Changes in PSD-95 expression in environmentally enriched rat somatosensory cortex

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Independent Project for ISCI 3A12

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Introduction

Dendritic spines are the actin-rich, dynamic postsynaptic sites of most excitatory synapses[1–3]. Changes in dendritic spine shape have been shown to occur rapidly and actin polymerization-dependently[4]. Dendritic spines are closely associated with the postsynaptic density (PSD): an electron-rich, membrane-associated disc that consists of the receptors, channels, and structural proteins required for neurotransmission[5].

The main scaffolding protein of the glutamatergic postsynaptic density is postsynaptic density protein 95 (PSD-95)[6]. The PSD typically contains 200-300 PSD-95 molecules, which play a key role in anchoring and stabilizing both N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors at glutamatergic synapses[6,7]. NMDA receptors are tetramers of two obligatory GluN1 subunits and two additional GluN2 (or GluN3) subunits, where the two main types of GluN2 subunits are GluN2A and GluN2B[8]. Different combinations of subunits result in different receptor properties. PSD-95 binds both GluN2A and GluN2B NMDA receptor subunits[9–11], and has been shown to block NMDA receptor internalization by binding NR2B and causing clustering of the receptor at the PSD[12]. Furthermore, PSD-95 binds stargazin, through which it interacts indirectly with AMPA receptors[13]. In fact, the stability of AMPA receptors at the PSD is dependent on PSD-95 binding to stargazin, while disruption of the interaction between PSD-95 and stargazin prevents AMPA receptor accumulation at the postsynaptic density[14]. Furthermore, mutation of the PSD-95-binding domain of stargazin downregulates hippocampal AMPA receptor mEPSCs[15]. As such, PSD-95 plays a key role in stabilizing both AMPA and NMDA receptors at the PSD of the glutamatergic synapse.

Changes in NMDA and AMPA receptor numbers at the PSD are a mechanism for synaptic plasticity[16]. At negative potentials, NMDA receptors are blocked by Mg^{2+}, but release of glutamate from the

presynaptic terminal as a result of high-frequency stimulation will open AMPA receptors, causing an influx of Na^+ and K^+, and increasing the potential at the postsynaptic membrane. This relieves the Mg^{2+} block, and NMDA receptors can then open upon binding glutamate. The opening of NMDA receptors allows Ca^{2+} to enter the postsynaptic neuron, where it acts as a second messenger and causes the addition (in long-term potentiation, LTP) or removal (in long-term depression, LTD) of AMPA receptors from the postsynaptic density via complex signalling pathways[16].

LTP has been shown to potentially involve calcium-calmodulin-dependent kinase II phosphorylation of stargazin following Ca^{2+} entry into the cell via open NMDARs[17]. Phosphorylation of stargazin results in increased association of stargazin with PSD-95, thus decreasing internalization of AMPARs and stabilizing them at the postsynaptic density[17]. Therefore, PSD-95 may potentially be used as a signal molecule for LTP induction.

Environmental enrichment has very distinct effects on neuronal morphology. Environmental enrichment has been shown to enhance dendritic branching in hippocampus[18], primary auditory cortex (A1)[19], and primary somatosensory cortex (S1)[20]. In A1, environmental enrichment has also been shown to increase strength of auditory cortex responses in both juvenile and adult rats, and can induce remodeling of cortical responses in even under two weeks[21]. In all areas, cortices of rats housed in enriched environments exhibit greater numbers of active spines, enlarged spine heads, and more double-headed spines[22]. Spine density and turnover rate on dendrites of the pyramidal cells of the somatosensory cortex in both infant-enriched and adult-enriched rats have been shown to increase following environmental enrichment[23]. Therefore, environmental enrichment has a clear effect on the properties of dendritic spines in many areas of the brain.

Long-term potentiation (LTP) as well as long-term depression (LTD) has been shown to increase in rats following five weeks of environmental enrichment[24]. Furthermore, PSD-95 has been shown to

localize to the dendrites of central visual neurons activity-dependently due to eye opening, while binding stargazin equally, NR2A-rich NMDARs more, and NR2B-rich NMDARs less than before eye opening[25]. PSD-95 expression has been shown to increase in response to environmental enrichment in multiple major brain regions, as well[26].

PSD-95 brightness in GFP studies has been shown to be correlated with PSD size, and subsequently spine volume[27]. As such, PSD-95 expression can be used to quantify spine volume.

Given that PSD-95 expression can be used as a marker of postsynaptic density size, which is correlated with dendritic spine volume, PSD-95 is a protein of interest for studying changes in dendritic spines in response to environmental enrichment. Furthermore, because PSD-95 may mediate some aspects of long-term potentiation or long-term depression by regulating AMPA and NMDA receptors, studying its expression could indicate changes in synaptic plasticity that occur at dendritic spines in response to environmental enrichment.

As such, the current study investigated PSD-95 expression in rat somatosensory cortex in response to long-term and short-term environmental enrichment. Using Western blotting to quantify PSD-95 expression following long-term and short-term environmental enrichment compared to control, we found no significant differences in expression of the protein in long-term enrichment or short-term enrichment compared to control. This may indicate that no significant changes in dendritic spine volume or density, PSD size, or synaptic plasticity, occur at the synapse. However, because not all available samples have been immunoblotted and analyzed, increasing sample size may show that the current results are because of a type II error, and therefore changes in PSD-95 expression following environmental enrichment may yet be observed following addition of samples to the dataset.

Methods

Animals

Long Evans rats (n=22) were reared in three groups: control (n=11), long-term enrichment (LTE, n=5), and short-term enrichment (n=6). All animals were weaned and lived in standard conditions until P64.

LTE rats were all placed in a large, four-levelled enriched cage, with three ramps, and toys of different shapes and colours that were replaced weekly. Food and water locations within the cage were also changed weekly. LTE rats lived in the enriched housing from P64 until sacrifice at P490.

STE rats were placed in the enriched housing at P490, and sacrificed two weeks later, at P504.

Control rats lived in standard conditions (two rats to a cage, no enrichment, same water and food locations) until sacrifice at P490.

All animals were assigned sample IDs, as per **Table 1.**

Table 1. Total dataset sample IDs.

Sample ID	Condition
E08	Normal
E12	Normal
E13	Normal
E17	Normal
E06	Normal
E07	Normal
E10	Normal
E11	Normal
E15	Normal
E16	Normal
E18	Normal
LE01	Long-term enrichment
LE03	Long-term enrichment
LE04	Long-term enrichment
LE05	Long-term enrichment
LE02	Long-term enrichment
SE19	Short-term enrichment
SE22	Short-term enrichment
SE23	Short-term enrichment
SE24	Short-term enrichment
SE20	Short-term enrichment
SE25	Short-term enrichment

Tissue collection and preparation

Animals were euthanized using Euthanyl (150 mg/kg) and transcardial perfusion with cold 0.1 M PBS cleared the circulating fluid. The brain was removed from the skull and immersed in cold PBS. A 2 mm by 2 mm sample of somatosensory cortex was identified and cut from each hemisphere of the brain. The samples were frozen on dry ice and stored at -80ºC.

Tissue samples were suspended in cold homogenization buffer inside a Lysing Matrix-D tube (Cat. #116913050, MP Biomedicals, Santa Ana, CA, USA). Once loaded into the FastPrep-24 Tissue and Cell Homogenizer (Cat. #116004500, MP Biomedicals, Santa Ana, CA, USA), a vertical velocity of 6 m/s was used for 40 s to homogenize the samples. 10% sodium-dodecyl-sulfate (SDS) was added to each sample. Each sample was then heated to 90ºC for 10 minutes. Samples were stored at -80ºC.

Western blotting

Tissue samples (25 μL) were separated on polyacrylamide gels (Mini-PROTEAN TGX Precast Protein Gels) (Bio-Rad Laboratories Inc, Hercules, CA, USA) (and another type) for two hours. Samples were then transferred to polyvinylidene difluoride (PVDF-FL) blots (Imobilion-FL). Each sample was run 2-4 times, along with a control which contained a small amount of each sample. Western blotting was done in two series: pilot data (**Table 2**) and additional data (**Table 3**), each with their own control samples consisting of samples from each member of the series. Because the pilot data and additional data had different controls, an additional blot was also run with both controls (3 runs) in order to find a conversion factor between datasets.

Table 2. Pilot dataset sample IDs.

Sample ID	Condition
E08	Normal
E12	Normal
E13	Normal
E17	Normal
LE01	Long-term enrichment
LE03	Long-term enrichment
LE04	Long-term enrichment
LE05	Long-term enrichment
SE19	Short-term enrichment
SE22	Short-term enrichment
SE23	Short-term enrichment
SE24	Short-term enrichment

Table 3. Additional dataset sample IDs.

Sample ID	Condition
E06	Normal
E07	Normal
E10	Normal
E11	Normal
E15	Normal
E16	Normal
E18	Normal
LE02	Long-term enrichment
SE20	Short-term enrichment
SE23*	Short-term enrichment
SE24*	Short-term enrichment
SE25	Short-term enrichment

**Additional runs of sample IDs included in the pilot study.*

Blots were blocked in PBS/Licor Blocking Buffer (1:1) for one hour, and were then incubated in (anti-PSD-95 primary) antibodies overnight. After being washed with PBS-Tween three times following 1^{0} antibody incubation, blots were incubated with 2^{0} antibody for one hour, and washed three times in PBS-Tween.

Blot quantification

Blots were scanned using the Odyssey Licor Inc. scanner, and optical density was quantified using Odyssey 3.0 software. To obtain protein expression, each band's optical density was divided by the width of the band, and normalized to control for each run. To quantify the "additional data" in terms of the "pilot data", pilot data control sample expression was divided by the additional data control sample expression, obtaining a conversion factor of 0.8488. The additional data sample expression was then multiplied by this conversion factor in order to obtain a larger dataset consisting of "pilot data" and "additional data".

Outliers for each run were determined quantitatively, by graphing each run and eliminating aberrant values. The average optical density of the 2-4 runs for each sample was calculated. The mean PSD-95 expressions of each group (Normal, LTE, STE) were compared using a one-factor analysis of variance (ANOVA), as well as individual two-tailed, homoscedastic sample t-tests.

Results

No statistically significant differences in PSD-95 expression between the normal, long-term enrichment, or short-term enrichment conditions were observed. In order to compare expression of PSD-95 between the normal, LTE, and STE conditions, protein expression in all three of the conditions was calculated as a percentage of normal PSD-95 expression (control=100%, SE=11.99%) (**Figure 1**).

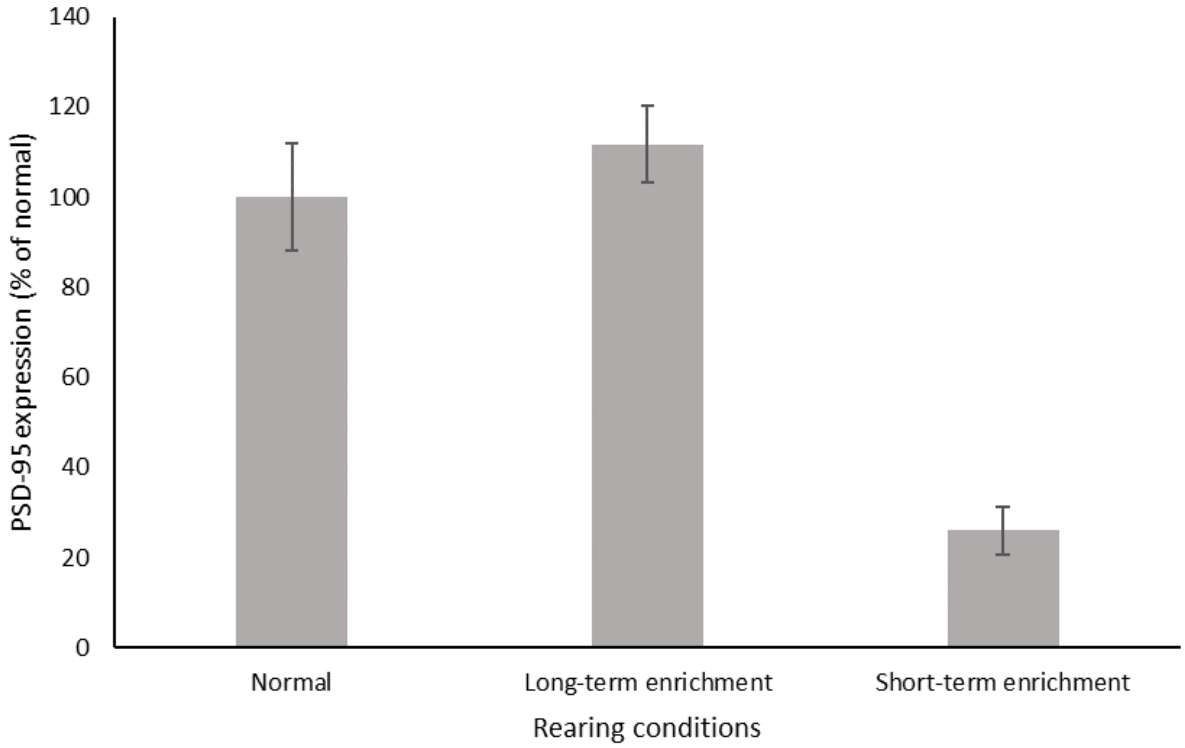

Figure 1. PSD-95 expression in long-term environmental enrichment, short-term environmental enrichment, and normal rearing conditions in percentage of PSD-95 expression in normal rearing conditions.

LTE expression was 111.71% of control (SE=8.56), showing an 11.71% increase. STE expression was 26.01% of control (SE=5.32%), showing a 73.99% decrease from control. Homoscedastic sample t-tests showed that the difference between the mean normal and LTE expression, and between the mean normal and STE expression, was not significant ($p>0.05$). However, a t-test comparing the mean protein expression between the STE and LTE conditions showed that LTE protein expression was significantly elevated from STE ($p=0.022$). However, a subsequent single-factor ANOVA comparing the means of all three groups revealed no significance ($F<F_{crit}$), indicating that none of the sample means differed significantly from one another.

Discussion

The current study reports no statistically significant changes in PSD-95 expression in somatosensory cortex of environmentally enriched rats. This may be because not all available samples have been immunoblotted. Running those samples would increase the sample size of the groups being analyzed and increase the power of the statistical analysis, therefore decreasing the probability of a type II error, or false negative. Given that previous studies have shown activity- and environmental enrichment-dependent increases in PSD-95 expression, this is likely the case[25,26].

However, if we assume that the current results are not a false negative, there may be several explanations why PSD-95 expression is not increasing in response to environmental enrichment. Firstly, previous studies on changes in PSD-95 and dendritic branching in somatosensory cortex have used a shorter environmental enrichment time (3 weeks to 30 days) compared to the current long-term exposure time of 426 days (≈14 months), and greater than the short-term exposure time of 2 weeks, which may indicate that the effects of environmental enrichment either do not persist or become ineffective over time[22,26]. Furthermore, positive effects of environmental on LTP at hippocampal synapses have been shown to persist only for a few weeks following exposure[24], suggesting that LTP-related effects on PSD-95 expression might be temporally limited. However, three-hour-long daily exposure to environmental enrichment for 18 months, a comparable time period to this study, has nevertheless been associated with improved memory, increased neural proliferation, and increased synaptophysin expression in the hippocampus[28]. The current study would therefore imply that long-term continuous exposure to environmental enrichment, as opposed to a three-hour-long daily exposure, abolishes increases in PSD-95 expression due to environmental enrichment. Though the aforementioned study did not measure PSD-95 expression levels, synaptophysin expression has been shown to increase alongside PSD-95 in response to a shorter time period of environmental enrichment in rat somatosensory cortex[26]. Thus, the current results suggest that there

may be a time limit on environmental enrichment's effect on PSD-95 expression levels. It would therefore be of interest to study changes in expression levels while varying environmental enrichment exposure timeframes, to isolate how much exposure is required for the previously observed changes in PSD-95 expression.

As PSD-95 is a marker for the postsynaptic density, it is possible that increases in dendritic spine density and size in response to environmental enrichment may be occurring without changing the size of the PSD. Though persistent dendritic spines almost always bear a PSD (as marked by PSD-95), bearing a PSD does not guarantee that a spine will grow or persist[27]. As such, the current results imply that environmental enrichment, and thus, dendritic spine growth may occur without changing the size of the PSD. However, the current study only implies that no changes in PSD size occur in environmental enrichment. Further morphological studies, involving immunohistochemically staining for PSD-95, as well as dendritic spine markers, would be required in order to examine this possibility.

The lack of change in PSD-95 expression following environmental enrichment observed in the current study may also have implications for AMPA and NMDA receptor-dependent synaptic plasticity in environmentally enriched rats. A lack of change in PSD-95 expression implies that AMPA and NMDA receptor-dependent synaptic plasticity is not occurring in the somatosensory cortex following environmental enrichment, meaning that there is no change in the number of AMPA receptors at the postsynaptic density and therefore no LTP or LTD occurring. However, this is unlikely, given that environmental enrichment has been shown to enhance LTP in rat primary somatosensory cortex (S1) NMDA receptor-dependently[29]. Therefore, because PSD-95 is an AMPA and NMDA receptor stabilizing protein[12,13] and as such can be used as a measure of AMPA and NMDA receptor numbers at the PSD, the current data suggest that the net amount of PSD-95 does not change during environmental enrichment. Because LTP does occur during environmental enrichment in rat

somatosensory cortex, an increase in AMPA receptors and therefore PSD-95 might be occurring at the same time as an equivalent decrease in NMDA receptors and therefore a decrease in PSD-95, resulting in a net zero change in PSD-95 expression. *In vivo* molecular studies, with labelled NMDARs, AMPARs, and PSD-95 would need to be conducted to examine receptor dynamics at the postsynaptic density following environmental enrichment, to determine whether a zero net change in PSD-95 expression actually occurs.

Though no changes in PSD-95 occurred in response to environmental enrichment in this study, quantifying and analyzing the rest of the available samples that have not been yet analyzed may reveal significant changes in PSD-95 expression in response to environmental enrichment.

An increase in PSD-95 expression following long-term environmental enrichment could imply a larger PSD, and could indicate an increase in dendritic spine volume, as well as synaptic strengthening[30]. As such, an increase in PSD-95 would confirm that environmental enrichment causes increases in dendritic spine volume and increases the size of the PSD. An increase in PSD-95 expression could also suggest increased stabilization and reduced internalization of AMPA and NMDA receptors at the synapse following environmental enrichment due to increased association of PSD-95 with stargazin and the NR2B NMDA receptor subunit. Therefore, increased PSD-95 in response to environmental enrichment could imply PSD-95 as a marker for experience-driven LTP and synaptic strengthening as a result of dendritic spine growth, but further morphological and immunohistochemical studies would be required in order to confirm this.

However, if PSD-95 expression were to decrease significantly from normal following environmental enrichment, this could imply that PSD size may be decreasing despite dendritic spine volume increasing, or perhaps that dendritic spine volume may be decreasing. Decreased PSD-95 may indicate increased dendritic pruning as a result of environmental enrichment, potentially caused by LTD.

Experience-dependent LTD could therefore be mediated by PSD-95 in environmental enrichment, where a decrease in PSD-95 would cause destabilization of AMPA and NMDA glutamate receptors at glutamatergic synapses in the somatosensory cortex, therefore causing removal of AMPA and NMDA receptors from the PSD and a long-term decrease in neuronal excitability.

Conclusion

In conclusion, the current study found no significant differences in expression of PSD-95 in the somatosensory cortex between normal, long-term enriched, or short-term enriched rats. This lack of change implies that extended long-term exposure to environmental enrichment may not have as pronounced of an effect on PSD-95 expression as shorter exposure times. As well, these results suggest that even though LTP is thought to occur following environmental enrichment, the increased expression of AMPA receptors at the PSD following LTP could coincide with an equivalent decrease in NMDA receptors at the PSD, but further morphological studies would need to be conducted in order to support this hypothesis. However, because PSD-95 expression for all available samples has not yet been quantified and analyzed, addition of these samples to the current dataset could still show changes in PSD-95 expression in the somatosensory cortex of environmentally enriched rats. In addition, further morphological studies on the neuronal structure of the somatosensory cortex, as well as immunohistochemical analysis of PSD-95 expression and colocalization with glutamate AMPA and NMDA receptors might shed light on the effects of environmental enrichment on synaptic transmission and function.

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