**Laminar-specific distribution of zinc: Evidence for presence of layer IV in
forelimb motor cortex in the rat**

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Running title: Layer IV and zinc distribution in motor cortex in rats

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Abstract

The rat is the most widely studied pre-clinical model system of various neurological and neurodegenerative disorders affecting hand function. Although brain injury to the forelimb region of the motor cortex in rats mostly induces behavioral abnormalities in motor control of hand movements, behavioral deficits in the sensory-motor domain are also observed. This questions the prevailing view that cortical layer IV, a recipient of sensory information from the thalamus, is absent in rat motor cortex. Because zinc-containing neurons are generally not found in pathways that run from the thalamus, an absence of zinc (Zn) in a cortical layer would be suggestive of sensory input from the thalamus. To test this hypothesis, we used synchrotron micro X-ray fluorescence imaging to measure Zn distribution across cortical layers. Zn maps revealed a heterogeneous layered Zn distribution in primary and secondary motor cortices of the forelimb region in the adult rat. Two wider bands with elevated Zn content were separated by a narrow band having reduced Zn content, and this was evident in two rat strains. The Zn distribution pattern was comparable to that in sensorimotor cortex, which is known to contain a well demarcated layer IV. Juxtaposition of Zn maps and the images of brain stained for Nissl bodies revealed a “Zn valley” in primary motor cortex, apparently starting at the ventral border of pyramidal layer III and ending at the close vicinity of layer V. This finding indicates the presence of a conspicuous cortical layer between layers III and V, i.e. layer IV, the presence of which previously has been disputed. The results have implications for the use of rat models to investigate human brain function and neuropathology, such as after stroke. The presence of layer IV in the forelimb region of the motor cortex suggests that therapeutic interventions used in rat

models of motor cortex injury should target functional abnormalities in both motor and sensory domains. The finding is also critical for future investigation of the biochemical mechanisms through which therapeutic interventions can enhance neural plasticity, particularly through Zn dependent pathways.

Key words: Motor Cortex, Zinc, Layer IV, micro X-ray Fluorescence Spectroscopy, Synchrotron

1 Introduction

The structural plan of the cerebral cortex is comparable across the whole mammalian class. There are six basic, tectogenetic layers based on cytoarchitectonics of the cortex. The cellular elements that make up the cortex reveal a series of layers in brain sections taken perpendicular to the cortical surface. The individual layers, labelled from outside to inside, are: I – molecular layer; II – outer granular layer; III – pyramidal layer; IV – inner granular layer; V – ganglion cell layer; and VI – spindle cell layer (Brodmann, 1909). Nevertheless, the mammalian cerebral cortex undergoes regional modification in cellular lamination during ontogeny and maturation. This process of architectonic transformation is essentially the same for the whole mammalian class (Brodmann, 1909).

Layer IV, the inner granular layer in the motor cortex, undergoes one of the greatest maturational modifications in both humans and animals (Brodmann, 1909). This layer receives thalamic inputs (Donoghue and Wise, 1982; Welker, 1976) and contributes to sensory-motor integration. Complete regression of layer IV has been documented for primary motor cortex in the adult human and primate brain, which contributes to hand function (Brodmann, 1909; Lewis, 1878; Lewis and Clarke, 1878). Nevertheless, the existence of layer IV in the “hand” motor cortex in the adult rat is controversial. Some studies report the absence of layer IV (Donoghue and Wise, 1982; Moroni et al. 2009; Sanderson et al. 1984, Zilles, 1980) whereas others report a small but distinct layer IV (Aldes et al. 1988, Krieg, 1946 a, 1946b; Skoglund et al. 1997; Yazdan-shahmorad et al. 2011). Moreover, behavioral deficits associated with the sensory system have been observed following lesions to rat motor cortex. For example, Gharbawie and Whishaw (2006) reported that rats neglected a food pellet after it was grasped by hand and

withdrawn from a shelf in order to place it in the mouth. Diminished responsiveness to tactile stimulation, measured using Von Frey hair/filaments, was also observed after stroke in the motor cortex in the rat (personal observation of MA). The fact that histology is traditionally used as the "gold standard" for identification of cortical tissue layers may be one of the reasons for the conflicting results. Using these methods, important elemental or molecular characteristics of tissue layers may be missed.

Thus, the aim of the present study was to explore the presence of layer IV in the rat motor cortex in two strains of rat via direct mapping of the element zinc (Zn). The rationale behind the study design was based on the previous observation that Zn-containing neurons, a subset of glutamergic neurons with a high concentration of labile Zn, are generally not found in pathways that run from the thalamus (Casanovas-Aguilai et al. 1995; Dyck et al. 1993; Frederickson et al. 2000). The sensory-motor cortex, since it has a well demarcated layer IV, was used as a positive control region. Therefore, it was hypothesized that the existence of layer IV would be portrayed by a region of decreased Zn concentration situated between layers III and V in both motor and sensory-motor cortices. This study employed X-ray fluorescence imaging at the micron level (XFI) using a synchrotron generated X-ray source, a technique previously utilized to study tissue architecture on the basis of elemental composition (e.g. Chwiej et al. 2011; James et al. 2011; Leskovjan et al. 2009; Linkous et al. 2008; Miller et al. 2006; Popescu et al., 2009). Since rat model systems are among the most widely used to study neurological and neurodegenerative disorders affecting hand function, clarification of this question is critical to both mechanistic understanding and therapeutic development.

2. Material and methods

2.1 Subjects

Male, young adult (90–100 days old) Long Evans hooded (n=4) and Sprague Dawley rats (n=4) were obtained from Charles River (QC, Canada). 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. Rats were housed in Plexiglas cages (36 cm long, 20 cm wide, and 21 cm deep) 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 h) with controlled temperature and humidity.

2.2 Experimental design

This study utilized a subset of rats from a larger investigation on the plasticity-associated changes in Zn levels in the cortex and the relationship between these changes and outcome after ischemic stroke. To serve the research aims of the larger investigation, the rats used in the current study were trained to learn a skilled reach-to-eat movement in a single pellet reaching task (Alaverdashvili and Whishaw, 2013; Alaverdashvili et al. 2008; Whishaw and Pellis, 1990). The rats were trained for 3 weeks until each rat achieved a 50% or higher success rate. Briefly, the dominant paw used by each rat was identified during pre-training sessions. Each rat was trained to reach with its dominant paw through a narrow slit for a single pellet positioned on a shelf outside the reaching box; the rat should grasp the food pellet within its paw and bring it to its mouth for eating. However, to avoid the possible confounding effect of training-associated changes

in Zn concentration, only the untrained hemisphere, i.e. that ipsilateral to the trained “hand”, was analyzed in this study. In addition, to allow for resolution of the neuroanatomy of the motor cortex without confounding influences, no other behavioral or surgical manipulations were performed on the rats used in the current study.

To investigate if the presence of layer IV is a “universal” feature of the rat forelimb motor cortex, we examined it in two strains of rat, Sprague Dawley and Long Evans. These strains were chosen because they show comparable proficiency in a single pellet reaching task and comparable limb representation in the motor cortex (Whishaw et al. 2003).

To determine if the presence of layer IV is also a common feature across the forelimb motor cortex, two loci, 2.7 mm and 0.7 mm anterior to bregma, were analyzed. These loci were chosen because they contain both the sensorimotor cortex (which contains a well demarcated layer IV) and the motor cortex (for which the existence of layer IV is under question). Both loci belong to the caudal region of motor cortex (Donoghue and Wise, 1982).

Thus, brain sections were collected at 2 specific regions of forelimb motor cortex for each rat (Sprague-Dawley, n=4; Long Evans, n=4). The collection of duplicate (adjacent) sections at each location allowed for both Zn mapping by XFI and assessment of cortical morphology by cresyl violet staining in the untrained hemisphere.

2.3 Brain harvesting and sectioning

Animals were anesthetized with 4% isoflurane (1L/min O₂), and humanely sacrificed through decapitation, with the head immediately flash frozen in liquid nitrogen

and stored at -80°C. The frozen brains were subsequently chiseled out from the frozen heads on dry ice and stored at -80°C until sectioning. This method, in which brain tissue is not perfused or fixed, has been shown to best preserve the biochemical composition of brain tissue closest to that present in vivo (Hackett et al. 2012b; Hackett et al. 2011). Brain tissue sections were cut at a thickness of 30 µm on a cryomicrotome at -16 to -18°C.

To summarize sample size, for each of the 4 rats sampled from each of the 2 rat strains, duplicate adjacent sections were collected at each of 2 forelimb motor cortex loci, 2.7 mm and 0.7 mm anterior to bregma. One of the sections was melted onto a sulfur- and metal-free Thermanox plastic coverslip (Thermo Scientific) for Zn mapping and subsequent to the Zn mapping, this section was stained with cresyl violet. The second section was mounted onto a Superfrost slide (VWR) for cresyl violet (Nissl substance) staining only. The sections were air dried and stored in a dessicator in the dark at room temperature prior to analyses. Zn maps were collected within 7 to 10 days of tissue sectioning.

2.4 X-ray fluorescence imaging

Large scale elemental maps of the entire sensory-motor and motor cortex were collected using XFI at the micron level at the Stanford Synchrotron Radiation Lightsource (SSRL) experimental station 10-2 (<http://www-ssrl.slac.stanford.edu/beamlines/bl10-2/>). The energy of the incident X-ray beam was 13 keV. Incident X-ray intensity was measured using a nitrogen gas-filled ion chamber. A monocapillary optic (XOS, East Greenbush, N.Y., USA) was used to generate a microfocussed incident beam of approximately 25 µm

at the sample when positioned 1 mm away from the optic. The sample was mounted at 45° to the incident beam and rastered through the beam, with a beam exposure time of 100 ms per 20 µm step. A 4-element Vortex® silicon drift detector at 90° to the incident beam collected the X-ray fluorescence spectra from the brain tissue. The detector readout was synchronised to the stage movement speed, and data collected continuously, such that the full emission spectrum was collected every 100ms, for an average stage movement of 20 µm. This method of "rapid scanning" has been described previously (Popescu et al. 2009). The full emission spectrum via multichannel analysis (MCA) was recorded at each pixel, in addition to single channels for P, S, Cl, K, Ca, Fe, Cu, and Zn fluorescence lines, and inelastic scatter. In addition to measurements of brain sections, emission spectra were collected from a thin film of ZnTe, with a concentration of 45 µg/cm², on 6µm of mylar foil (Micromatter, Vancouver).

A higher resolution image was collected from the motor cortex of a representative tissue section subsequent to its analysis at 10-2. The higher resolution image was collected at experimental station 2-3, using Kirkpatrick-Baez mirror optics to produce a micro-focused X-ray beam, with approximate *x,y* dimensions of 2 µm × 2 µm. The energy of the incident X-ray beam was 13 keV, and the sample was mapped with 10 µm steps and a dwell time of 500 ms per point. The incident X-ray intensity was measured using a nitrogen gas-filled ion chamber.

2.5 Cresyl violet staining

Sections were fixed prior to cresyl violet staining using a vapor fixation method (Hackett et al. 2013a). The slides were placed in a desiccator containing desiccant and

paraformaldehyde and heated in an oven at 80°C for one hour to generate formaldehyde vapor to fix the tissue. Thereafter, the slides were cooled and stained for Nissl substance. The slides were immersed for 2 min each in 70%, 95% $\times$ 2, and 100% $\times$ 2 ethanol. The slides were rehydrated in 100% $\times$ 2, 95% $\times$ 2 and 70% ethanol for 2 min each. They were then dipped in distilled water and stained in 0.25% cresyl violet for 15 min. The slides were differentiated in distilled water for 1 min, rinsed in distilled water, and then dehydrated through 70% ethanol and 95% ethanol for 2 min each. The slides were dehydrated again through 95% (30 sec) and 100% ethanol (1 min), and then put in xylene and coverslipped with cytosol.

2.6 Quantitative analysis of Zn

X-ray fluorescence imaging data were viewed and analysed using Sam's Microanalysis Toolkit (<http://smak.sams-xrays.com/>). Zn maps were converted from fluorescence intensity to Zn concentration ($\mu\text{g}/\text{cm}^2$), using a Zn reference standard, as previously described (Webb, 2011). Overlay of Zn images with histological images allowed the cortical layers (the dorsal border of layers I-III and V-VI) to be defined according to cell somata distribution identified by Nissl staining. Regions of interest (ROI) were outlined (layer I, layer II/III, layer IV, layer V, layer VI, corpus callosum), and the average amount of Zn within each region ($\mu\text{g}/\text{cm}^2$) was calculated.

The method described yields the amount of zinc expressed as a function of unit area within the ROI. In addition, these values were converted to determine effective hydrated tissue concentrations, in units of $\mu\text{g}/\text{g}$ and $\mu\text{mol}/\text{L}$, for the fresh frozen tissues prior to air drying. To quantify Zn in $\mu\text{g}/\text{g}$, the effective hydrated tissue volume

corresponding to a unit area (cm^2) was determined ($\text{area [1 cm}^2\text{]} \times \text{hydrated tissue thickness [30 }\mu\text{m}]$). The hydrated mass of tissue per unit area in the observation was determined by multiplying this volume by the brain hydrated tissue density. Zn in $\mu\text{g/g}$ is thus the mass of Zn per unit area [$\mu\text{g/cm}^2$] divided by this tissue mass per unit area. The motor cortex (gray matter) and corpus callosum (white matter) hydrated mass was estimated considering the specific gravity for gray (~ 1.045) and white (~ 1.041) matter (Olson et al. 1990). In order to convert the amount of Zn per unit area ($\mu\text{g/cm}^2$) to a molar concentration of Zn per hydrated tissue volume ($\mu\text{mol/L}$), the latter was quantified as a function of hydrated tissue thickness (30 μm) and the molar mass of Zn (65.38 g). These calculations assumed that although the tissue thickness decreased during air-drying of the tissue section (Dorph-Petersen et al. 2001), the lateral distribution of the elements does not change (no shrinkage in X, Y spatial domains). This assumption has been previously shown correct in mid-IR imaging studies of mouse cerebellum tissue (Hackett et al. 2012a).

As described above, the average Zn concentration was calculated for each ROI in each layer of the motor cortex, and Zn concentration was compared between cortical layers. Zn concentration was also measured in the corpus callosum in the untrained hemisphere from each brain section for an individual rat. The average Zn concentration of each cortical layer was then normalized to the Zn concentration of the corpus callosum from the same section, by expressing the results as a ratio. Normalizing the average Zn concentration of each cortical layer reduces data variability caused by any variation in tissue thickness (see Supplementary Information). The corpus callosum was chosen for this purpose because it contains no glutamergic neurons of high Zn

concentration (Cole et al. 1999) and has a uniform composition such that the main fluctuation in Zn concentration observed between tissue sections would be expected to come from variation in tissue thickness.

The radial profile of Zn concentration as a function of depth from the cortical surface was measured across the motor cortex along a 250-300 μm wide rectangle parallel to radial elements of the cortex (from cortical surface to white matter i.e. corpus callosum), and a plot profile was created and plotted. The radial profiles were generated from Zn images imported into Image J JV1.36 (<http://rsb.info.nih.gov/ij/download.html>). To simplify visualization of the layered Zn distribution more clearly, a Zn trendline was determined with a moving average with a period of 3. In addition to Zn, a radial profile was also constructed from the inelastic scatter map to examine tissue density, and to determine if variation in Zn content was correlated to tissue density.

2.7 Statistics

Power analysis was performed to determine that the sample size of $n=4$ was adequate to make statistical comparisons between the cortical layers within each rat strain (see Supplementary Information). An assessment of inter- and intra-animal variability in Zn concentration can be found in the Supplementary Information.

Statistical analysis was performed using SPSS 13 (Field, 2005). **A two-tailed paired t-test was used to determine whether there was a significant difference in the absolute Zn concentration of layer IV relative to layers II/III and V. The Supplementary Information shows parallel statistical analysis for relative Zn concentration. For all analyses, $p < 0.05$ was considered significant.**

3 Results

Figure 1 summarizes morphological features of primary (M1) and secondary (M2) motor cortices and the sensory-motor cortex (SM) visualized by cresyl violet staining. A higher magnification image of layer IV (Figure 1B) highlights the numerous granule neurons that allow clear demarcation of layer IV in the SM region; most importantly, Figure 1B highlights that this demarcation is absent in M1 or M2 regions.

3.1 Distribution of Zn in the forelimb region of cortex

Laminar-specific distribution of Zn was observed in the forelimb region of motor cortex (Figure 2A). Representative histology of the region defined by the white box in Figure 2A is presented in Figure 2B and a higher spatial resolution image (10 μm pixel size) of the Zn distribution is presented in Figure 2C. Zn was heterogeneously distributed in the forelimb region of motor cortex (Figures 2A and 2C) and three major “bands” of varying Zn concentration (i.e., a laminar profile) were noticeable on Zn maps to span both motor and sensory-motor cortex areas (Figures 2A and 2C). Specifically, two wider bands with increased Zn levels were separated by a narrower band of lower Zn content. The location of the region with lower Zn concentration observed in Figure 2C correlates with the ventral border of layer III and the dorsal border of layer V observed by cresyl violet staining (Figure 2B); however, the histology does not provide any evidence of a well demarcated layer IV. A radial profile of the Zn concentration as a function of distance from the cortical surface (Figure 2E) identified that local maxima in Zn concentration are present at $\sim 350 \mu\text{m}$ and $\sim 700 \mu\text{m}$ below the cortical surface, and a

local minimum in Zn concentration is present ~ 550 -600 μm below the cortical surface. The position of the local maxima in Zn concentration corresponds to the validated and widely accepted location of layers II/III and V in the rat motor cortex (Paxinos and Watson, 2004). Therefore, the results from XFI analysis of Zn distribution in the rat motor cortex highlight that a “valley” of relatively reduced Zn concentration occurs between regions of higher Zn concentration in layer II/III and V, i.e., a conspicuous layer IV. Despite the laminar variation in Zn concentration, no corresponding variation in tissue density (inelastic scatter radial profile) was observed (Figure 2D and 2F). To allow comparison of the results of this study to other studies), the absolute concentration of Zn is summarized across laminas of the primary motor cortex and corpus callosum in Table 1.

3.2 The radial profile of Zn within the primary motor cortex, secondary motor cortex and sensorimotor cortex

Figure 3 summarizes the radial profile of Zn distribution in M1, M2, and SM regions of the rat cortex. Representative examples of the radial profile of Zn as a function of distance from the cortical surface in M1, M2 and SM regions (Figure 3D) demonstrate local maxima in Zn concentration at the ventral border of pyramidal layer III and the dorsal border of layer V, and a local minimum in Zn concentration between these layers.

The profiles of the distribution of Zn in M1 and M2 are consistent with that observed in SM, which has a well-accepted layer IV. The “Zn valley” in the sensory-motor cortex aligned with the known location of layer IV (~ 650-700 μm below the cortical surface), demonstrating that Zn mapping could detect layer IV in this positive

control region. In addition, the regions of high Zn concentration aligned with layers II/III and V, which are known to be enriched in Zn in other cortical regions (Frederickson et al. 2000; Sorensen et al. 1998).

The radial distribution of Zn in M1, M2 and SM followed the same pattern across the anterior-posterior axis of the motor cortex (Figure 4). The Zn signal reached a minimum around 600 μm from the cortical surface both in anterior (2.7 mm anterior to bregma; data not shown) and central regions (0.7 mm anterior to bregma) of forelimb motor and sensory-motor cortices. The “Zn valley” was observed in both Long Evans and Sprague Dawley rats. Although there were insufficient tissue replicates to make statistical comparisons of the Zn distribution across cortical layers between the two rat strains and between the two motor cortex coordinates, it is of note that every rat analyzed under any studied condition had an apparent “Zn valley” between layers II/III and V.

Figure 5 shows the cortical layers of the rat motor cortex within which ROI were identified and used to quantify the average Zn concentration. **The results of statistical comparisons of the absolute Zn concentration in layer IV as compared to layer II&III and layer V of primary motor cortex for each of the 4 subgroups studied (2 brain loci and 2 rat strains) are summarized in Figure 6.** The decline in Zn concentration in layer IV compared to layer II&III was statistically significant at both loci of the forelimb region of motor cortex in Sprague Dawley (2.7 mm anterior to bregma: $t = 7.42$; $p < 0.01$; 0.7 mm anterior to bregma: $t = 3.81$; $p < 0.05$) and Long Evans rats (2.7 mm anterior to bregma: $t = 15.47$; $p < 0.001$; 0.7 mm anterior to bregma: $t = 11.36$; $p < 0.001$). The average Zn concentration in layer IV compared to layer V was significantly lower at both loci of the forelimb region of motor cortex in Sprague Dawley

(2.7 mm anterior to bregma: $t = 3.95$; $p < 0.05$; 0.7 mm anterior to bregma: $t = 4.64$; $p < 0.05$) and Long Evans rats (2.7 mm anterior to bregma: $t = 22.75$; $p < 0.001$); 0.7 mm anterior to bregma: $t = 3.82$; $p < 0.05$).

Statistical analysis on relative Zn concentration is summarized in Figure S2 (Supplementary Information). Briefly, the decline in relative Zn concentration in layer IV compared to layers II&III and V was comparable to that observed for absolute Zn concentration. However, the level of statistical significance for the difference in Zn amount between cortical layers was increased since the normalization procedure used in this study reduced variability in the amount of Zn within each cortical layer (see Supplementary Information).

4 Discussion

Rats are the most widely studied species in pre-clinical model systems of neurological disorders affecting hand function. After a lesion to the forelimb area of the motor cortex, rats exhibit motor deficits in skilled hand use (Alaverdashvili and Whishaw, 2013; Karl and Whishaw, 2012; Klein et al, 2012; Kolb and Tees, 1990). For example, the rats' success in reaching for a food item and bringing it to their mouths drops remarkably; moreover, the way they reach for a food item is abnormal even when they can complete the task (Alaverdashvili and Whishaw, 2013). Alternatively, sensory neglect has been reported after stroke to the motor cortex of the rat (Gharbawie and Whishaw, 2006). Diminished haptic sense using the von Frey hair pinch test has also been observed after a stroke in the motor cortex. It is puzzling that a lesion to layer IV of the motor cortex, which is believed to lack sensory inputs, can lead to sensorimotor

abnormalities. This suggests the existence of layer IV, a recipient of sensory information, in the motor cortex of the rat. Since neurons containing a high Zn concentration (a subset of glutamergic neurons enriched in labile Zn) are generally not found in sensory pathways that run from the thalamus (Casanovas-Aguilai et al. 1995; Dyck et al. 1993; Frederickson et al. 2000), we hypothesized that the presence of a cortical layer IV, although not evident on routine histological assessment, would be portrayed by a cortical band of reduced Zn concentration between layers III and V. XFI was employed in this study to measure the amount of Zn across different layers of forelimb motor cortex.

Zn showed a pattern of uneven distribution in the forelimb region of primary and secondary motor cortices that was similar to that in the sensory-motor cortex, which contains a well demarcated layer IV. Two wider bands with higher Zn content were separated by a narrow band of lower Zn content in both motor and sensory-motor areas. Juxtaposition of Zn maps and the images of brain sections stained for Nissl bodies revealed that the “Zn valley” in primary motor cortex started at the bottom border of pyramidal layer III and ended close to the vicinity of pyramidal layer V. We found that, while the histological data did not differ sufficiently to reveal the presence of layer IV in forelimb regions of the rat motor cortex in the rat, the Zn content differed significantly to indicate its presence. Thus, there is a conspicuous cortical layer IV between layers III and V.

The significant decrease in Zn content in layer IV relative to layers II, III and V observed in this study was consistent in both Long Evans and Sprague Dawley rat strains. This finding is reminiscent of the similarities reported in forelimb representation in motor cortex between those strains studied (Whishaw et al. 2003). However, it would be

interesting to investigate the laminar-specific distribution of Zn in the forelimb region of motor cortex in other strains of rats with pronounced strain-related differences in the microstimulation-topographic map of this brain region (VandenBerg et al., 2002).

The major finding of the present study on the presence of layer IV in the forelimb region of the motor cortex in the adult rat is in agreement with some previous reports. In 1946, Krieg (1946 a, b) described the forelimb motor cortex of the rat as a six-layered structure with a distinct and conspicuous layer IV. Krieg's (1946 b) description, based on the cell pattern analysis on thionin-stained brain specimens, was later confirmed by others. A genuine layer IV consisting of densely packed small neurons in rat primary motor cortex was revealed using a computerized analysis system based on the "optical dissector" (Skoglund et al. 1997). A thin layer IV can also be recognized in motor cortex stained for layer IV specific transcription factor *Ror- β I* (Moroni et al. 2009). Recently, electrophysiological laminar analysis in rats (Yazdan-Shahmorad et al. 2011) also confirmed the presence of layer IV in the forelimb region of motor cortex. Nevertheless, some studies report the absence of layer IV (Donoghue and Wise, 1982; Moroni et al. 2009; Zilles, 1980).

The laminar-specific distribution of Zn identified by XFI in the forelimb region of the motor cortex in adult rats is in agreement with some previous observations made on histochemically stained sections for other cortical regions with thalamocortical sensory input (Frederickson, 2000; Slomianka et al. 1990; Sorenson et al. 1998). However, in contrast, no "Zn valley" was observed by Perez-Clausell (1996) in the hindlimb area of motor cortex, where a well demarcated layer IV occurs, suggesting that variation in the

sensitivity and specificity of staining methods could obscure neuroanatomical features of brain tissue.

Since X-ray fluorescence is an atomic property, Zn measured in this study includes all pools of Zn in brain tissue, which is in contrast to previous studies on cortical Zn distribution in which only labile Zn was measured. Total Zn includes the Zn bound to proteins (participating in chemical catalysis or maintaining protein structure and stability) as well as vesicular Zn, synaptically released Zn, and transmembrane intracellular Zn. Zn-rich cortical layers in the present study most likely correspond to brain tissue containing higher amounts of vesicular Zn. Two lines of evidence support this hypothesis. Firstly, it was reported that Zn-enriched cortical regions detected by XFI correspond to ZnT3 rich terminal boutons (Linkous et al. 2008). Secondly, coincident accumulation of Zn in the vesicles with a zinc-specific pump called ZnT3 has been shown via juxtaposition of histochemically, histo(cyto)fluorescently and immunofluorescently stained brain specimens (Lee et al. 2011; Palmiter et al. 1996; Shen et al. 2007). Although vesicular Zn, the “labile” Zn pool detected by histo-fluorescence methods, accounts for only approximately 23% of total Zn in cortex (Cole et al. 1999), it is considered to represent 95% of the entire histofluorescently reactive Zn pool (Lee et al. 2011). Moreover, the pattern of distribution of vesicular Zn is striking relative to the more evenly distributed protein-bound Zn.

A tightly controlled and well validated sample preparation protocol (Hackett et al. 2012a; Silasi et al. 2012) was employed in this study to avoid the potential confounding effects associated with other techniques that stain the labile Zn pool. As described above, great variation is often observed between independent studies of the labile Zn distribution

(Frederickson, 2000; Perez-Clausell, 1996). In addition, although Zn stains aim to stain a more specific pool of Zn than “total” Zn as revealed by XFI, recent studies have shown that Zn stains may also stain other divalent metal cations, including Ca^{++} (Danscher and Zimmer, 1978; Nowakowski and Petering, 2011; Stork and Li, 2006). Further, sample preparation has been shown to create huge variation in the extent of staining observed with various Zn stains (Flinn et al. 2005; Hackett et al. 2011). The process of perfusion that is required for most Zn staining protocols re-distributes Zn as we have recently shown (Hackett et al. 2012b; Hackett et al. 2011). Indeed, the perfusion-fixation process affects not only the distribution of Zn measured in staining studies, but also that measured by all elemental imaging techniques. For example, recently published three-dimensional Zn maps of the rat cortex in perfusion-fixed brains obtained by laser ablation-inductively coupled plasma mass spectrometer (LA-ICPMS) lack a laminar-specific pattern of Zn distribution not only in the forelimb region of motor cortex, but also in other, widely recognized six-layer regions of neocortex (Hare et al, 2012).

It is noteworthy that differences in sample preparation can affect the measured concentrations of Zn in brain sections. Such factors include: i) the way the tissue was harvested (e.g. frozen versus fixed tissue) (Hackett et al. 2011); (ii) tissue preparation (e.g. hydrated vs. air vs freeze-dried) (Flinn et al. 2005) and (iii) the tissue processing method (histochemical, histo(cyto)fluorescent and immunofluorescent staining vs. unstained specimens) (Cole et al. 1999; Danscher, 1981; Frederickson et al. 2000; Frederikson et al. 1989; Lee et al. 2011; Palmiter et al. 1996). Additionally, variability in tissue thickness associated with sectioning can affect the measured Zn level and distribution. Our study has demonstrated that normalizing the average Zn concentration

in each cortical layer to that of the corpus callosum in the same brain section reduces inter- and intra-animal variability between tissue sections (see Supplementary Information). Most importantly, the Zn concentration determined for each tissue layer of the cortex in this study is consistent with some previous studies (Chwiej et al. 2011; Frederickson, 2000; Hare et al. 2010; Lee et al. 2011; Wang et al. 2009) and falls within the normal physiological range. Likewise, the ratio of bulk Zn concentration in cortex and corpus callosum is also in general agreement with previous findings (Chwiej et al. 2011; Sergeant et al. 2005).

Laminar-specific distribution of Zn in the forelimb region of motor cortex has several significant implications for motor cortex physiology and pathophysiology. Overlap between motor and sensory representation in forelimb motor cortex implies that motor cortex can receive cutaneous input from the thalamus (ventral complex) in the rat. Alternatively, it can be explained by direct sensory collaterals that have been eliminated in more complex brains due to the longer circuiting of the stimuli (Krieg, 1946 a, b). Although the six-layered forelimb motor cortex in rats contrasts with the five-layered primary motor cortex in high order primates, the species-specific features of cortical lamination could be the result of heredity and adaptation to cerebral activity during ontogeny and evolution (Broadmann, 1909; Kaas, 2004).

There are many similarities in the motor system of the human and rat, but there are also many differences. Although skilled “hand” use in rodents and humans is homologous (Sacrey et al. 2009) due to conserved specific motor pathways inherited from a common ancestor during evolution, not all aspects of sensory-motor control in rats correspond to that of the human. For example, among the differences are extensive direct

connections of cortico-spinal neurons onto the motor neurons of the spinal cord in recent members of the primate lineage that are absent in rodents. It is proposed that the direct connections mediate the fractionated movements of the limbs and digits. It is possible that layer IV in the rat motor cortex improves sensory control to shape the digits for grasping of food items.

Thus, the presence of layer IV in forelimb motor cortex indicates the importance of tactile guidance for “hand” movements, especially for the distal limb (e.g. digits) in rats that use senses other than vision for skilled “hand” movements (Whishaw et al. 2005). The results are instructive for designing pre-clinical studies that utilize rodent models to address abnormalities of upper extremity functions that occur with injury to the motor cortex, such as stroke and traumatic brain injury. Our results support the idea that therapeutic interventions being tested in such models should target behavioral abnormalities in both the motor and sensory domains. The findings are also critical for future investigations of the biochemical mechanisms through which therapeutic interventions can enhance neural plasticity, particularly through Zn dependent pathways.

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Figure legends

Figure 1. (A) Representative image of cortical layers in a brain section stained with cresyl violet (4x). (B) Higher magnification histology (20x) shows the numerous small granular neurons that comprise the well-defined layer IV of the sensory-motor cortex (SM), which are largely absent at the location where layer IV would occur in primary (M1) and secondary (M2) cortices. The existence of layer IV in the SM is widely accepted (indicated by check symbol), but is disputed in M1 and M2 (indicated by question mark).

Figure 2. Investigation of the laminar distribution of Zn in the forelimb region of primary motor cortex using X-ray fluorescence imaging. (A) Zn distribution in the rat motor cortex measured with a 20 μm pixel size. (B) Representative histology of the region defined by a white box in A, highlighting the location of cortical layers and corpus callosum (CC). Black arrowheads indicate the beginning and end of each layer. (C) Zn distribution of the region defined by the white box, measured with higher resolution (10 μm pixel size). (D) Scatter map of the region defined by the white box, measured with higher resolution (10 μm pixel size). (E) Zn profile as a function of distance perpendicular from the cortical surface (0 μm), generated from the image present in C. (F) Scatter profile as a function of distance perpendicular from the cortical surface (0 μm), generated from the image present in D. Scale bar in A = 500 μm , Scale bar in C = 100 μm . Intensity scale in A, C and E, I and Zn axes in C and E represents the Zn concentration in $\mu\text{g}/\text{cm}^2$. The scattered intensity represents inelastic scattered counts normalized to the incident intensity. The figure highlights the presence of layer IV in the

forelimb region of the primary motor cortex (**A-C**). It also demonstrates that the Zn valley does not correlate with scatter intensity (**D-F**).

Figure 3: Representative images of a (**A**) brain section stained with cresyl violet, (**B**) Zn map ($\mu\text{g}/\text{cm}^2$) and (**C**) scatter map using X-ray fluorescence imaging (20 μm pixel size); (**D**) Profile of Zn concentration ($\mu\text{g}/\text{cm}^2$) as a function of distance from the cortical surface for secondary motor cortex (M2), primary motor cortex (M1) and sensorimotor cortex (SM) (0.7 mm anterior from bregma) as defined by the white boxes in **Figure 3B**. The scattered intensity represents inelastic scattered counts normalized to the incident intensity. Plots were created with a 3 point smoothing average.

Figure 4: Zn distribution in the primary motor cortex (M1), secondary motor cortex (M2) and sensorimotor cortex (SM) in representative (**A, D**) Sprague Dawley rats and (**G, J**) Long Evans rats. A ~ 250 μm wide band with low Zn concentration situated between two bands of higher Zn concentration. LH – Left hemisphere; RH - Right hemisphere; CC - Corpus callosum. Scale bars = 500 μm . The laminar-specific distribution of Zn is consistent between strains and at two loci in the forelimb region of motor cortex. White arrow heads in **A, D, G and J** indicate location of Zn “valley”. Units for scale bar colour intensities are $\mu\text{g}/\text{cm}^2$. **Corresponding scatter (B, E) and histological (cresyl violet) (C, F) maps are shown for Sprague Dawley rats. The corresponding scatter (H, K) and histological (I, L) maps are also provided for Long Evans rats.**

Figure 5: A representative image of Zn distribution in the forelimb region of motor cortex with a line sketch (not drawn to scale) of the cortical layers of rat motor cortex within which regions of interest were identified and used to generate Zn maps and quantify the average Zn concentration. Regions of interest drawn (from top to bottom) correspond to the cortical layers I, II & III, IV, V, VI, and corpus callosum (CC). Scale bar = 500 μm . Units for scale bar colour intensities are $\mu\text{g}/\text{cm}^2$.

Figure 6: The average ($\pm$ SD) absolute Zn concentration ($\mu\text{g}/\text{cm}^2$) was significantly decreased in layer IV relative to layers II/III and V in the forelimb region of primary motor cortex. The following symbols denote a significant difference in the Zn concentration of layer IV relative to layer II/III (*, ** or ***) and between layer IV relative to layer V († or †††) within the subgroup. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $^\dagger p < 0.05$, $^\dagger\dagger\dagger p < 0.001$.

Table 1. Zn amount (Mean $\pm$ SEM) in coronal sections of the primary motor cortex

Cortical layers (L)	Zn per cortical area ($\mu\text{g}/\text{cm}^2$)	Zn per cortical mass ($\mu\text{g}/\text{g}$)	Zn per cortical volume (mmol/L)
Long Evans			
At 0.7 mm anterior to bregma*			
LI	0.630 $\pm$ 0.014	20 $\pm$ 4	0.32 $\pm$ 0.07
LII-III	0.111 $\pm$ 0.021	35 $\pm$ 3	0.57 $\pm$ 0.05
LIV	0.090 $\pm$ 0.017	29 $\pm$ 3	0.46 $\pm$ 0.05
LV	0.096 $\pm$ 0.019	30 $\pm$ 3	0.49 $\pm$ 0.05
LVI	0.087 $\pm$ 0.010	27 $\pm$ 3	0.44 $\pm$ 0.06
CC	0.060 $\pm$ 0.010	27 $\pm$ 3	0.30 $\pm$ 0.02
At 2.7 mm anterior to bregma*			
LI	0.066 $\pm$ 0.025	21 $\pm$ 8	0.34 $\pm$ 0.13
LII-III	0.115 $\pm$ 0.018	36 $\pm$ 6	0.59 $\pm$ 0.09
LIV	0.097 $\pm$ 0.018	31 $\pm$ 6	0.49 $\pm$ 0.09
LV	0.111 $\pm$ 0.018	35 $\pm$ 6	0.57 $\pm$ 0.09
LVI	0.097 $\pm$ 0.016	31 $\pm$ 5	0.50 $\pm$ 0.08
CC	0.074 $\pm$ 0.013	31 $\pm$ 5	0.38 $\pm$ 0.07
Sprague Dawley			
At 0.7 mm anterior to bregma*			
LI	0.078 $\pm$ 0.020	25 $\pm$ 7	0.39 $\pm$ 0.10
LII-III	0.131 $\pm$ 0.018	42 $\pm$ 6	0.67 $\pm$ 0.09
LIV	0.104 $\pm$ 0.011	33 $\pm$ 4	0.53 $\pm$ 0.06
LV	0.113 $\pm$ 0.013	36 $\pm$ 4	0.58 $\pm$ 0.07
LVI	0.103 $\pm$ 0.013	32 $\pm$ 4	0.53 $\pm$ 0.07
CC	0.085 $\pm$ 0.011	33 $\pm$ 4	0.44 $\pm$ 0.05
At 2.7 mm anterior to bregma*			
LI	0.023 $\pm$ 0.008	7 $\pm$ 3	0.11 $\pm$ 0.04
LII-III	0.073 $\pm$ 0.007	23 $\pm$ 2	0.37 $\pm$ 0.04
LIV	0.059 $\pm$ 0.005	19 $\pm$ 2	0.30 $\pm$ 0.03
LV	0.069 $\pm$ 0.007	22 $\pm$ 3	0.35 $\pm$ 0.04
LVI	0.064 $\pm$ 0.005	20 $\pm$ 2	0.33 $\pm$ 0.03
CC	0.046 $\pm$ 0.004	21 $\pm$ 2	0.24 $\pm$ 0.02

*n=4; CC – Corpus callosum.

Fig.1

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6