Increased expression of the growth-associated protein-43 gene after
primary motor cortex lesion in macaque monkeys

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Abstract

We recently showed that changes of brain activity in the ipsilesional ventral premotor cortex (PMv) and perilesional primary motor cortex (M1) of macaque monkeys were responsible for recovery of manual dexterity after lesioning M1. To investigate whether axonal remodeling is associated with M1 lesion-induced changes in brain activity, we assessed gene expression of growth-associated protein-43 (GAP-43) in motor and premotor cortices. Increased expression was observed in the PMv during the period just after recovery and in the perilesional M1 during the plateau phase of recovery. Time-dependent and brain region-specific remodeling may play a role in functional recovery after lesioning M1.

Keywords:

functional recovery; *in situ* hybridization; perilesional area; plastic changes; ventral premotor cortex

In a series of studies in macaque monkeys conducted in our laboratory, we showed that a lesion of the primary motor cortex (M1) initially caused flaccid paralysis in the contralateral hand, which was followed by functional recovery of hand movements over the course of 1–2 months of post-lesion motor training (Murata et al., 2008, 2015). Functional and structural reorganization in a part of the undamaged brain may be associated with such functional recovery (Nudo et al., 1996; Friel et al., 2000; Frost et al., 2003; Dancause et al., 2005). Our recent brain imaging study revealed changes in the activities of the ipsilesional ventral premotor cortex (PMv) at 1–2 months and the perilesional M1 at 3–4 months after the M1 lesion (Murata et al., 2015). Pharmacological inactivation of these areas by muscimol suggested that they were causally involved in the functional recovery. Although anatomical tracer studies are needed to confirm changes of neural structures that underlie the observed changes of brain activity during functional recovery, such studies only identify axons that originate from the area where the tracer is injected. In the present study, as a first step toward surveying the cortical areas and pathways where axonal remodeling occurs after an M1 lesion, we focused on growth-associated protein-43 (GAP-43), the expression of which has been shown to be related to axonal remodeling (Benowitz and Routtenberg, 1997; Denny, 2006; Tetzlaff et al., 1991; Van der Zee et al., 1989).

Brain tissue was obtained from eight rhesus and three Japanese monkeys randomly assigned to one of three groups given different periods of recovery after an M1 lesion (see below): 2 weeks, 1–1.5 months, and 3–4 months (Table 1). Brain tissues were also obtained from eight intact monkeys (three rhesus and five Japanese monkeys, five males and three females, 2.2–7.0 kg) used in our previous study (Higo et al., 2009). The

data were pooled across species because neither the behavioral analyses nor the *in situ* hybridization (ISH) analyses (described below) differed between the two species (Mann–Whitney U test, $P > 0.2$). The monkeys were at least two years old and either purchased from a local provider or bred in the Primate Research Institute (PRI) of Kyoto University. The animal use protocol was approved by the Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology (AIST), the National Institutes of Natural Sciences, and PRI. Adequate measures were taken to minimize pain or discomfort in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 80-23; revised 1996).

An artificial lesion was induced in the hand digit area of M1 as described previously (Murata et al., 2008); after constructing a somatotopic motor map of M1 according to the intracortical microstimulation technique, ibotenic acid was injected intracortically to destroy the digit region of M1. After the M1 lesion, the monkeys received post-lesion training as described previously, and daily performance of hand movements before and after lesion were evaluated by means of a small-object retrieval task (Murata et al., 2008, 2015). Although the hand movements of the monkeys used in the present study gradually recovered during the first month after M1 lesion, the mean success rate of the small-object retrieval task was below the 95% confidence level for pre-lesion performance at 1–2 months post-lesion, as was observed previously (Murata et al., 2015). At 3–4 months post-lesion, the mean success rate increased to within the 95% confidence level for pre-lesion performance and behavioral recovery reached a plateau. Lesion locations were also determined as described previously (Murata et al., 2008). Coronal sections (16- μ m thickness) were made at both the level through the superior

genu of the central sulcus, which included the hand area of M1, and the level through the caudal part of the arcuate sulcus, which included the supplementary motor area (SMA), dorsal premotor area (PMd), and PMv. Images of the Nissl-stained sections of M1 were digitized with an image analysis system (MCID; Imaging Research, St. Catharines, ON, Canada). The lesioned area was defined as the area of gliosis as shown by Nissl staining, and unbiased volumes of the lesioned region and M1 digit region were calculated based on Cavalieri's principle (Mayhew, 1992; Table 1).

ISH was performed as described previously (Higo et al., 2000). Sections from as many different groups as possible were hybridized at the same time to minimize intrinsic variation between different ISH experiments. The specificity of the probe was confirmed by Northern blot analysis, in which a single specific band for GAP-43 mRNA was observed (Murata et al., 2005). In addition, control sections using the sense probe showed no specific signal. We quantified the signal intensity of GAP-43 mRNA in layer V pyramidal cells by measuring the optical density (OD) in 30 positive neurons from each cortical area by using MCID (Higo et al., 2009). We also quantified laminar expression as described previously (Higo et al., 2004a, b). Briefly, we overlaid hybridized sections onto adjacent Nissl-stained sections and identified each cortical layer. The OD was measured for each layer of a 300- μ m-wide column that included all the layers of the cortex (OD_{signal}). The OD of the background staining of each section was measured in the subjacent white matter (OD_{background}). At least five columns from each cortical area were measured for each monkey. We calculated the normalized OD from hybridized sections (in situ OD_{signal} – in situ OD_{background}). We also calculated the normalized OD from Nissl-stained sections (Nissl OD_{signal} – Nissl OD_{background}), which

represented the cell density of each region. To compensate for differences in cell density between the layers, we determined the ratio according to the following formula:

$$\text{expression level} = (\text{in situ ODsignal} - \text{in situ ODbackground}) / (\text{Nissl ODsignal} - \text{Nissl ODbackground}).$$

No significant differences in the OD from Nissl-stained sections were observed among the intact, ipsilesional, and contralesional cortical areas (Kruskal–Wallis one-way ANOVA, $P > 0.3$).

In addition, to investigate whether GAP-43 mRNA was co-expressed with the α -subunit of calcium/calmodulin-dependent protein kinase II (CAMKII- α ; a marker for excitatory neurons) (Jones et al., 1994) or gamma-aminobutyric acid (GABA) in M1 and PMv, we conducted double-labeling experiments as described previously (Higo et al., 2009; Higo et al., 2000). The sections were incubated with the primary antibody: a mouse monoclonal antibody to CAMKII- α (clone number, 6G9; catalog number, sc-32288; Santa Cruz Biotechnology, Santa Cruz, CA), or a mouse monoclonal antibody to GABA (clone number, GB69; catalog number, A0310; Sigma, St. Louis, MO), and then incubated in the secondary antibody. After digitizing the image by using MCID, the sections were hybridized with the probes for GAP-43. The specificity of each primary antibody was described previously (Higo et al., 2009; Higo et al., 2000).

No GAP-43 mRNA expression was observed in the lesioned M1 area because there were no neurons, whereas prominent expression was observed in layers II–V of the perilesional area (Fig. 1A–C). We previously reported weak expression of GAP-43 mRNA in layers II and III as well as moderate expression in layers V and VI of the motor cortex of normal intact monkeys (Higo et al., 2007). The present analysis indicated that

GAP-43 mRNA expression in the M1 of lesioned monkeys was higher than that in M1 of normal intact monkeys (Fig. 2A). The increased expression was most prominent in layer III of the perilesional M1 and layer V of the contralesional M1 at 3–4 months after the lesion (Kruskal–Wallis one-way ANOVA with Dunn’s test, $P < 0.01$). Increased GAP-43 expression was also observed in the ipsilesional PMv (Figs. 1F and 2B; Kruskal–Wallis one-way ANOVA with Dunn’s test, $P < 0.05$), whereas the increase was less prominent in the contralesional PMv (Figs. 1G and 2B).

To determine which population of M1 and PMv neurons expresses GAP-43 mRNA, we then performed double-labeling of GAP-43 mRNA and CAMKII- α or GABA. GAP-43 mRNA was expressed in nearly all CAMKII- α -positive excitatory neurons in M1 and PMv of the lesioned monkeys, but not in GABA-positive inhibitory interneurons (Fig. 1H–K). Moreover, the large pyramidal cells in layer V of the ipsilesional PMv frequently showed strong signals for GAP-43 mRNA, in contrast to the weak signals in those of the intact PMv (Fig. 1L and M). Therefore, the relationship between the size of neuronal cell bodies in layer V and the signal intensity of GAP-43 mRNA was quantitatively analyzed. This analysis indicated that the large pyramidal cells in layer V of PMv had weaker hybridization signals than the smaller cells in intact monkeys ($P < 0.01$, linear regression analysis; Fig. 2C), although no significant relationship was found after the M1 lesion (Fig. 2D). The composite data for each size range of layer V neurons indicated that the signal intensities in both medium-sized (area, 200–500 μm^2) and large pyramidal cells (area, $>500 \mu\text{m}^2$) at 1–1.5 months after the M1 lesion were significantly stronger than those in intact monkeys (Fig. 2E; $P < 0.001$ and $P < 0.01$, Kruskal–Wallis one-way ANOVA with Dunn’s test). No significant change in

GAP-43 expression between the intact and M1-lesioned monkeys was observed in the PMd or SMA (Fig. 3).

Because previous studies have confirmed that the expression of GAP-43 protein is regulated by mRNA stability and the protein expression in the axon terminals is correlated with mRNA expression in the cell body (Federoff et al., 1988; Perrone-Bizzozero et al., 1993; Van der Zee et al., 1989; Tetzlaff et al., 1991; Linda et al., 1992; Bendotti et al., 1997), the increased GAP-43 mRNA expression in the large layer-V pyramidal cells of the ipsilesional PMv suggests that GAP-43 protein levels in descending axons including those of the corticospinal tract are also increased. As GAP-43 immunoreactivity is reported to increase in association with sprouting of spinal cord axons (Weaver et al., 1997), the present finding suggests that sprouting occurs in the axons descending from the ipsilesional PMv. We previously showed that GAP-43 mRNA increases in large layer-V pyramidal cells of the M1 after a spinal cord hemisection (Higo et al., 2009), and suggested that sprouting occurs in the axons descending from the M1, a conjecture that was subsequently proven correct (Rosenzweig et al., 2010). A recent study showed that PMv contains the projection neurons giving rise to the corticospinal tract that indirectly control hand muscle motoneurons (Borra et al., 2010). Thus, alternative motor output pathways may be recruited from PMv by strengthening this indirect connection after an M1 lesion. A further study investigating the localization of GAP-43 protein is needed to clarify the projections where GAP-43-mediated axonal remodeling occurs.

The present results in M1 are similar to but different from those of previous cortical lesion studies in the rat, which showed increased GAP-43 expression in the

perilesional area for several weeks after the lesion (Carmichael et al., 2005; Stroemer et al., 1995). The present study in the macaque monkey showed increased GAP-43 expression in perilesional M1 at 3–4 months after the lesion, when behavioral recovery reached a plateau (Murata et al., 2015). GAP-43 mRNA expression, which increased even after functional recovery had been attained, may be related to the consolidation of newly established projections. Our previous brain imaging study showed that activity of the ipsilesional PMv at 3–4 months was lower than at 1–2 months after M1 lesion (Murata et al., 2015), suggesting that a higher level of brain activity may be needed during the period when a small number of new projections are established (but not yet consolidated) to compensate for the function of the lesioned neural circuit.

The present study also showed increased GAP-43 expression in the contralesional M1 in addition to the perilesional M1. This result is consistent with a previous study conducted in cortically damaged rats, which showed structural changes of layer V neurons in the contralesional motor cortex (Biernaskie and Corbett, 2001), suggesting that GAP-43-mediated axonal remodeling may also occur in the contralesional hemisphere in the present monkey M1-lesion model. Further anatomical studies are needed to confirm the anatomical changes suggested by the present data.

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Figure legends

Fig. 1. Change in the gene expression of growth-associated protein-43 (GAP-43) during functional recovery. **A**, Nissl-stained section of a lesional/perilesional area in the primary motor cortex (M1). The boundary of ibotenic acid-lesioned and perilesional areas is indicated by the double-headed arrow. **B**, Expression of GAP-43 mRNA in the section adjacent to **A**. The arrowhead indicates the neurons showing the prominent signals in layer III of the perilesional M1. **C**, **D**, High-magnification photomicrographs of neurons showing hybridization signals for GAP-43 mRNA in layer III of the perilesional M1 (**C**, Peri-M1) and contralesional M1 (**D**, Co-M1) at 3 months post-lesion. **E-G**, GAP-43 expression was higher in the ipsilesional ventral premotor cortex (**F**, Ip-PMv) at 1.5 months post-lesion than those in the intact (**E**) and contralesional PMv (**G**, Co-PMv). **H**, **I**, Expression of GAP-43 mRNA (**I**) in CAMKII- α -immunoreactive neurons (**H**) in layer III of the Ip-PMv. Most of the CAMKII- α -immunoreactive neurons (arrowheads) expressed GAP-43 mRNA. **J**, **K**, Absence of GAP-43 mRNA (**K**) in GABA-immunoreactive neurons (**J**, arrows) in layer III of the Ip-PMv. **L**, **M**, High-magnification photomicrographs of neurons showing hybridization signals for GAP-43 mRNA in layer V of the intact PMv (**L**) and Ip-PMv (**M**) at 1.5 months post-lesion. Double arrowheads indicate large pyramidal neurons, in which weak GAP-43 mRNA expression was observed before, and intense expression after, the M1 lesion. Scale bars: **A**, **B** = 1 mm; **C**, **D** and **H-M** = 50 μ m; **E-G** = 200 μ m.

Fig. 2. A, B, Quantitative analysis of growth-associated protein-43 (GAP-43) mRNA expression in the primary motor (**A**, M1) and ventral premotor (**B**, PMv) cortices. The median values of normal intact monkeys ($n = 8$) for each cortical layer are shown as white bars, and the interquartile ranges of all intact monkeys are shown as error bars. Similarly, the median values of the ipsilesional and contralesional hemispheres of the M1-lesioned monkeys ($n = 11$) for each cortical layer are shown as dark- and light-grey bars, respectively, and the interquartile ranges of all lesioned monkeys are shown as error bars. In the ipsilesional M1, GAP-43 mRNA expression in the perilesional M1 area, within 5 mm from the border of the lesional area, was quantified. A significant difference in expression was observed between layer III of the normal intact and ipsilesional PMv ($*P < 0.05$; Kruskal–Wallis one-way ANOVA with Dunn’s test). The median values for the three recovery stages—at 2 weeks, 1–1.5 months, and 3–4 months after the M1 lesion—are indicated by circles, triangles, and squares, respectively. When the differences between expression in the normal intact monkeys and that in the lesioned monkeys at each recovery stage were evaluated by using Kruskal–Wallis one-way ANOVA with Dunn’s test, a significant increase was verified in layer III of the perilesional M1 and layer V of the contralesional M1 at 3–4 months, and layer III of the contralesional PMv at 2 weeks after M1 lesion (arrowheads, $P < 0.01$). **C, D,** The scattergrams show the relationships between the sizes of the neuronal cell bodies in layer V of the intact (**C**) and ipsilesional PMv (**D**), and the signal intensity of GAP-43. Composite data for all monkeys are shown in both intact and M1-lesioned monkeys. The data for the three recovery stages after the M1 lesion are indicated by circles, triangles, and squares. **E,** The median optical densities in intact monkeys for each size of layer V

neuron in PMv are shown as white bars, and the interquartile ranges of all intact monkeys are shown as error bars. Similarly, the median optical densities in the ipsilesional PMv of the M1-lesioned monkeys for each size of layer V neuron are shown as gray bars. The interquartile ranges of all monkeys at each recovery stage are shown as error bars. Differences were evaluated by using Kruskal–Wallis one-way ANOVA with Dunn’s test ($*P < 0.01$, $**P < 0.001$). The signal intensities of larger pyramidal cells (area, 200–500 μm^2 and $>500 \mu\text{m}^2$) in the ipsilesional PMv at 1–1.5 months after the M1 lesion was significantly stronger than those in intact monkeys.

Fig. 3. The gene expression of growth-associated protein-43 (GAP-43) in the dorsal premotor cortex (PMd) and the supplementary motor cortex (SMA). **A–C**, laminar expression of GAP-43 mRNA in PMd of a normal intact monkey (**A**) and that in the ipsilesional (**B**, Ip-PMd) and contralesional hemisphere (**C**, Co-PMd) of an M1-lesioned monkey at 1.5 months post-lesion. **D–F**, Laminar expression of GAP-43 mRNA in SMA of a normal intact monkey (**D**) and that in the ipsilesional (**E**, Ip-PMd) and contralesional hemisphere (**F**, Co-PMd) of an M1-lesioned monkey at 1.5 months post-lesion. (**G**, **H**) Quantitative analysis of GAP-43 mRNA expression in PMd (**G**) and SMA (**H**). The median values of normal intact monkeys ($n = 8$) for each cortical layer are shown as white bars, and interquartile ranges of all intact monkeys are shown as error bars. Similarly, the median values of the ipsilesional and contralesional hemispheres of M1-lesioned monkeys ($n = 11$) for each cortical layer are shown as dark- and light-grey bars, respectively, and interquartile ranges of all lesioned monkeys are shown as error bars. Median values for the three recovery stages—at 2 weeks, 1–1.5 months, and 3–4

months after the M1 lesion—are indicated by circles, triangles, and squares, respectively.

Scale bars: **A–F** = 200 μm .

	Postlesion survival period	Sex	Weight, kg	Lesion Volume, mm ³ , % Whole digit area
Monkey Ky	2 weeks	male	2.7	88.7, 60.2
Monkey Po	2 weeks	female	2.9	164.3, 89.9
Monkey Se	2 weeks	female	3.2	138.6, 79.1
Monkey Tr	2 weeks	male	4.6	137.3, 85.9
Monkey Hc	1 month	male	2.6	82.8, 76.1
Monkey Si	1 month	male	7.1	64.8, 53.4
Monkey Ro	1.5 months	female	5.8	101.0, 89.2
Monkey Bl	3 months	male	3.8	108.9, 66.7
Monkey Sa	3 months	female	3.4	101.7, 68.3
Monkey Ya	3 months	male	2.8	75.2, 67.7
Monkey Ni	4 months	female	6.8	125.5, 95.2

Table 1. List of the primary motor cortex (M1)-lesioned monkeys studied

Figure 1

Figure 2

Figure 3