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Research report

Mapping the dynamics of cortical neuroplasticity of skilled motor learning using micro X-ray fluorescence and histofluorescence imaging of zinc in the rat

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HIGHLIGHTS

- Moderate and distributed practice in skilled reaching promotes neural plasticity.
- c-Fos upregulation parallels the improved proficiency in skilled forelimb use.
- Gluzinergic neurons may contribute to maintenance of the learned motor skills.
- μXFI of cortical Zn doesn't detect neural plasticity after skilled motor learning.
- Multimodal imaging should be applied to studies on post-stroke brain plasticity.

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ABSTRACT

Synchrotron-based X-ray fluorescence imaging (XFI) of zinc (Zn) has been recently implemented to understand the efficiency of various therapeutic interventions targeting post-stroke neuroprotection and neuroplasticity. However, it is uncertain if micro XFI can resolve neuroplasticity-induced changes. Thus, we explored if learning-associated behavioral changes would be accompanied by changes in cortical Zn concentration measured by XFI in healthy adult rats. Proficiency in a skilled reach-to-eat task during early and late stages of motor learning served as a functional measure of neuroplasticity. c-Fos protein and vesicular Zn expression were employed as indirect neuronal measures of brain plasticity. A total Zn map ($20 \times 20 \times 30\ \mu m^3$ resolution) generated by micro XFI failed to reflect increases in either c-Fos or vesicular Zn in the motor cortex contralateral to the trained forelimb or improved proficiency in the skilled reaching task. Remarkably, vesicular Zn increased in the late stage of motor learning along with a concurrent decrease in the number of c-fos-ip neurons relative to the early stage of motor learning. This inverse dynamics of c-fos and vesicular Zn level as the motor skill advances suggest that a qualitatively different neural population, comprised of fewer active but more efficiently connected neurons, supports a skilled action in the late versus early stage of motor learning. The lack of sensitivity of the XFI-generated Zn map to visualize the plasticity-associated changes in vesicular Zn suggests that the Zn level measured by micro XFI should not be used as a surrogate marker of neuroplasticity in response to the acquisition of skilled motor actions. Nanoscopic XFI could be explored in future as a means of imaging these subtle physiological changes.

1. Introduction

Zinc (Zn) is implicated in regulating neuroplasticity. Zn modulates brain functions, and brain activity modulates Zn level in the brain. Activity-dependent decreases [5] and increases [4,9] in Zn in the barrel cortex of adult mice were observed in response to prolonged sensory (i.e. whisker) stimulation and deprivation, respectively. Exposure of mice to an enriched environment further enhanced the sensory-deprivation induced increase in Zn [37]. Zn also regulates expression of immediate early genes (e.g. c-Fos) in response to brain and behavioral activity through zinc finger transcription factors [18]. Abnormally low amounts of Zn in the brain can also induce learning and memory deficits, although

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results are not consistent. Whereas administration of Zn chelators affected acquisition and consolidation of hippocampal- and amygdala-dependent memory formation [11,17,27,33,37,46,54], intact spatial learning, memory, and sensorimotor functions have also been reported in mice with depleted vesicular Zn [6].

There is controversy about methodologies used to detect the Zn involved in brain plasticity. Zn can be visualized using various staining techniques. Histochemical [7] and immunofluorescent staining methods [34,39,44] visualize primarily labile Zn. However, these methods also stain other divalent metal cations, including Ca^{+2} [10,38,47], which can overestimate the role of Zn in brain plasticity. A second disadvantage is that the perfusion of a rat as used for most Zn staining procedures can generate artifacts by re-distributing Zn within the tissue [20]. An alternative approach, X-ray fluorescence imaging at the micron level (micro XFI) using a synchrotron generated X-ray source, can provide Zn-specific maps [1,21]. However, since this technique visualizes all zinc, including labile Zn (vesicular and transmembrane intracellular) and Zn bound to proteins participating in chemical catalysis and maintaining protein structure and stability [14,17], this lack of specificity may limit its utility for detecting changes in brain plasticity.

Methodology employing micro XFI has been recently implemented to understand the pathophysiological mechanisms of stroke [3,45,51,55] and the efficiency of various therapeutic interventions that target post-stroke neuroprotection and neuroplasticity, such as hypothermia and physical rehabilitation [3,45]. Although Zn detected by micro XFI has been employed as a plasticity marker after stroke [3,45], it is uncertain if this technique can resolve those Zn alterations due to neuroplasticity. Thus, in the present study, we explored if learning-associated upregulation of c-Fos would be accompanied by changes in Zn concentration measured by two complementary techniques. The *N*-(6-methoxy-8-quinolyl)-*para*-toluenesulfonamide (TSQ) histofluorescence method was used to label vesicular Zn, and synchrotron-based micro XFI was employed to detect all forms of Zn. Proficiency in a skilled reach-to-eat task was employed as the behavioral measure of neuroplasticity. This behavioral paradigm has been widely used to evaluate neuroplasticity both before and after stroke [2,53]. Skilled reaching, the conventional term for the reach-to-eat act, is a form of prehension in which a forelimb is used to reach and grasp a food item and place it into the mouth for eating. Corticospinal pathways in the rat consist of a large crossed pathway, and therefore motor cortex contralateral to the forelimb used for the reach-to-eat act controls and undergoes major plastic changes when a rat is trained for skilled reaching. The structural changes of brain plasticity in neural populations underlying skilled motor action might have a different temporal profile both in magnitude and direction [22,24,28,35,36] when a skill becomes more automated and efficient. Since skilled motor learning advances through early and late stages of motor learning, a skilled reach-to-eat task is an ideal behavioral paradigm to study neural changes accompanying tuning of neural activity in a particular neural (e.g. Zn rich glutamatergic) population. Therefore, the changes in total and vesicular Zn, and c-Fos were analyzed during the early and late stages of learning of the skilled reaching action. Brain correlates of skilled reaching were studied 15 min after the last training session, since c-Fos protein can be detected within 15–30 min of stimulus application [31].

2. Experimental procedures

2.1. Subjects

Male, Sprague-Dawley (SD) (n = 6) and Long-Evans (LE) rats (n = 10) of comparable age (90–100 days old) were obtained from Charles River (QC, Canada). Rats were housed in Plexiglas cages (36 cm × 20 cm × 21 cm) with absorbent bedding (hardwood and softwood shavings), in groups of two or three in a colony room maintained on a 12 h light/12 h dark cycle (07:00–19:00) with controlled temperature and humidity. This work was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Fig. 1. A reaching box. A representative rat reaches through a narrow slot for a single pellet.

2.2. Experimental design

Two groups of rats were trained for a skilled reach-to-eat movement [2]. The 20-trial session per day training in a single pellet reaching task (Fig. 1) was for either nine days (early learning stage group; n = 6) or sixteen days (late learning stage group; n = 6). Each of these groups was comprised of 3 LE and 3 SD rats. Each rat was trained to reach with its dominant paw [2] through a narrow slit for a single banana-flavoured sugar pellet (Bio-Serv #F05986) positioned on a shelf outside the reaching box. To be successful, the rat must grasp the food pellet within its paw and bring it to its mouth for eating. The reaching behavior was quantified as the percent of total trials (20 trials) on which a food pellet was successfully obtained. A third group, serving as the control group, was comprised of LE rats (n = 4) exposed for nine days to the same experimental room and the same banana-flavoured sugar pellets; for the latter, control rats were provided the amount of the pellets, that the other rats were successful at eating during training or testing session in the home cages for ~10–15 min on each day. Only four animals were assigned to the control group, because the rats in this group were not subjected to any behavioral manipulation and we expected less variability in measures of brain plasticity. Rats were killed humanely 15 min after the last training session.

Learning-associated neural changes were measured at early and late stages of motor learning. The early stage of learning was defined as the period when success level to retrieve a single pellet was at least 50% for 3 consecutive days of training. The late stage of learning was considered to be the period when the success level was at least 50% for 5–6 days during the second and third week of training.

Learning-associated neural changes were measured only in the caudal area of the forelimb motor cortex, since intensive and massed practice for a skilled reaching action does not induce any significant upregulation of Arc (learning associated immediate early gene) in the trained (vs. nontrained) hemisphere in the rostral area of the forelimb motor cortex [24]. Both layers II/III and