, the pool of synapsin present in the total cortical homogenate, but not enriched in the synaptoneurosome preparation, increases over the course of development, and is targeted by protein kinases activated by the non-deprived input following monocular deprivation in adults. The pool of synapsin present in total cortical homogenate is also regulated by age and visual experience. Monocular deprivation in adults induced an increase in the phosphorylation of synapsin in the binocular visual cortex dominated by synapses serving the non-deprived eye. The increase in phosphorylation of site 1 (PKA site) was paralleled by an increase in phosphorylation at site 3 (CaMKII site). As CaMKII is present only in excitatory neurons, the activity-dependent increase in site 3 phosphorylation observed in homogenate, but not synaptoneurosomes, may represent soluble synapsin released from synaptic vesicles by activity-dependent phosphorylation, which does not sediment with synaptoneurosomes. We propose that the parallel increase we observe in activity-dependent increase in site 1 phosphorylation is occurring in the same population of synapsin present in excitatory axons but is not isolated by the synaptoneurosome procedure. However we cannot rule out the possibility that the increase in synapsin phosphorylation at site 1 in the ipsilateral homogenate following monocular deprivation in adults occurs at inhibitory synapses (Gitler et al., 2004), which are not enriched in the synaptoneurosome preparation.

There are several ways that a non-synaptoneurosome population of synapsin could influence synaptic strength. Regulation of putative synapsin functions via activity-dependent phosphorylation/dephosphorylation of key serine/threonine residues is well-documented. Phosphorylation of sites 1 and 3, which are targeted *in vivo* by PKA and CaMKII respectively, inhibits the binding of synapsins to the lipid bilayer of synaptic vesicles and decreases the affinity of synapsins for actin (Bahler and Greengard, 1987). Presynaptic calcium influx, and the resultant activation of PKA and CaMKII, has been implicated in triggering the dispersion of synaptic vesicles from the reserve pool and facilitation of the synaptic response evoked by synaptic activity (Schiebler et al., 1986; Hosaka et al., 1999; Parfitt et al., 1991; Hilfiker et al., 2005). If synapsin maintains a reserve pool of synaptic vesicles, phosphorylation would release synapsin from synaptic vesicles, releasing the synaptic vesicles from the reserve pool. An increase the number of synaptic vesicles at the active zone is known to increase in the probability of neurotransmitter release. This model is consistent with the contribution of PKA-dependent phosphorylation of synapsin at site 1 to post-tetanic potentiation (Fiumara et al., 2007) and may underlie the PKA- and CaMKII-dependent increase in the amplitude of evoked EPSCs (Nayak et al., 1996; Menegon et al., 2006). Phosphorylation of synapsin would also decrease the size of the reserve vesicle pool, resulting in an enhanced depression in response to tetanic stimulation. The increase in synapsin phosphorylation observed in the ipsilateral visual cortex following monocular deprivation in adults may also reflect an activity-dependent increase in the number of synapses serving the non-deprived input, consistent with strengthening of the non-deprived input which is characteristic of ocular dominance shifts in the adult visual cortex (Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Prusky et al., 2006; Fischer et al., 2007; Sato and Stryker, 2008).

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Figure 1. Developmental increase in synapsin IIa and IIb in the rat visual cortex

A. No difference in the expression of synapsin isoforms (Ia/b, IIa or IIb) phosphorylation at site 1 in homogenate prepared from juvenile (J = postnatal day 28) versus adult (A = postnatal day 70-90) visual cortex. Optical density (O.D.) for each immunoreactive band is expressed as the % of the level observed in the average of juvenile homogenates (dashed line) run on the same gel. Two lanes of a representative immunoblot show the level of each protein in juvenile (J) and adult (A) homogenate prepared from the visual cortex.

B. Significant increase in the expression of synapsin IIa and IIb, but not synapsin Ia/b in homogenate prepared from adult (A) relative to juvenile visual cortex. $*=p<0.05$, unpaired t-test adult versus juvenile.

C. No difference in the proportion of synapsin phosphorylated on site 1 (phosphorylated synapsin/total synapsin) in homogenate prepared from juvenile (J) versus adult (A) visual cortex.

Figure 2. Differential distribution of phosphorylated synapsin in juvenile versus adult synaptoneuroosomes

A. Synaptoneuroosomes prepared from the juvenile visual cortex have a significantly reduced level of synapsin phosphorylated at site 1 relative to an equal concentration of homogenate. Optical density (O.D.) for each immunoreactive band is expressed as the % of the level observed in homogenate (dashed line) prepared from the same subject run on the same gel. Two lanes of a representative immunoblot show the level of each protein in a synaptoneurosome (S) and homogenate (H) prepared from the juvenile visual cortex. *= $p < 0.05$ paired t-test S versus H, each isoform calculated separately.

B. No difference in the level of total synapsin in synaptoneuroosomes versus an equal concentration of homogenate prepared from the juvenile visual cortex.

- C. The proportion of synapsin phosphorylated at site 1 (phosphorylated synapsin/total synapsin) is significantly lower in synaptoneurosomes than homogenate prepared from juvenile visual cortex. $*=p<0.05$ paired t-test S versus H, each isoform calculated separately.
- D. No difference in the level of synapsin phosphorylated at site 1 in synaptoneurosomes compared to homogenate prepared from adult visual cortex.
- E. No difference in the level of total synapsin in synaptoneurosomes versus an equal concentration of homogenate prepared from the adult visual cortex.
- F. No difference in the proportion of synapsin phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in synaptoneurosomes versus homogenate prepared from the adult visual cortex.

Figure 3. Monocular deprivation in juveniles does not regulate synapsin phosphorylation

A. No change in the proportion of synapsin Ia/b phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in homogenate prepared from juvenile visual cortex following monocular deprivation. Optical density (O.D.) of the ratio of phosphorylated/total synapsin expressed as % of the level observed in homogenate prepared from normal-reared controls run on the same gel. Two lanes of a representative immunoblot show the level of phosphorylated synapsin Ia/b (P-synapsin Ia/b) and total synapsin Ia/b (T-synapsin Ia/b) in a homogenate prepared from the juvenile visual cortex contralateral (C) and ipsilateral (I) to the deprived eye.

B-C. No change in the proportion of synapsin IIa or IIb phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in homogenate prepared from the visual cortex following monocular deprivation in juveniles.

D-F. No change in the proportion of any of the synapsin isoforms phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in synaptoneurosomes prepared from the visual cortex following monocular deprivation in juveniles.

Adult Homogenate Site 1 Phosphorylation

Figure 4. Monocular deprivation in adults induces an increase in synapsin phosphorylation at site 1 in homogenate prepared from the ipsilateral visual cortex

A. Monocular deprivation in adults induces a significant increase in the proportion of synapsin Ia/b phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in homogenate prepared from the visual cortex ipsilateral to the deprived eye (A₁ **p*<0.05; unpaired t-test versus control). No change in synapsin Ia/b phosphorylation was observed in sham-operated adults (A₂). Optical density (O.D.) of the ratio of phosphorylated/total synapsin is expressed as the % of the level observed in homogenate prepared from normal-reared controls run on the same gel. Two lanes of representative immunoblot show the level of phosphorylated synapsin Ia/b/ (P-synapsin Ia/b) and total synapsin Ia/b (T-synapsin Ia/b)

in homogenate prepared from the adult visual cortex contralateral (C) and ipsilateral (I) to the deprived eye.

B. Monocular deprivation in adults induces a significant increase in the proportion of synapsin IIa phosphorylated at site 1 in homogenates prepared from the visual cortex ipsilateral to the deprived eye (B_1 ; $*p<0.05$; unpaired t-test versus control). No change in synapsin IIa phosphorylation was observed in sham-operated adults (B_2).

C. Monocular deprivation in adults induces a significant increase in the proportion of synapsin IIb phosphorylated at site 1 in homogenates prepared from the visual cortex ipsilateral to the deprived eye (C_1 ; $*p<0.05$; unpaired t-test versus control). No change in synapsin IIb phosphorylation was observed in sham-operated adults (C_2).

Adult SN Site 1 Phosphorylation

Figure 5. Monocular deprivation in adults induces does not regulate synapsin phosphorylation in synaptoneurosomes

A. No differences in the proportion of synapsin Ia/b phosphorylated at site 1 (phosphorylated synapsin/total synapsin) in synaptoneurosomes prepared from adult visual cortex following monocular deprivation (A₁) or sham-operated controls (A₂). Optical density (O.D.) of the ratio of phosphorylated/total synapsin is expressed as the % of the level observed in synaptoneurosomes prepared from normal-reared controls run on the same gel. Two lanes of a representative immunoblot showing the level of phosphorylated synapsin Ia/b/ (P-synapsin Ia/b) and total synapsin Ia/b (T-synapsin Ia/b) of synaptoneurosomes prepared from the adult visual cortex contralateral (C) and ipsilateral (I) to the deprived eye.

B. Monocular deprivation in adults (B_1) and sham operated controls (B_2) does not regulate the proportion of synapsin IIa phosphorylated at site 1 in synaptoneurosomes prepared from adult visual cortex contralateral or ipsilateral to the deprived eye.

C. Monocular deprivation in adults (C_1) or sham operated controls (C_2) does not regulate the proportion of synapsin IIb phosphorylated at site 1 in synaptoneurosomes prepared from the visual cortex contralateral or ipsilateral to the deprived eye.

Figure 6. Monocular deprivation in adults induces an increase in synapsin phosphorylation at site 3 in homogenate prepared from the ipsilateral visual cortex

A. Monocular deprivation in adults induces a significant increase in the proportion of synapsin Ia/b phosphorylated at site 3 (phosphorylated synapsin/total synapsin) in homogenate prepared from the visual cortex ipsilateral to the deprived eye (A_1 * $p < 0.05$; unpaired t-test versus control). No change in the phosphorylated synapsin Ia/b was observed in sham-operated controls (A_2). Optical density (O.D.) of the ratio of phosphorylated/total synapsin is expressed as the % of the level observed in homogenate prepared from normal-reared controls run on the same gel. Two lanes of representative immunoblot show the level of phosphorylated synapsin Ia/b (P-synapsin Ia/b) and total synapsin Ia/b (T-synapsin Ia/b)

in homogenate prepared from the adult visual cortex contralateral (C) and ipsilateral (I) to the deprived eye.

B. No differences in the proportion of synapsin Ia/b phosphorylated at site 3 (phosphorylated synapsin/total synapsin) in synaptoneurosomes prepared from adult visual cortex following monocular deprivation.

C. Monocular deprivation in juveniles does not regulate the proportion of synapsin Ia/b phosphorylated at site 3 in homogenates prepared from the ipsilateral relative to contralateral visual cortex.

D. No differences in the proportion of synapsin Ia/b phosphorylated at site 3 (phosphorylated synapsin/total synapsin) in synaptoneurosomes prepared from juvenile visual cortex following monocular deprivation.