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Lesion size-dependent synaptic and astrocytic responses in cortex contralateral to infarcts in middle aged rats

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Abstract

In young adult rats, unilateral lesions of the sensorimotor cortex lead to neuronal structural plasticity and synaptogenesis in the contralateral motor cortex, which is connected to the lesion site by transcallosal fibers. The contralesional neural plasticity varies with lesion size and results from the convergence of denervation-induced reactive plasticity and behavioral asymmetries. It was unknown whether similar effects occur in older animals. Furthermore, the coordination of synaptic responses with that of perisynaptic astrocytes had not been investigated. In this study, middle-aged rats (14–16 months old) were given sham-operations or unilateral ischemic lesions of the sensorimotor cortex. Fifty days later, numerical densities of neurons and synapses and morphological characteristics of astrocytic processes in layer V of the contralesional motor cortex were measured using stereological light and electron microscopy methods. Lesions resulted in behavioral asymmetries, but no significant synapse addition in the contralesional motor cortex. Synapse number per neuron was negatively correlated with lesion size and reduced opposite larger lesions compared with smaller ones. Astrocytic changes were also lesion size-dependent. Astrocytic hypertrophy was observed only after smaller lesions and was associated with greater coverage and greater numbers of synapses. These findings are consistent with those in younger rats indicating an inverse relationship between lesion size and adaptive neuronal restructuring in denervated cortex. However, they indicate that the synaptogenic reaction to this lesion is relatively limited in older animals. Finally, the results indicate that structural plasticity of perisynaptic astrocytes parallels, and could play a role in shaping, synaptic responses to post-ischemic denervation.

Keywords

stroke; denervation; forelimb behavior; perisynaptic astrocytes; stereology

INTRODUCTION

Animal models of CNS damage have been used for decades to understand how surviving neurons reorganize their connectivity after losing input from the site of an injury. It is hoped that such an understanding will facilitate efforts to improve adaptive neural restructuring and behavioral outcome. A prominent focus of animal brain injury models has been on neural changes relevant to hand and arm function because upper extremity impairments are a leading long-term disability resulting from stroke (Hendricks et al., 2002; Nudo, 2007). In

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rats, unilateral lesions of the forelimb representation regions of the sensorimotor cortex (FLsmc) result in sensory and motor deficits in the contralateral forelimb and a compensatory reliance on the less-affected (ipsilateral) forelimb. These and similarly placed injuries have been found to increase neuropil volume (Hsu and Jones, 2005; Jones and Schallert, 1992), dendritic arborization of layer V pyramidal neurons (Adkins et al., 2004; Biernaskie and Corbett, 2001; Jones et al., 1996; Jones and Schallert 1992; Voorhies and Jones, 2002), neuronal plasticity-related molecules (Hughes-Davis et al., 2005; Stroemer et al., 1995), neuronal excitability (Witte et al., 2000), and synapse number per neuron (Hsu and Jones, 2005; Jones et al., 1996; Jones, 1999; Luke et al., 2004) in the contralesional cortex. The neuronal structural plasticity reflects both denervation-triggered and experience-dependent plasticity (Bury et al., 2000a; Jones and Schallert, 1994) and it accompanies functional changes in the ipsilesional limb (reviewed in Allred and Jones, 2008). It is important to note, however, that most of these studies of unilateral cortical lesion effects were conducted with young adult animals (~3–5 months of age). Given that stroke incidence increases with age, it seems pertinent to investigate these events in older animals.

Aging involves microanatomical brain changes that include regressive alterations in dendritic arbor and spine numbers (Anderson and Rutledge, 1996; Duan et al., 2003), reductions in synaptic density (Peters et al., 1998), demyelination of axons (Peters and Sethares, 2002), and increased vulnerability in synaptic connectivity (reviewed in Hof and Morrison, 2004). Such age-related changes might modify the brain's response to injury. For example, Yurek and Fletcher-Turner (2000) found that young rats had increased expression of brain-derived neurotrophic factor (BDNF) in the striatum following a unilateral 6-hydroxydopamine lesion, but that aged rats did not. Astrocytic alterations in the aged brain may play a critical role in how it responds to trauma. Astrocytic reactivity is increased with aging (Cotrina and Nedergaard, 2002) and excitotoxicity, an important contributor to cell death following brain injury, induces early astrogliosis in the aged brain (Castillo-Ruiz et al., 2007). Furthermore, early and extensively elevated glial reactivity in response to a stroke-like lesion has been correlated with reduced functional recovery in aged rats (Badan et al., 2003; Popa-Wagner et al., 2007). Because astrocytes also play many critical roles in synaptic plasticity (Jones and Greenough, 2002; Stevens, 2008), it seems important to further characterize the coordination of astroglial and synaptic responses to injury in older brains.

The goals of this study were: (i) to determine whether unilateral ischemic sensorimotor cortex (SMC) lesions in middle aged rats result in synaptic structural changes in the contralesional homotopic cortex, as many previous studies conducted with young adult rats have shown; (ii) to investigate whether synaptic alterations are accompanied by significant morphological changes in astrocytic processes and their contact with synaptic elements; (iii) to assess whether lesion size correlates with differential responses. We used endothelin-1 (ET-1), a vasoconstrictive peptide, to make unilateral focal ischemic lesions in the FLsmc. Asymmetrical forelimb use was measured with the Schallert cylinder test. Stereological measures and light and transmission electron microscopy (TEM) were used to measure quantities of neurons, degenerating neurons, synapses, astrocytic processes and synaptic-astrocytic contact in layer V of the contralateral homotopic motor cortex 50 days after the lesions.

MATERIALS AND METHODS

Animals

Thirty-four male Long-Evans hooded rats were used. Rats were 14–16 months old at the time of surgery (mean $\pm$ SEM weight = 686.0 ± 19.5 g). This strain was chosen for comparison purposes with numerous previous studies of SMC lesion effects in young adults

of the same strain (e.g., Adkins et al., 2004; Hsu and Jones, 2005; Jones et al., 1996; Jones, 1999; Jones and Schallert, 1992; Luke et al., 2004). For the TEM study, 24 rats, obtained from Charles River Laboratories (CR; n = 10, retired breeders) and from the Animal Resources Center at the University of Texas at Austin (ARC; n=14), were used. Rats were randomly assigned in equal proportions from each breeder source to two groups of 12 (lesion and sham). Another ten rats (15 months old) from Harlan were used for the analysis of neurodegeneration with Fluoro-Jade B. All animals were received at least ten months before the onset of experimental procedures and were made tame by handling to reduce the effects of stress during behavioral assessment. Frequent handling was maintained throughout the course of the study. Rats were housed in pairs on a 12:12 hour light/dark cycle and received food and water *ad libitum*. All animal use was in accordance with a protocol approved by the Animal Care and Use Committee of the University of Texas at Austin.

Surgical procedures

Rats received either sham-operations or unilateral ischemic lesions to the forelimb representation region of the sensorimotor cortex (FLsmc). Rats were anesthetized with either Equithesin (150 mg/kg chloral hydrate and 37.5 mg/kg pentobarbital), with atropine sulfate (1.25 mg/kg) used to counteract respiratory depression, or with a cocktail of ketamine (100 mg/kg) and xylazine (10–13 mg/kg). Lesions and lesion-induced behavioral effects are similar with these anesthetics (Adkins et al., 2004, 2006; Hsu and Jones 2005; Luke et al., 2004). The skull and dura were removed between 0.5 mm posterior and 1.5 mm anterior to bregma and 3.0 to 4.5 mm lateral to midline. Ischemic damage to the SMC was then created by topical application of 1.5 µL of ET-1 (120 pmol, 0.2g/L in sterile saline) aimed at the overlapping primary somatosensory and motor cortical representation regions of the forelimb (i.e., the forelimb “overlap zone”; Donoghue and Wise, 1982). ET-1 application results in a potent vasoconstriction followed by gradual reperfusion over 24 h (Biernaskie et al., 2001) and this method was chosen for its ability to produce highly localized cortical infarcts (Adkins et al., 2004; Fuxe et al., 1997; Gilmour et al., 2004). Ten minutes after ET-1 administration, the scalp was sutured. Sham-operated animals received identical treatment up to, but not including, removal of the skull, since it has been previously demonstrated that skull removal can cause behavioral and neurochemical asymmetries (Adams et al., 1994).

Schallert cylinder test

Animals were tested for behavioral asymmetries once pre-operatively and at multiple time points post-operatively. The Schallert cylinder test (Schallert et al., 1997, 2000) was used to detect decreased use of the impaired limb (contralateral to the lesion) for postural support behaviors. Rats were videotaped as they explored the walls of a transparent cylinder. The first 30 instances of sole use of either forelimb (ipsilateral or contralateral to the lesion) or simultaneous bilateral forelimb use for upright support against the cylinder wall were recorded from slow-motion playbacks of each session. The percentage of impaired limb use was calculated using the formula: % (contralateral support observations)/(total forelimb use observations). The data were analyzed as differences between post- and pre-operative scores. For post-operative data, the scores for every two to three time points were pooled into the following three categories: Early (pooled over three time points during the first two weeks post-surgery), Mid (pooled over three time points during weeks 3–5), and Late (pooled over two time points during weeks 6–7).

Stereological light and electron microscopy

Tissue processing—The time point chosen for the analysis of astrocytic and synaptic changes was 50 days after the lesions. In younger animals, neuronal structural changes in the cortex opposite FLsmc lesions have been reported at time points between 7–120 days

postlesion (e.g., Jones and Schallert, 1992; Jones et al., 1996; Hsu and Jones, 2005). Synapse addition here takes time to occur, but then it appears to be relatively enduring. Using TEM methods similar to the present study, significant increases in synapse number per neuron were evident at 25, 30 and 46 days, but not yet at 18 days, in younger animals (Hsu and Jones, 2005; Jones et al., 1996). We chose 50 days to allow for the potential age-related slowed reactive synaptogenic responses to denervation, which has been found in other systems (e.g., Schauwecker et al., 1995; Scheff, 2003).

Rats were anesthetized with a lethal dose of sodium pentobarbital (100mg/kg) and transcardially perfused with 0.1 M phosphate buffer with heparin sodium salt (0.05 g/L). This was followed by a fixative solution made of 2% paraformaldehyde and 2.5% glutaraldehyde in the same buffer. Brains were extracted and sliced with a Leica VT1000S vibratome within 24 h of perfusion. Coronal sections were collected in alternating sets of 200 μ m, 100 μ m, and two sets of 50 μ m thicknesses. For volume measurement of the remaining cortex and lesion reconstruction, 50 μ m-thick sections were Nissl stained with Toluidine Blue.

For TEM, 200 μ m-thick sections were collected into the same fixative solution used for perfusion until they were osmicated. From these 200 μ m-thick sections, the primary motor cortex (MI) in the contralesional hemisphere, inclusive of the forelimb overlap zone, was dissected under a stereomicroscope. The region was identified based on macrostructural landmarks and unique cytoarchitectural characteristics that are evident in unstained sections, as previously described (Jones et al., 1996). In the rat, the forelimb representation includes an “overlap zone” of the primary somatic sensory (SI) and MI cortex which is easily identifiable in coronal sections. The lateral agranular cortex (AGl) medial to the forelimb overlap zone is reliably the non-overlapping forelimb MI representation area (Donoghue and Wise, 1982). However, the anterior and posterior borders of the forelimb MI lack as clear anatomical boundaries. Since this identifiable region of interest is reliably found in about two of the 200 μ m-thick sections, these two sections were processed for TEM. A limitation of this strategy is that the anterior regions (and to lesser extent, the posterior region) of the forelimb MI and overlap zone are undersampled. The advantage is that the forelimb region is reliably sampled in each animal.

The samples were osmicated (2% osmium tetroxide in 0.05 M cacodylate buffer with 0.75% potassium ferrocyanide), stained *en bloc* with 2% uranyl acetate, dehydrated in ascending ethanol and acetone, sandwich embedded in resin and polymerized in a 60°C oven. One of two sections was randomly selected for stereological analysis. The sections were mounted on a resin block, and serial coronal semithin (0.8 μ m) and ultrathin (70 nm) sections were made using a Leica Ultracut R microtome. Samples were coded so that data collection was blind to experimental conditions. Semithin sections were mounted onto gelatin-coated slides, stained with Toluidine Blue, and used to identify and to measure neuronal densities in layer V of the motor cortex using light microscopy. After collecting semithin sections, the resin blocks were trimmed to retain only layer V of the motor cortex and then used to obtain serial ultrathin sections. These were mounted onto formvar coated slotted copper grids and stained with lead citrate.

Estimation of changes in synapse number per neuron—Synapse number per neuron is a measure sensitive to quantitative changes in the total number of synapses when neuron number is stable (e.g., Jones et al., 1996; Kleim et al., 2004; Luke et al., 2004; Turner and Greenough, 1985). In contrast, the numerical density of synapses may not reflect a change in synapse number when neuropil volume also changes, a potential concern in this study because the contralateral homotopic cortex was previously found to have increased dendritic arborization (Biernaskie and Corbett, 2001; Jones et al., 1996; Jones and Schallert

1992) and reactive astrocytic responses (Bury et al., 2000b) in younger animals. Synaptic density and neuronal density were both estimated from the same resin embedded samples, using unbiased stereological analysis (described below). Synapse number per neuron was calculated by dividing synaptic density by neuronal density.

Neuronal density (Nv_{neuron}) and neuropil volume per neuron—In the presence of stable neuronal number the volume of neuropil can be estimated as the inverse of neuronal density. Neuronal density (Nv_{neuron}) was measured using the physical disector method (Sterio, 1984). Digital images of semithin sections were obtained using a high-resolution digital camera (DVC Co., Austin, TX) and a Nikon Optiphot-2 light microscope equipped with a rotating stage (1140 $\times$ final magnification; 40 $\times$ objective). The sampling strategy and disector pairings were the same as previously described (Hsu and Jones, 2005), except that greater numbers of samples were used for estimates of neuronal density. This was done based on pilot data which indicated that a larger sample was needed to reduce the coefficient of error (West and Gundersen, 1990) and compensate for an expected higher level of sample-to-sample variation in the older brains used in the study. Briefly, two sample fields in layer V of each section (0.7–1.0 mm apart medially to laterally) were selected with systematic random sampling. Images within the same sample field were obtained from five alternating semithin sections. Adjacent sections served as disector pairs using the following combinations of reference and look-up sections: sections 1 and 2, 2 and 3, 3 and 4, 4 and 5, 5 and 4, 4 and 3. Ten sets of samples were taken so that a total of 50 images and 60 disector pairs were used per animal. Neuron nuclei were counted when they appeared in a reference section and disappeared in a look-up section. The coefficient of error (CE) of the neuronal density estimates from individual brains ranged from 0.030 to 0.054 (median=0.041) and mean CE's were similar between groups (Sham: 0.038; Lesion: 0.041). Neuronal density was calculated by the formula: $Nv = \Sigma Q^- / \Sigma v(\text{frame})$, where ΣQ^- is the sum of neurons counted per brain and $\Sigma v(\text{frame})$ is the sum of the sample volume (4,828,800 μm^3), which was calculated as the product of the area of one sample frame (50,300 μm^2), distance between section planes (1.6 μm), and number of disector pairs (60). As described below, the pattern of results suggests that the changes in neuronal density observed in the present study primarily reflect changes in neuropil volume. Thus, these data are reported as the neuropil volume per neuron.

Synaptic density ($Nv_{synapse}$)—Axodendritic synaptic density was also estimated using the physical disector method. Four sets of four serially positioned digital electron micrographs per brain were obtained using a Philips EM 208 transmission electron microscope with an AMT advantage HR 1MB digital camera (14,000 $\times$ image capture magnification; digitally viewed at a final magnification of 42,000 $\times$). All images were coded to insure the measurer was blind to experimental conditions. A total of 24 disector pairs were used per brain because every section for synaptic density was used as both a reference and a look-up section. Synaptic density was calculated with the formula: $Nv_{synapse} = \Sigma Q^- / \Sigma v(\text{frame})$, where ΣQ^- is the sum of synapses counted per brain and $\Sigma v(\text{frame})$ is the sum of the sample volume (188.0 μm^3), which was calculated as the product of the sample frame area (111.9 μm^2), the section thickness (70 nm), and the number of disector pairs (24). Two principle criteria were used to identify a synapse: the presence of (i) a postsynaptic density and (ii) at least three presynaptic vesicles in the apposing presynaptic process. All synapses formed between a bouton and a dendritic process (spines and shafts) were included in the measures (Fig. 1A). Thus, the estimates include intrinsic and transcallosal cortical connections, thalamic projections and other connections. Synapses were also classified as being formed by multiple synaptic or single-synaptic boutons and as possessing perforated or simple postsynaptic density, as described previously (Hsu and Jones, 2005). However, the data on these synaptic types are not reported herein since there were no lesion-related effects

in middle-aged rats. Synapse number per neuron was determined using the formula: $(Nv_{synapse})/(Nv_{neuron})$.

Estimation of astrocytic volume fraction, astrocytic volume per neuron, and percentage of synapses with astrocytic contact—To determine whether astrocytic hypertrophy occurs in the contralesional motor cortex, the layer V volume fraction of astrocytic processes was estimated. One electron micrograph from each of the four series used for the synaptic measures was chosen. Astrocytic processes were identified by their relatively transparent cytoplasm, irregular and sheet-like shape, absence of dendritic and axonal characteristics (e.g., highly ordered microtubules, small vesicle clusters, myelination) and, occasionally, the presence of intermediate filaments (Peters et al., 1991). The software program RECONSTRUCT (Fiala, 2005; available from <http://synapses.clm.utexas.edu/tools/index.stm>) was used to trace astrocytic processes and estimate their areas. The volume fraction of astrocytic processes was calculated as astrocytic process area divided by sample area. The volume of astrocytes per neuron was calculated by dividing the astrocytic volume fraction by neuronal density, as previously described (e.g., Kleim et al., 2007).

The percentage of synapses with astrocytic apposition was estimated using the same micrographs that were used to estimate astrocytic volume fraction. When astrocytic processes were found in direct contact with pre- and/or postsynaptic elements, the location of this contact was noted. This included astrocytic processes positioned at the (i) axon-dendrite interface of the active zone, (ii) the presynaptic element only, (iii) the postsynaptic element only, or (iv) both pre- and postsynaptic elements, but not the axon-dendrite interface.

Lesion analyses

Lesion sizes were estimated by calculating the volume differences between injured and intact hemispheres. Estimation of volume was based on the Cavalieri principle (Gundersen et al., 1988). The perimeters of Nissl-stained coronal sections were traced using Neurolucida software (MicroBrightField, Colchester, VT) to estimate the area of cortex in each section. Moving caudally, the first section was selected when forceps minor corpus callosum appeared (~2.7 mm anterior to bregma). An additional five more caudal sections (800 μ m apart) were then traced so that a total of six sections were used for estimation of cortical volume. Volume was calculated by the formula: $V = \Sigma A \times T$, where ΣA is the sum of the area of all the sections and T is the distance between section planes (800 μ m). This strategy focuses the volume estimation in the region of the sensorimotor cortex and the lesions, which permits more sensitive estimation of inter-animal variability in lesion volume than estimation of the entire cortex. As described below, animals with lesions were divided into two groups, one with smaller lesions (Small Lesion) and one with larger lesions (Large Lesion). Additionally, to characterize the approximate lesion placement and extent, lesions were reconstructed onto schematic coronal section templates adapted from Paxinos and Watson (1986).

Fluoro-Jade B neurodegeneration labeling

As described above, when neuronal number is stable the volume of neuropil can be estimated using neuronal density. Although these cortical lesions have not been found to result in significant neuronal death in the contralesional cortex in younger animals, this had not been established in older rats. Fluoro-Jade B (FJB) labeling was used to detect presumed degenerating neurons in the perilesion and contralesional FLsmc. For FJB analysis, the same surgical procedures for lesion and sham operations were used as in rats used for TEM analysis. Rats were sacrificed at 24 h or 3 days after surgery (one sham-operate, four rats

with lesions per time point). These time points were based on a previous study which found extensive neuronal degeneration in perilesion cortex at 24 h after similar lesions and more subtle degeneration 3 and 14 days post-lesion (Adkins et al., 2006). Rats were anesthetized with a lethal dose of sodium pentobarbital as described above and transcardially perfused with 0.1 M sodium phosphate buffer, followed by a 4% paraformaldehyde solution in the same buffer. Extracted brains were stored in the fixative solution at 4°C for at least 24 hours. Coronal sections (50 μ m) were then collected and stored in cryoprotectant solution at -20°C. The staining of tissue using FJB (Chemicon, Temecula, CA) and the quantification of degenerating neuron density has been previously described in detail (Adkins et al., 2006). Briefly, slides were treated with sodium hydroxide and potassium permanganate followed by 0.0004% FJB solution, dried overnight and coverslipped using Krystalon (EMD Chemicals, Gibbstown, NJ). In three sections between 0.2 and 1.2 mm anterior to bregma, the sample areas of the ipsilesional cortex and the contralesional homotopic cortex were outlined using Neurolucida (39 $\times$ magnification). All FJB positive cells within the outlined areas (all layers) were then counted (622 $\times$). The density of FJB positive neurons was calculated by the formula: $N_v = \Sigma Q^- / \Sigma v$, where ΣQ^- is the sum of FJB positive neurons and Σv is the sum of the sample volume, which was calculated as the product of the estimated area of the outlined sample region of each section and the thickness of the section (50 μ m). Sham results from 24 h and 3d after surgery were pooled since there was no difference between these two time points.

Statistical analysis

The Statistical Package for the Social Science (SPSS) general linear model procedure (GLM) for one-way analyses of variance (ANOVA) was used to compare means. Bivariate correlations were used to examine relationships between anatomical variables and lesion size. To further characterize lesion-size dependent effects, a median split was used to subdivide the lesion group (as described below) and pair-wise planned comparisons (Sham vs. Small Lesion; Sham vs. Large Lesion; Small vs. Large Lesion) were used to assess differential responses due to lesion size. For Fluoro-Jade B data, planned comparisons were used to determine (i) the evident difference in neuronal degeneration in the ipsilesional cortex compared with the contralesional side at both time points and (ii) the time-dependent effect on neuronal degeneration in each side. The Schallert cylinder data were analyzed using SPSS repeated measures ANOVA, and Fisher Least Significant Difference (LSD) post hoc analyses were used to further analyze group differences when needed.

RESULTS

Synapse number per neuron in the contralesional motor cortex was negatively correlated with lesion size

Synapse number per neuron ($N_{v_{\text{synapse}}} / N_{v_{\text{neuron}}}$) was estimated using the physical disector method. Fifty days after an ischemic lesion, synapse number per neuron in layer V of the contralesional homotopic cortex was not significantly different in middle-aged rats with SMC lesions compared with sham-operates (mean $\pm$ SEM synapses/neuron = 20,829 $\pm$ 896 in Sham and 21,788 $\pm$ 1381 in Lesion). However, as shown in Figure 1B, synapse number per neuron in the contralesional cortex was negatively correlated with lesion size as estimated by the volume difference between the two cortices ($r = -0.86$; $p = 0.001$, two-tailed), excluding one outlier (with the outlier included, the correlation coefficient was $r = -0.21$; $p = 0.51$, two-tailed). This outlier was discarded based on particularly unusual values on regression diagnostic statistics: a Studentized residual of 2.87, Cook's distance of 3.16 and a leverage of 0.353. There was no correlation between interhemispheric volume difference and synapse number per neuron in sham-operates ($r = -0.27$; $p = 0.39$, two-tailed).

Since these results suggested an effect of lesion size on plasticity of the microanatomy of the contralesional homotopic cortex, we probed this effect further by dividing the lesion group into two using a median split: (1) one with smaller amounts of tissue loss ($n=6$), (2) one with larger amounts of tissue loss ($n=5$, excluding the outlier). The lesion sizes between these two subgroups were significantly different (mean $\pm$ SEM lesion size = $10.7 \pm 1.29 \text{ mm}^3$ in Small and $19.0 \pm 1.90 \text{ mm}^3$ in Large; $F(1,9) = 13.96$, $p = 0.005$). Furthermore, these mean lesion sizes approximate those that were found to result in differential contralesional neuronal structural plasticity in younger rats (Hsu and Jones, 2006). Thus, we consider results with respect to these small and large lesion subgroups below. Representative Nissl-stained sections and schematic lesion reconstructions are shown for the two lesion groups in Figure 1C.

Unilateral lesions induced behavioral asymmetries in postural support behavior

Animals had a significant post-lesion decrease in use of the impaired forelimb for postural support behavior in the Schallert cylinder test. When comparing the Sham and two Lesion groups (small and large, excluding the outlier, Fig. 1D), there was a significant main effect of Group ($F_{(2,20)} = 4.04$, $p=0.034$). In *post hoc* multiple comparisons (LSD), the Large Lesion group was found to have significantly greater forelimb use asymmetries than the Sham group ($p = 0.02$). The Small Lesion group tended to have greater forelimb use asymmetries than the Sham group, but the results failed to reach significance ($p = 0.057$). There was no significant difference in forelimb use asymmetries between the Small and Large Lesion groups ($p>0.05$). The asymmetrical behavioral was evident throughout the time span examined (7 weeks post-lesion). There was neither a significant interaction effect of Group by Time post-lesion nor a main effect of Time post-lesion.

Layer V neuropil volume per neuron and synapse number per neuron were significantly decreased after larger lesions compared to smaller lesions

As with synapse number per neuron, when all lesions were considered together, there was no change in neuropil volume in layer V of the contralesional motor cortex compared with sham operates (mean $\pm$ SEM neuropil volume per neuron in $\text{mm}^3 = 26.6 \pm 0.89$ in Sham and 27.1 ± 1.02 in Lesion before median split). However, neuropil volume per neuron was significantly greater in rats with smaller compared with larger lesions ($F_{(1,9)}=5.82$; $p=0.039$, Fig. 2A). Neuropil volume per neuron in the Sham group was not significantly different from that of the Small or the Large Lesion group ($F_{(1,16)} = 2.18$, $p=0.159$; $F_{(1,15)} = 1.31$, $p=0.271$, respectively). Despite a significant difference in the neuropil volume between the two lesion groups, there was no significant difference in synaptic density (Table 1). This resulted in the Large Lesion group having significantly fewer synapses per neuron than the Small Lesion group ($F_{(1,9)}=7.68$; $p=0.022$, Fig. 2B). There was no significant difference in synapse number per neuron between Sham and Small Lesion groups ($F_{(1,16)}=2.55$; $p=0.130$). Although the Large Lesion group had the fewest synapses per neuron on average, this failed to reach significance when compared with the Sham group ($F_{(1,15)}=3.10$; $p=0.099$).

Degenerating neurons were increased in the ipsilesional, but not contralesional, cortex

To determine whether a unilateral FLsmc lesion results in major degeneration of neurons in the contra- as well as the ipsilesional cortex in middle-aged rats, we quantified neurons labeled by Fluoro-Jade B (FJB) in both hemispheres (Fig. 3). There were few clearly FJB labeled neurons in the contralesional homotopic cortex at either 24 h or 3 days after lesions and the subtle labeling was similar to that found in sham-operates. In contrast, in the peri-lesion cortex, there was abundant labeling at either time point. The density of FJB labeled cells was significantly increased in the ipsilesional cortex compared with the contralesional cortex ($p<0.001$ at both time points). As was shown in a previous study with young adults (Adkins et al., 2006), the density of FJB positive neurons in the peri-lesion cortex at 24h

post-lesion was considerably higher than that at 3 days post-lesion ($F_{(1,6)}=145.64$; $p<0.001$). There was no significant difference in the density of FJB stained neurons in the contralesional side between the two time points ($F_{(1,6)}=2.63$; $p=0.16$). These data do not rule out the presence of subtle contralesional neuronal loss, but they do suggest that it is unlikely to be a major contributor to lesion-induced structural differences in the contralesional cortex.

Astrocytic hypertrophy in the contralesional homotopic cortex was observed in the Small, but not Large, Lesion group

As shown in Fig. 4A, astrocytic processes were identified in electron micrographs and the volume fractions of astrocytes (V_v) were estimated. Astrocytic volume per neuron was then determined ($V_v/N_{v_{neuron}}$) as a measure of astrocytic hypertrophy (e.g., Kleim et al., 2007). While synapse numbers in the Small Lesion group were significantly different only from the Large Lesion group (Fig. 2B), astrocytic volume per neuron in the contralesional homotopic cortex was significantly increased in the Small Lesion group compared to both the Sham ($F_{(1,16)}=4.89$; $p=0.042$) and the Large Lesion groups ($F_{(1,9)}=17.51$; $p=0.002$) (Fig. 4B). In contrast, the Large Lesion group's astrocytic volume per neuron was not significantly different from the Sham group ($F_{(1,15)}=0.77$; $p=0.395$). The volume fraction of astrocytes (i.e., astrocytic volume per unit volume of neuropil) was also significantly increased in the Small Lesion group compared to the Sham group ($F_{(1,16)}=4.56$; $p=0.048$), but not to the Large Lesion group ($F_{(1,9)}=0.56$; $p=0.47$), as shown in Table 1.

Astrocytic hypertrophy in the contralesional homotopic cortex was highly correlated with synapse number per neuron

To investigate whether astrocytic hypertrophy is associated with synaptic responses to lesions, correlations between synapse number per neuron and astrocytic volume per neuron were examined. Astrocytic volume per neuron was highly correlated with synapse number per neuron in the Lesion group (large and small lesions together, $r=0.745$; $p=0.008$, two-tailed, Fig. 5A). Note that Large and Small lesion animals near the center of the scatter plot in Fig. 5A are also close to the median split shown in Fig. 1B. The Sham group did not have a significant correlation ($r=0.369$; $p=0.237$, two-tailed, Fig. 5B).

The Small Lesion group had more synapses with astrocytic apposition than the Large Lesion group

Table 2 shows the percentage of synapses with astrocytic contact subdivided by the location of the contact. In all groups, astroglia processes most frequently contacted the axon-dendrite interface (ADI). The Small Lesion group tended to have more frequent astrocytic contact at the ADI when compared to the Large Lesion group, but this failed to reach statistical significance ($F_{(1,9)}=4.32$; $p=0.067$). However, the total percentage of synapses with any direct astrocytic apposition was significantly increased in the cortex contralateral to small lesions compared to the cortex contralateral to large lesions ($F_{(1,9)}=5.37$; $p=0.046$, Table 2).

DISCUSSION

Although many previous studies have found that unilateral brain damage results in lesion-induced neural restructuring in the contralesional cortex of young adult rats (e.g., Biernaskie and Corbett, 2001; Hsu et al. 2005; Jones, 1999; Jones and Schallert, 1992; Luke et al., 2004; Stroemer et al., 1995), the present study suggests that, at least at the synaptic level, this lesion-induced plasticity is relatively limited and markedly sensitive to lesion sizes in middle-aged (14–16 months old) rats. Although the middle-aged rats of this study had similar lesion-induced behavioral asymmetries as those found in young adults, there was no corresponding increase in synapse number per neuron in the contralesional cortex, in contrast to findings in younger rats. This reduced neuroplastic response is consistent with

evidence that aging attenuates the deafferentation- and lesion-induced expression of neurotrophic factors (Woods et al., 1998, Yurek and Fletcher-Turner, 2000). However, it is in contrast to findings that even older animals are capable of a significant, though slowly developing, reactive synaptogenic response in the denervated hippocampus (Scheff, 2003), as discussed more below. Despite the lack of lesion-induced synapse addition in middle-aged rats of the present study, it is notable that quantities of synapses, astrocytic processes, and astrocyte-synapse contacts in the contralesional cortex varied with lesion size. There was a negative correlation between lesion size and synapse number per neuron in layer V of the contralesional cortex, with larger lesions resulting in reduced synapse numbers per neuron compared to smaller lesions. Furthermore, astrocytic hypertrophy in the same cortical region was observed only after smaller lesions and was highly correlated with synapse number per neuron after lesions, but not in sham-operates. This result suggests a lesion-induced alteration in the structural relationship between synapses and astrocyte.

In young adults, neuroplastic changes in the contralesional cortex have been found to depend upon at least two events: lesion-induced partial denervation and increased reliance on the ipsilesional forelimb (Adkins et al., 2002; Bury et al., 2000a; Jones and Schallert, 1994). The motor cortices of the two hemispheres are interconnected by transcallosal fibers, and unilateral FLsmc lesions result in a loss of some of these connections with the contralesional motor cortex. The degeneration of transcallosal projections is believed to trigger growth-promoting changes associated with reactive synaptogenesis that, in turn, enhance the sensitivity of cortical pyramidal neurons in the contralesional cortex to experience-dependent plasticity (reviewed in Allred and Jones, 2008). In the presence of transcallosal denervation, increased activity and learning with the ipsilesional forelimb results in greater neuronal structural plasticity in the motor cortex than can be found with similar experiences in intact animals (Adkins et al., 2002; Bury et al., 2000a; Bury and Jones, 2004). We found that middle-aged rats were similar to younger rats in their development of lesion-induced behavioral asymmetries and in the absence of significant neural death in the contralesional homotopic cortex, as indicated by Fluoro-Jade B staining, but not in their synaptic response. This suggests that aging limits this aspect of post-injury experience-dependent plasticity in the contralesional cortex. This could be due to age-related changes in either degeneration-triggered plasticity, as previously reported in hippocampus (e.g., Hattiangady et al., 2008; Schauwecker et al., 1995; Shetty and Turner et al., 1999), or in experience-dependent plasticity.

A feature of the contralesional events in younger animals is that synapse numbers are increased relative to intact animals (e.g., Hsu and Jones, 2005; Jones et al., 1996), indicating that this type of reactive synaptogenesis is not merely replacing synapses lost to denervation but also adding more than the equivalent amount of lost synapses (Cotman et al., 1981). The failure of the lesions to result in significant change in synapse number per neuron compared to sham-operates in the present study suggests that any synaptogenesis that did occur in response to denervation in the contralesional cortex was sufficient only to replace those lost. Furthermore, the reduction in synapse number per neuron in large versus small lesion groups suggests that this response could be limited in the presence of greater tissue damage.

The inverse relationship between lesion size and neuronal structural plasticity is consistent with previous findings in younger rats. In young adult rats (3–4 months of age), smaller FLsmc lesions result in greater dendrite addition in the contralesional cortex than do larger lesions (Hsu and Jones, 2006). The small (~8 mm³) versus large (~22 mm³) lesions in this previous study were similar to those of the present study. However, young adults showed considerable neuronal structural growth in the contralesional cortex regardless of lesion size, whereas the middle-aged rats of the present study did not, at least as evidenced by synapse numbers per neuron in layer V. Moreover, in the younger rats, the dendritic and synaptic

plasticity found in contralesional cortex are linked with functional changes in the ipsilesional forelimb, including enhancement in its rate of skill learning (Allred and Jones, 2008; Hsu and Jones, 2006). Additional work is needed to determine whether these behavioral correlates of the contralesional plasticity are also lost in middle-aged rats.

It is well established that astrocytes play an active role in regulating the microenvironment around neurons (Araque et al, 1999) and in modulating neuronal activities, synaptic transmission and synaptogenesis (Ullian et al., 2001). Astrocytes in the sensorimotor cortex react to both denervation resulting from callosal transections and increased use of the ipsilesional limb, and these astrocytic reactions are exaggerated when denervation and behavioral asymmetries are combined (Bury et al., 2000b). Aged brains have elevated astrocytic reactivity to lesions and denervation (Badan et al., 2003; Gordon et al., 1997; Goss and Morgan, 1995). Considering this and the interactive relationship between astrocytic and synaptic plasticity, we hypothesized that any synaptic structural changes in the contralesional cortex might be associated with a corresponding change in perisynaptic astrocytic processes. In fact, astrocytic volume per neuron was tightly correlated with synapse number per neuron in the contralesional cortex of both lesion groups, but not in the cortex of sham-operates. Furthermore, the hypertrophy of astrocytic processes was linked with their greater coverage of synapses. The failure to find similar astrocytic hypertrophy after larger lesions could mean that the greater degeneration resulting from larger lesions overwhelmed the capacity, or prolonged the time course, of the astrocytic reactions. Alternatively, it could indicate a diminished astrocytic responsiveness that contributed to both greater lesion size and fewer synapses in the contralesional cortex in comparison to the small lesion group.

The present study focused on a relatively late stage of remote neural adaptation to injury and it is likely that examination of earlier time points will be needed to understand the age-related differences in response to the injury. For example, ischemic lesions result in severe oxidative stress and induction of the 70 kD heat shock proteins (Hsp 70) helps counter the oxidative damage (Sharp et al., 2000). Young adults have dramatic Hsp 70 induction in perilesion cortex at one day after focal ischemic cortical lesions and this response is comparatively limited in aged rats, despite a greater extent of oxidative damage (Li et al., 2005). The reduced neuroprotective response to lesions coincides with a time point in which remaining tissue is vulnerable to further insults (Weimar et al., 2002). Furthermore, the induction of the 72 kD Hsp in perilesion cortex precedes functional improvements (Fredduzzi et al., 2001) and massive protein synthesis in synaptosomes (Marriucci et al., 2007). Induction of Hsp 72 expression also occurs in the contralateral homotopic cortex at a delayed time point compared with perilesion cortex. Microglia are also principal contributors to inflammatory, neuroprotective and regenerative responses, e.g., they contribute to spine turnover in after cortical ischemia (Wake et al., 2009) and the removal of synapses after inflammatory lesions (Trapp et al., 2007), and their function is compromised with age (Streit et al., 2008). Such age-related differences in early neuroprotective responses, even in the contralateral cortex, may contribute to differences in subsequent neuroregenerative responses.

Given the analysis of a single time point (50 days post lesion), it remains possible that synapse addition in middle-aged rats occurs at a later time point after the lesions. The slower course of reactive synaptogenesis in hippocampus (Scheff, 2003) and other slowing of neural plastic responses in older animals (Burke and Barnes, 2006) were considerations in the selection of the 50 day time point of the present study. Dendritic arborization in the contralesional cortex has been found to be increased at time points between 14 days and 9 weeks after the lesion (Jones and Schallert, 1992; Biernaskie and Corbett, 2001) and greater numbers of synapses have been observed at 25, 30 and 46 days post lesion, but not at 18

days post lesion in younger rats (Hsu and Jones, 2005; Jones et al., 1996). Thus, the process would have to be extremely delayed, or transient, relative to younger animals for it to have been missed in the present study.

In summary, we found that the neuroplastic changes previously reported to occur in the contralesional cortex of younger adult rats following a unilateral ischemic lesion do not similarly occur in middle-aged rats. Furthermore, we found that lesion size limits the extent of reactive synaptogenesis and astrocytic hypertrophy in middle-aged rats and that the number of synapses was tightly correlated with astrocytic plasticity as a response to lesions. Taken together with previous findings, these results suggest that middle-aged rats have a reduced capacity for lesion-induced synaptogenesis in the contralesional homotopic cortex and suggest that denervation extent strongly interacts with astrocytic reactivity to influence recuperative synaptogenesis.

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Fig. 1.

Axodendritic synapses (white arrows) were identified in transmission electron micrographs (A, scale bar=500nm). Synapse number per neuron was negatively correlated with lesion size (B). A median split divided the lesion group into two subgroups. Representative Nissl stained coronal sections and lesion reconstructions are shown for the two lesion sizes (C). The open rectangle in the contralesional cortex of the photomicrographs indicates the layer V sample region for the synaptic and neuronal density estimates. Numbers are coordinates in mm relative to bregma. Scale bar = 1 mm. In C, lesion reconstructions from different animals are overlaid so that the darker color indicates greater overlap of lesion territory between brains. In the Schallert cylinder test (D), the Large Lesion group had significantly reduced use of the impaired limb compared to the Shams (* $p < 0.05$).

Fig. 2.

Neuropil volume per neuron (A) and synapse number per neuron (B) in the contralesional motor cortex and in one hemisphere of sham operates. Both neuropil volume per neuron and synapse number were significantly decreased in the Large Lesion group compared to the Small Lesion group ($*p < 0.05$). Neither lesion group was significantly different from Sham.

Fig. 3.

Fluoro-Jade B (FJB) staining for degenerating neurons in the contralesional (Contra), ipsilesional (Ipsi) and sham-operated (Sham) cortices. The open rectangle in each lower magnification image (A–D, I) indicates the region shown in the accompanying higher magnification image (E–H, J). FJB (+) neurons were abundant in the peri-infarct area on post-lesion day 1 (B and F). They were less abundant by post-lesion day 3 (D and H). Clearly labeled neurons were rarely found in either the contralesional cortex (E and G) or in sham-operates (J). In quantitative analyses (K), there were significantly more FJB (+) neurons in the peri-infarct region at each time point compared with the contralateral side (* $p < 0.001$ vs. Contra). There were significantly fewer FJB+ cells in peri-infarct cortex at Day 3 compared with Day 1 († $p < 0.001$ vs. Day 1 Ipsi). There was no significant difference in the contralesional cortex between two time points.

Fig. 4. Astrocytic hypertrophy in the contralesional homotopic cortex. Astrocytic processes (asterisks) were identified in electron micrographs (A, scale bar=500nm). Increased astrocytic process volume per neuron was observed only in the Small Lesion group compared with Sham (B, * $p < 0.05$, † $p < 0.01$).

Fig. 5. Correlations between astrocytic hypertrophy and synapse numbers in the contralesional homotopic cortex. Astrocytic process volume per neuron was significantly correlated with synapse number per neuron in the Lesion group (A, $p < 0.01$), but not in Sham (B).

Table 1

Synaptic density and astrocytic volume fraction

	Sham	Small Lesion	Large Lesion
Synaptic density (per $10^3\mu\text{m}^3$)	786.79 ± 27.07	814.72 ± 45.64	724.46 ± 37.66
Astrocytic volume fraction	0.0717 ± 0.0032	$0.0836 \pm 0.0045^*$	0.0787 ± 0.0045

* $p < 0.05$ vs. Sham. Data are means $\pm$ S.E.

Table 2

Percent of Synapses with Astrocytic Contact

Synaptic Position of Astrocytic Process				
	ADI	Pre only	Post only	Pre + Post
Sham	49.41 ± 2.21	10.31 ± 0.99	13.16 ± 1.15	6.29 ± 0.67
Small Lesion	55.26 ± 2.03	10.86 ± 0.97	12.86 ± 1.05	4.49 ± 0.96
Large Lesion	48.37 ± 2.70	10.27 ± 1.87	11.26 ± 1.13	5.85 ± 0.62

ADI, axon-dendrite interface.

[†]Total % of synapses with any astrocytic apposition. Data are means ± S.E.

* p<0.05 vs. Large Lesion