Brief Communications

Enhanced Plasticity in Zincergic, Cortical Circuits after Exposure to Enriched Environments

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Despite the plethora of reports that demonstrate plasticity in the mammalian cerebral cortex, the characterization of the cellular mechanisms that mediate it is sparse. Here, we show that the magnitude of the experience-dependent regulation of vesicular zinc is significantly increased through enriched-environment housing. Mice were reared either in a deprived environment and subsequently housed in deprived, minimally enriched, or enriched conditions after the removal of the c-row of vibrissae or reared in an enriched environment before and after vibrissae removal. Levels of vesicular zinc were assessed in deprived and nondeprived barrels 6 h to 14 d after vibrissae removal. We found that housing in enriched environmental conditions resulted in a greater change in vesicular zinc levels than did other housing conditions; however, this effect was dependent on both the magnitude and duration of enrichment. Our data indicate that enriched-environment housing has profound effects on the regulation of vesicular zinc that occurs concurrently with experience-dependent plasticity, suggesting a role for zinc in the multitude of cortical modifications associated with enriched environments.

Key words: zinc; barrel cortex; cortical plasticity; enrichment; somatosensory cortex; synaptic plasticity

Introduction

The divalent cation zinc is an essential element, having a multitude of general and specialized functions in the body. In the brain, after transport across the blood—brain barrier, the majority of zinc is bound to proteins having regulatory and structural functions. However, 15–20% of zinc in the brain is found within synaptic vesicles in a subset of glutamatergic neurons (Frederickson, 1989; Beaulieu et al., 1992) and is released in a voltage- and activity-dependent manner (Assaf and Chung, 1984; Howell et al., 1984). Zinc can potently alter the functioning of a variety of neurotransmitter receptors, as well as modulate second messenger systems (Westbrook and Mayer, 1987; Paoletti et al., 1997; Huang et al., 2008). Furthermore, the experimental induction of plasticity, long-term potentiation and depression (LTP and LTD, respectively), has been shown to be robustly affected by zinc concentrations (Li et al., 2001; Izumi et al., 2006; Huang et al., 2008).

Conversely, experience-dependent plasticity produced through the manipulation of sensory input is accompanied by changes in vesicular zinc levels. For example, the removal or stimulation of vibrissae is a well characterized method that results in considerable plasticity within the barrels of the somatosensory cortex (Glazewski and Fox, 1996; Bender et al., 2006). Such manipulations have been shown to result in a rapid increase or decrease (with vibrissae removal or stimulation, respectively) in vesicular zinc levels within the corresponding barrels (Czupryn

and Skangiel-Kramska, 2001b; Brown and Dyck, 2002, 2005). Changes in vesicular zinc levels that occur concurrently with experience-dependent plasticity, together with the regulatory effects of zinc on neurotransmission, are suggestive of a modulatory effect of zinc on this type of cortical plasticity.

To expand on these findings, we have examined how exposure to enriched environments altered the dynamics of vesicular zinc regulation in experience-dependent plasticity generated by vibrissae plucking. Housing laboratory animals in enriched environments that promote sensory, motor, and social stimulation has robust effects on the structure and function of the cortex. For instance, enriched environmental housing promotes improvements in behavioral tasks (Venable et al., 1988; Leggio et al., 2005), leads to alterations in the morphology and physiology of cortical neurons, and, in certain brain regions, increases neurogenesis (Sirevaag and Greenough, 1988; Kempermann et al., 1997; Leggio et al., 2005). Electrophysiologically, enriched environments can induce fundamental alterations in the induction and maintenance of LTP/LTD (Foster et al., 1996; Foster and Dumas, 2001; Abraham et al., 2002; Artola et al., 2006; Irvine et al., 2006). Thus, housing in enriched conditions results in a multitude of cortical changes, some of which may involve a zincergic component.

Utilizing different magnitudes and durations of enrichment, we examined how enriched environments affected the experience-dependent regulation of vesicular zinc after vibrissae removal. Our results demonstrate that a greater increase in vesicular zinc staining is observed within barrels deprived of sensory input when mice are housed in enriched conditions, indicating that changes in vesicular zinc regulation may contribute to the changes in plasticity that occur with enriched-environment housing.

Materials and Methods

All mice were provided food and water *ad libitum* and were housed in standard laboratory housing under a 12 h light/dark cycle for the duration of the experiment. All procedures were authorized by the Animal Care Committee of the University of Calgary and mice were handled in accordance with the guidelines dictated by the Canadian Council for Animal Care.

In total, four groups of different environmental manipulations were used to examine the effect of enriched housing conditions on the regulation of vesicular zinc after the vibrissae plucking (Fig. 1). These groups were chosen to delineate the effect of differing degrees of enrichment on the vesicular zinc response. The first three groups examined the effect of environment after the removal of vibrissae. In these groups, male C57BL/6 mice were housed in a deprived environmental condition after weaning, consisting of a standard shoebox cage (13 × 17.5 × 28 cm) with mice housed two to a cage. At 12 weeks of age, the c-row of vibrissae was plucked under light isoflurane anesthesia in a manner that prevented follicle damage. After plucking, the mice were separated into three groups. The first, referred to as "deprived," consisted of mice that were simply returned to their original home cage. Mice in the "minimally enriched" group were returned to their home cage but also had a novel item added, stimulating vibrissae use but not necessarily having the broad effects of multiple levels of enrichment. The third group of mice, "deprived to enriched," were placed in an enriched environment after vibrissae removal. The enriched environment consisted of a large, bilevel cage (61 × 60 × 60 cm) that contained items such as tunnels, wire mesh, and running wheels, and were housed six to eight mice to a cage. Finally, the effect of prolonged enrichment was investigated through a fourth "enriched" group, which consisted of mice that were housed in an enriched environment before and after vibrissae plucking.

Mice remained in their assigned housing conditions for 6 h, 48 h, 7 d, or 14 d ($n = 6$ per group per duration) after vibrissae plucking. Additionally, control mice that underwent the same protocols as above without vibrissae plucking were examined.

After the designated time had elapsed, the mice were prepared for histochemical staining of vesicular zinc by intraperitoneal administration of sodium selenite (15 mg/kg, i.p.; Sigma). After 1 h, mice were killed using an overdose of sodium pentobarbital. Brains were bisected after extraction and the cortices gently removed. To visualize the barrel cortex in a single plane, cortices were flattened between glass slides and then rapidly frozen. Sections were cut on a cryostat to 20 μm.

The Danscher autometallographic staining method was used to selectively visualize the histochemically reactive zinc present within vesicles of axon terminals (Danscher, 1982). To do this, sections were thawed at room temperature, fixed in 95% ethanol for 15 min, hydrated in a descending series of ethanol and water (75%, 2 min; 50%, 2 min; distilled water 3 × 2 min), and then dipped in a 0.5% gelatin solution. Sections were placed in the developing solution consisting of 50% gum arabic (100 ml), 2.0 M sodium citrate buffer (25 ml), 0.5 M hydroquinone (30 ml), 37 mM silver lactate (30 ml), and 20 ml of distilled water for 1.5 h. After development, sections were rinsed under warm tap water (37°C, 10 min) and distilled water (2 × 2 min). Development was halted by immersing the sections in sodium thiosulfate (5%, 12 min). After rinsing (distilled water, 3 × 2 min), sections were fixed and dehydrated in an ascending series of ethanol (70%, 30 min; 95%, 5 min; 100%, 10 min), cleared in xylene (10 min), and coverslipped using Permount.

Images were captured digitally (Qimaging monobit 10-bit camera; Zeiss Axioskop2 microscope) and stored on an Apple iMac Intel Core Duo computer using Openlab imaging software (version 4.0.4, Improvision). All images were captured at a 256 grayscale.

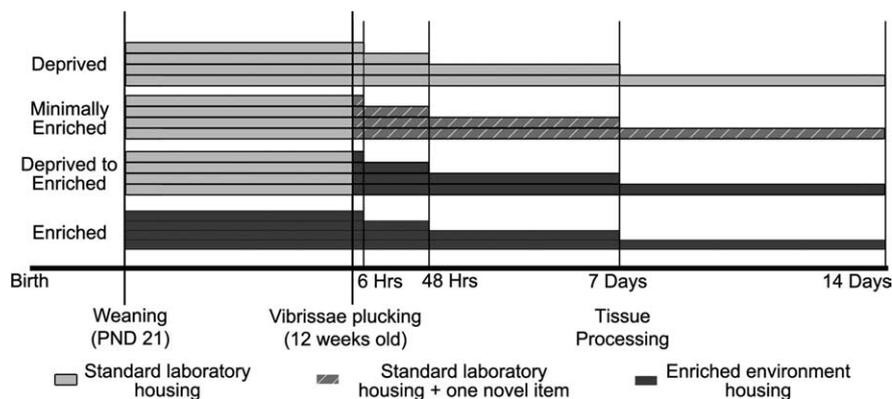Densitometric analysis was conducted using Openlab. Four to five

Figure 1. Schematic for experimental protocol. Mice were reared under deprived conditions for 12 weeks, and then the c-row of vibrissae was removed. Mice were then returned to their home cage (deprived), returned to their home cage with a novel item inside (minimally enriched), or placed in an enriched environment (deprived to enriched). Additionally, a subset of mice were reared in an enriched environment and placed back in these conditions after vibrissae removal (enriched). Mice remained in their postplucking environment for 6 h, 48 h, 7 d, or 14 d before being processed for histochemical analysis.

adjacent sections that represented the majority of the posterior medial barrel subfield were captured. To control for inherent, between-animal differences in staining intensity, a ratio was calculated that compared the mean grayscale values for the deprived row (c-row; numerator) and the adjacent nondeprived rows (b- and d-row; denominator). The staining intensity between the c-row and b/d-rows of barrels is not consistent in unmanipulated mice (Brown and Dyck, 2002), and to ensure that our environmental manipulations did not alter this basal difference and our results, we also calculated a ratio value for control mice that were exposed to our environmental manipulations but did not undergo vibrissae plucking. We then calculated a relative percentage difference in staining intensity by comparing the average ratio obtained in the experimental group with that obtained in the corresponding control group.

All image quantifications were conducted while the investigator was blind to the experimental conditions. The data were submitted to a 4 × 4 (group × time point) factorial ANOVA. Significant main effects and interactions were followed up using the Tukey test.

Results

Consistent with previous findings, the removal of vibrissae resulted in robust increases in vesicular zinc within the corresponding barrels (Czupryn and Skangiel-Kramska, 2001b; Brown and Dyck, 2002). Representative images can be found in Figure 2, while Figure 3 displays the quantified data.

The main effects of group ($F_{(3,176)} = 24.2, p < 0.001$) and time point ($F_{(3,176)} = 20.1, p < 0.001$) were significant, as was the interaction ($F_{(9,176)} = 4.26, p < 0.001$). The percentage increase in vesicular zinc staining was lowest in the deprived group at all time points examined. At the 6 h survival time point, a significantly greater increase in vesicular zinc staining was observed in the minimally enriched group compared with the deprived group, whereas at all other time points, no significant differences were found between these two groups. Although the increase in staining intensity of the deprived to enriched group was relatively low at the 6 and 48 h time points, at 7 d, a significantly greater increase in vesicular zinc staining was observed after vibrissae removal, compared with the deprived and minimally enriched groups. At 14 d, the deprived to enriched group had a significantly greater increase only when compared with the deprived group.

The enriched group consistently had an augmented increase in vesicular zinc staining of the barrels deprived of sensory input. At the 6 h time point, the increase was significantly different from the deprived to enriched group only, whereas at all other time
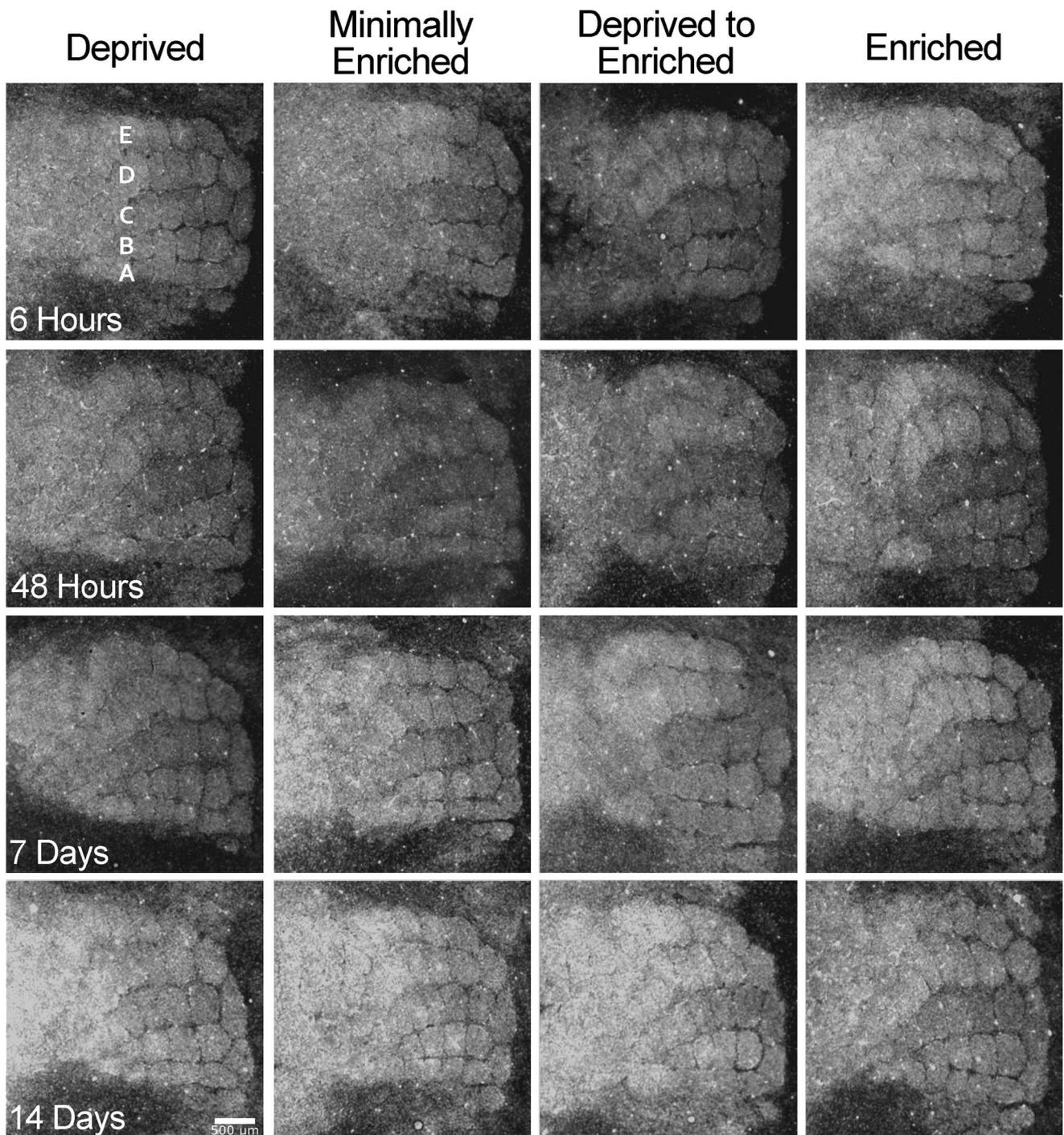
Figure 2. Experience-dependent regulation of vesicular zinc. The somatosensory cortex is well defined by vesicular zinc staining, and the five rows (A–E) of barrels corresponding to the vibrissae are easily resolved. Although an increase in zinc staining was observed in the c-row of all mice, the increases were noticeably augmented in the mice exposed to an enriched environment. Scale bar, 500 μm.

points examined, a significantly greater change was observed when comparing the enriched group with both the deprived and minimally enriched groups.

Discussion

In the present study, we explored whether manipulating the environmental housing conditions of mice would affect the regulation of vesicular zinc occurring concurrently with cortical plasticity induced by vibrissae plucking. We found that, in a manner dependent on the duration and degree of exposure, environmental enrichment led to a greater zincergic response to vibrissae plucking. Mice housed without enrichment were found to consistently have the lowest increase in vesicular zinc after vibrissae plucking. Conversely, mice housed in enriched conditions for the duration of the experiment had the greatest increase in vesicular zinc at all time points examined. Intermediate increases in vesicular zinc were observed with the minimal enrichment and deprived to enriched paradigms. Thus, we observe an increase in vesicular zinc within barrels deprived of sensory input that is correlated with the level of enrichment.

We found it interesting that minimal enrichment resulted in an immediate but brief amplification of the zincergic response to vibrissae plucking, whereas the deprived to enriched housing
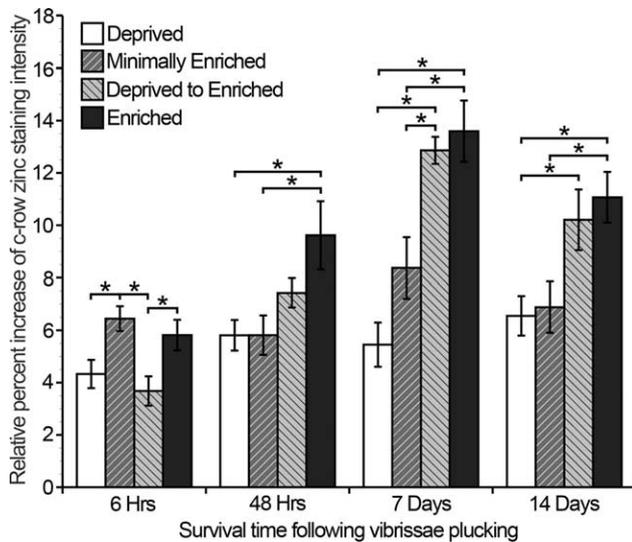
Figure 3. Quantification of changes in vesicular zinc staining in the barrel cortex after the plucking of the c-row of vibrissae in mice housed in enriched conditions. Percentage change in zinc staining reflects the difference in staining intensity ratio values between experimental (plucked) and control animals. Minimal enrichment had a small, but significant effect on the change in zinc staining only at the 6 h time point. Mice housed in the enriched environment consistently had a significantly greater change in zinc staining compared with the deprived condition at all time points. Exposure to an enriched environment after vibrissae removal (deprived to enriched) resulted in an augmented change compared with the deprived group, but only at the later time points. Error bars are ± SEM, *p < 0.05.

condition resulted in a large increase in vesicular zinc levels only at the longer survival time points. It is likely that these responses are reflective of the effectiveness of environmental manipulations. Minimal enrichment promotes activity in the short term, but it decreases rapidly as the uniqueness of the novel item fades. In the deprived to enriched condition, the exposure of the mice to a new environment with strange cohorts may initially induce a stress response that reduces activity and prevents immediate enrichment-related cortical changes from occurring. Alternatively, it is known from other studies that the effects of enrichment are not immediate, taking between 12 h and 4 d of exposure to enrichment to observe any consequences (Ferchmin et al., 1970; Diamond et al., 1976; Henderson, 1976; Rema et al., 2006).

Environmental conditions that promote activity through physical, mental, and social stimulation lead to a number of alterations in the morphology and physiology of the cortex (Nithianantharajah and Hannan, 2006). Many of the changes involve factors that are important for cortical plasticity, such as alterations in NMDA receptor subunit expression (Tang et al., 2001), production of growth factors like brain-derived neurotrophic factor (BDNF) (Ickes et al., 2000), and the expression of other genes and gene products associated with synaptic plasticity (Rampon et al., 2000; McNair et al., 2007). These alterations in turn affect synaptic plasticity, because LTP/LTD expression is modified by enrichment (Li et al., 2006) and, presumably, cortical plasticity. Together with previous research that supports the contribution of zinc to cortical synaptic plasticity, our results indicate that the regulation of zinc levels provides another level of control, and that it is enhanced with enrichment.

A major consideration is whether the increase in vesicular zinc is integral to the changes in cortical plasticity caused by environmental enrichment, or whether our observations are epiphenomenal. We argue for the former based on the following arguments. First, the large increases in vesicular zinc levels we

observed were not passively caused by enrichment; instead, they also required modifications of sensory input in the form of vibrissae removal. Further support comes from previous studies showing that vesicular zinc levels rapidly increased or decreased in the barrel cortex depending on whether input from the vibrissae was reduced by plucking or enhanced by stimulation, respectively (Czupryn and Skangiel-Kramska, 2001a; Brown and Dyck, 2002, 2005). We take this to be indicative of a role for zinc in experience-dependent plasticity. Alternatively, it could be presumed that these modulations simply reflect changes in the activity of neurons within the barrels of interest. For example, after silencing vibrissal input, synaptic activity within the corresponding barrels would decrease, leading to the retention of zinc within presynaptic vesicles. Such a process would also require that uptake levels remained constant and independent of activity. However, in the context of the present results, it would imply that zincergic neurons in the enriched brain were less able to maintain zinc homeostasis. Although this has not been specifically examined, in light of the increased capabilities that enrichment confers on cortical function, it seems extremely unlikely.

Instead, we argue that it is more likely that the neuromodulatory effects of zinc are enhanced by enrichment and thereby exert a greater influence on the mechanisms of cortical plasticity. For instance, zinc can modulate NMDA and AMPA receptor activity (Peters et al., 1987; Westbrook and Mayer, 1987; Mott et al., 2008), alter levels of BDNF mRNA (Nowak et al., 2004), modify postsynaptic proteins (Baron et al., 2006), and directly influence the induction of LTP/LTD (Xie and Smart, 1994; Izumi et al., 2006; Huang et al., 2008). These processes have all been shown to be modified by enriched environments. By modifying them, changes in zinc signaling could influence the induction and expression of cortical plasticity in the enriched brain.

The most likely mechanism by which zinc modulates plasticity is through its actions on NMDA receptors. Zinc inhibits the activity of NMDA receptors in a concentration-dependent manner (Christine and Choi, 1990; Chen et al., 1997; Paoletti et al., 1997), and, as such, an increase in vesicular zinc levels would result in a greater inhibition of NMDA receptors. Because the dynamics of NMDA receptor activation are crucial components of many types of cortical plasticity (for review, see Buonomano and Merzenich, 1998; Malenka and Bear, 2004), reductions in the relative activity of these receptors would result in the weakening of synaptic connectivity associated with deprived inputs, and allowing adjacent inputs to encroach on them, thereby facilitating the reorganization of cortical circuitry in the enriched animal.

Based on our results, future studies should be directed at determining the precise role of vesicular zinc on the functioning of the enriched brain, especially in regard to cortical plasticity. Studies using the ZnT3 knock-out mouse, a transgenic line lacking the transporter responsible for packaging zinc into synaptic vesicles and, therefore, lacking vesicular zinc (Cole et al., 1999), would be extremely informative. Environmental enrichment of these mice might reveal those components of plasticity that are enhanced by enrichment and are mediated by zinc. For example, changes in synaptic plasticity, in the form of LTP/LTD, experience-dependent plasticity, in the form of map alterations after sensory manipulations, or learning, in the form of behavioral tests, after exposure to environmental enrichment in the ZnT3 knock-out mouse would be indicative of the integral involvement of vesicular zinc in enrichment-induced cortical modifications.

Cortical plasticity is dictated by experiential factors that drive neuronal activity, maintaining and changing the circuitry therein. In the present study, we showed that the regulation of

vesicular zinc changes significantly when the complexity of experiential features is manipulated. Although much remains to be ascertained regarding the functional significance of these alterations, given the multitude of effects zinc can have on neurotransmission and synaptic plasticity, it is likely that active modulations of zinc levels provide an additional mechanism by which cortical plasticity can be induced.

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