Efficient transduction of eleven poly-arginine peptide in an ischemic lesion of mouse brain

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Running title: Protein transduction in the ischemic brain

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Abstract

Direct intracellular delivery of intact proteins has been successfully achieved by tagging cell-penetrating peptide (CPP), which consists of short positively charged amino acids such as eleven poly-arginine (11R); however, *in vivo* delivery of the proteins to the brain has remained challenging, because it is unclear whether CPP would enable proteins to cross the blood-brain barrier (BBB). In this study, we conducted an *in vivo* kinetic study to investigate the efficiency of 11R-mediated peptide delivery in the normal and ischemic brain. The 11R was observed in the microvessels and neurons surrounding the microvessels throughout the brain 1 h after systemic administration but the signal of the peptide was faint after 2h. In a transient middle cerebral artery (MCA) occlusion mouse model, 11R was markedly enhanced and remained detectable in the cells on the ipsilateral side for as long as 8 h after administration compared with the contralateral side. There results suggest that 11R is capable of *in vivo* delivery to the brain by passing through the BBB. Furthermore, 11R-mediated protein transduction could be utilized for the delivery of therapeutic molecules in cerebral ischemia.

Keywords: Protein transduction, Poly-arginine, Brain, Ischemia

1. Introduction

Stroke is a leading cause of death and the principal cause of adult disability worldwide (1). To treat an ischemic stroke, current therapies utilize thrombolytic approaches to dissolve blood clots (2). These treatments restore blood flow and prevent further brain damage; however, there is no medicine that can treat brain damage and prevent neuronal cell death by directly targeting neuronal cells. One reason is the difficulty of crossing the blood-brain barrier (BBB), which is composed of a layer of specialized endothelial cells that segregate the brain from the circulating blood (3). Extensive efforts have been made to engineer and deliver biological active molecules to the brain across the BBB (4).

Among the delivery systems, a technology utilizing CPP has been emerging as a potential tool to pass though biological membranes (5-7). CPP consists of short peptides containing positively charged amino acids such as lysine and arginine. Protein transduction domains (PTDs) in the transactivator of transcription (TAT) protein of human immunodeficiency virus (HIV) and VP22 in herpes simplex virus 1 (HSV1) enable efficient intracellular transduction of peptides and proteins through a macropinocytosis-dependent mechanism (8). Subsequent studies have aimed to apply TAT-derived PTD for *in vivo* delivery

across the BBB, and it has been found that TAT-derived PTD is capable of transduction into the intact brain (9, 10); however, several groups have reported that TAT protein could not enter the intact brain using the same construct (11,12). Currently, whether TAT-derived PTD is capable of transduction to the intact brain is still controversial and improvement of the delivery system is required to achieve high efficiency. We previously reported that 11R was capable of intracellular delivery both *in vitro* and *in vivo*, such as to the mouse kidney and heart, more efficiently than TAT (13). Taken together, these studies suggest that 11R may be utilized as a potential tool to deliver biological active molecules to the brain across the BBB.

In this study, we investigated whether 11R could enter intact and ischemic brains across the BBB after systemic administration.

2. Materials and Methods

2.1. Peptide synthesis

All of the peptides used were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis on Rink amide resin, as described previously (14). Briefly, deprotection of the peptide and cleavage from the resin were achieved by treatment with a trifluoroacetic acid/ethanedithiol mixture (95:5) at room temperature for 3 h followed by reversed-phase high performance liquid chromatography (HPLC) purification. Fluorescent labeling of the peptides was conducted by treatment with fluorescein isothiocyanate (FITC; Invitrogen, Carlsbad, CA) in a dimethylformamide (DMF)/methanol mixture (1:1) for 1.5 h followed by HPLC purification. The structure of the products was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

The structure of each peptide was as follows:

1. 11R-FITC:

FITC-XRRRRRRRRRRR-amide X = ε-Acp

2. 11E-FITC

FITC-XEEEEEEEEEEE-amide X = ε-Acp

2.2. Animals studies

All animal procedures were approved by the Animal Ethics Committee of Kurume University and followed the Guide for the Care and Use of Experimental Animals of the university. C57BL/6 male mice aged 8 to 9 weeks old (20-24 g) were assigned to transient focal cerebral ischemia followed by systemic injection of peptides.

2.2.1. Systemic injection of peptides

Mice were anesthetized by inhaling 1.2% concentration of isoflurane in air under spontaneous breathing. 11R-FITC (0.2 μmol) or 11E-FITC (0.2 μmol) was dissolved in 20 μl saline and then injected into the right femoral vein.

2.2.2. Transient focal cerebral ischemia

Mice were anesthetized by inhaling 1.2% concentration of isoflurane in air under spontaneous breathing. The rectal temperature was controlled at 37.0 ± 0.5°C via a temperature-regulated heating pad during surgery. Focal cerebral ischemia was induced by occluding the middle cerebral artery (MCA) using the

intraluminal filament technique. The right common carotid artery (CCA) was exposed via a midline pretracheal incision. The external carotid artery (ECA) was ligated. The CCA was then ligated permanently and a small incision was made 1 mm distal to the ligation. A 6-0 nylon monofilament coated with silicone (Doccol Corporation, Sharon, MA, USA) was introduced through a small incision in CCA and advanced 9 mm distal to the carotid bifurcation. The wound was sutured and the animal returned to its cage. After a 120 min occlusion period, the mouse was reanesthetized and reperfusion was accomplished by withdrawing the intraluminal filament from the CCA. Immediately after reperfusion, 0.2 μmol 11R-FITC or 0.2 μmol 11E-FITC dissolved in 20 μl saline was injected into the right femoral vein.

2.2.3. Assessment of cerebral blood flow

To monitor regional cerebral blood flow (rCBF) continuously, a laser-Doppler flowmetry probe was fixed to the intact skull (2 mm posterior and 5 mm lateral to the bregma). Only mice whose rCBF showed a drop of over 70% from the baseline just after MCAO were included.

2.3. Immunohistochemistry

At 1, 2, 4, 8 h after peptide injection, mice were deeply anesthetized with an overdose of pentobarbital and perfused transcardially with heparinized physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, cut into 2 mm coronal sections, fixed in 4% paraformaldehyde in 0.1 M PBS for 4 h and immersed in 30% scrose in 0.1 M PBS overnight. Brains were embedded in OCT compound (Tissue-Tek; Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. Brain sections at a thickness of 10 μm were prepared using a cryostat. The sections were washed 3 times in 0.1 M PBS, and then immersed for 30 min in 1% vH_2O_2 and pre-blocked with 0.1%Ttriton X-100 and 1% bovine serum albumin (BSA) in PBS for 1 h. The brain sections were then incubated with goat anti-FITC polyclonal antibody (1:200; GeneTex, Irvine, CA, USA) overnight. Excess antibody was washed 4 times by PBS, and then the sections were incubated with biotinylated anti-goat IgG (H+L) (1:200; Vector Lab, Burlingame, CA, USA) for 2 h. The reaction products were visualized using an ABC kit (Nacalai Tesque, Kyoto, Japan) followed by DAB as a substrate. The livers were stained by the same immunohistochemical procedure.

For double-labeled immunofluorescence staining, sections were washed 3 times in 0.1 M PBS and pre-blocked with 0.1% Triton X-100 and 1% BSA in PBS for 1 h. The sections were then incubated with goat anti-FITC polyclonal antibody (1:200 dilution; GeneTex) and rabbit anti-NeuN polyclonal antibody (1:100 dilution; Bioss, Wobum, MA, USA) , rabbit ant-glial fibrillary acidic protein (GFAP) polyclonal antibody (1:1000 dilution; Imgenex; San Diego, CA, USA) or rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1) polyclonal antibody (1:1000 dilution; Wako; Osaka, Japan) overnight. Excess antibodies were washed 4 times, and then the sections were incubated with Alexa Fluor488 donky anti-goat IgG (H+L) (1:200 dilution; Life Tecnologies, Grand Island, NY, USA) and Alexa Fluor594 donky anti-rabbit IgG(H+L) (1:200; Invitrogen) for 2 h. Excess antibody was washed three times and the sections were observed under a confocal microscope (FV-1000; Olympus, Tokyo, Japan).

2.4. Optical density measurement

Quantification of the optical density (OD) of 11R-FITC immunoreactivity was performed using ImageJ software (National Institutes of Health, MD). The cortex and the striatum were outlined manually, and OD was automatically analyzed on

gray-scale images using a computer program. The baseline OD was quantified with brain slices of mice without 11R-FITC injection. Previous studies have shown that OD measurements reflect changes in protein expression parallel to those obtained using a biochemical protein assay (15, 16).

2.5. Statistical analysis

Data are shown as the mean (s.e.m.). Data were analyzed by ANOVA to compare means between multiple groups followed by post-hoc examination, and $p<0.05$ was considered significant.

3. Results

3.1. Transduction of 11R-FITC into the normal brain

To investigate whether 11R is capable of *in vivo* delivery into the intact brain across the BBB, mice were systemically administrated FITC-conjugated 11R (11R-FITC). Strong immunoreactivity was observed in the vessels of the cortex, striatum and thalamus and in the cells surrounding the vessels 1h after injection (Fig.1A). 11R-FITC was also efficiently delivered to the median eminence, where the BBB is not present (Fig.1A) (17, 18). A weak immunoreactive signal was observed in the cells of the cortex, striatum and thalamus at 4 h (Fig. 1B), and had almost disappeared throughout all regions in the brain 8 h after the injection (data not shown). To investigate whether the transduction of 11R-FITC was mediated by the poly-arginine motif, FITC-conjugated eleven poly-glutamate (11E-FITC) was systemically administrated into mice. 11E-FITC was not detected in any regions of the brain (Fig. 1C). These results suggest that 11R is capable of *in vivo* delivery to the cells surrounding vessels across the BBB, but cannot deliver extensively to the brain regions.

On the other hand, to observe the amount of trapped 11R-FITC in the liver, the liver was stained similarly to the brain. Strong immunoreactivity was

observed in the liver at 1, 2, 4, 8h after 11R-FITC administration (data not shown).

3.2. Transduction of 11R-FITC to neurons

To identify the cells receiving 11R-FITC in the normal brain, mice were systemically administrated 11R-FITC. At 4 h after injection, the localization of 11R-FITC was examined by immunostaining against a neuronal specific marker, NeuN. As expected, 11R-FITC was efficiently delivered to NeuN-positive neurons (Fig. 2A-C). Notably, 11R-FITC was also co-localized with GFAP, a marker protein of astrocytes in the striatum (Fig. 2D-F), suggesting that 11R could be delivered to both neuronal and non-neuronal cells across the BBB upon systemic administration. However, 11R-FITC was not co-localized with Iba1, a microglia/macrophage marker.

3.3. Transduction of 11R-FITC into the ischemic brain lesion

Upon successful delivery of 11R to the intact brain, we next investigated *in vivo* delivery into an ischemic lesion in the mouse brain (Fig. 3A). We performed MCA occlusion in mice for 2 h, and then systemically administrated 11R-FITC. At 1 h

after injection of 11R-FITC, strong immunoreactivity of 11R-FITC was observed in both contralateral and ipsilateral regions, such as the cortex and striatum of the brain (Fig. 3B); however, the localization of 11R-FITC was apparently different between the two regions. In the contralateral region, where the BBB was still intact after ischemia, 11R-FITC was mainly found in cells surrounding vessels, the same as in the normal brain, shown in Fig. 1 (Fig. 3B). On the other hand, on the ipsilateral side, where the BBB was opened by transient ischemia, 11R-FITC was intensively delivered to cells of the cortex and striatum in addition to the vessel areas (Fig. 3B). At 2 and 4 h after injection, strong immunoreactivity of 11R-FITC was observed in cells in the ipsilateral region, whereas 11R-FITC was faint on the contralateral side. Even at 8 h after injection, 11R-FITC was still detectable on the ipsilateral side with some leakage of 11R-FITC to the interstitium. In contrast, 11R-FITC had disappeared on the contralateral side 8 h after injection (Fig. 3B). To investigate whether 11R-FITC selectively crosses the BBB in a CPP-dependent manner, or just diffuses through altered the BBB during ischemic injury on the ipsilateral side, 11E-FITC was systemically administrated after transient ischemia. 11E-FITC was not detected in either contralateral or ipsilateral regions of the brain (Fig. 3C).

To confirm that 11R-FITC is not delivered to microglia/macrophage activated by ischemia, double-labeled immunofluorescence staining was performed with anti-Iba1 antibody. 11R-FITC was not co-localized with Iba1 at 4h after injection (Fig.4).

The kinetics of peptide transduction during ischemia was examined by measuring the optic density of 11R-FITC in cortex and striatum at each time point after the administration (Fig.5A-B). In both the cortex and striatum, immunoreactivity of 11R-FITC on the ipsilateral side was significantly higher than on the contralateral side ($p = 0.0043$ in cortex, $p = 0.0031$ in striatum). Although the time effect was not significant, on the ipsilateral side, 11R-FITC was at its highest at 1 h after injection in the cortex and remained detectable in the cortex and striatum for 8 h after administration.

4. Discussion

In the present study, we aimed to investigate whether 11R could cross the BBB and reach cells in a normal brain or with an ischemic injury. In the intact brain, 11R-FITC was observed throughout the brain, including the cortex, striatum and thalamus. Although 11R was highly localized in vessels, it was also detected in both neuronal and non-neuronal cells in the peripheral brain area surrounding vessels. These results suggest that 11R is capable of crossing the BBB and being delivered into the brain; however, the delivery efficiency of 11R in the intact brain is relative low when compared with that in non-neuronal regions (13, 19). The low efficiency of peptide delivery by 11R may be due to the unique biological properties of BBB as well as the pharmacokinetic properties of 11R. To prevent leakage of harmful molecules into the nervous system, the endothelial cells in the BBB are cemented together by tight junctions and have reduced macropinocytosis (20). Because CPP-mediated delivery is mainly through macropinocytosis (8), uptake of 11R would be thus inevitably attenuated in an intact BBB. In addition, a large amount of 11R may be metabolized by BBB-specific drug efflux transporters or peptidases (21). On the other hand, the low transduction efficiency of 11R could be also due to a decrease in the net

amount of circulating 11R, because we detected a considerable amount of 11R-FITC peptides trapped in the liver after systemic administration (data not shown). Nevertheless, the current results suggest that 11R-mediated delivery is a reliable tool for brain delivery compared with the controversial TAT-mediated delivery.

In this study, we confirmed the peptides composed of 11 amino acids conjugated only with FITC that molecular weight is 2239 Da to cross the BBB although it is generally accepted that a molecular weight threshold limiting BBB transport of molecules is 400-600 Da,. However, if some bigger therapeutic materials would be fused with 11R, they may exhibit a different kinetics. Therefore, respective researches whether they can cross the BBB are needed.

11R-mediated delivery was greatly enhanced in the ischemic model up to 8 h after systemic administration. Interestingly, 11R was not detected in large vessels on the ischemic side compared with the dense localization of 11R on the contralateral side. This may be caused by reduced cerebral blood flow on the ischemic side of the brain by microcirculatory disturbances and occlusion of CCA (22). The precise mechanism by which delivery of 11R was enhanced on the ischemic side is unclear. It is known that, in disease states, the BBB becomes

less restrictive to proteins or cells by enhanced transcytosis through altered tight junctions (20). Such leakage of the BBB could continue for up to several weeks following temporary focal ischemia (23). The enhanced uptake of 11R could be caused by a leaky BBB during transient ischemic injury, however, given the finding that poly-glutamate (11E) did not cross the BBB at all, it is unlikely that 11R crossed the BBB through a non-specific diffusion model. Rather, 11R was delivered through a highly regulated CPP-dependent manner in the ischemic brain.

In addition to the biological properties, the pathogenicity of PTD-mediated delivery needs to be taken into consideration for therapeutic utilization. Thus, consideration is particularly important when using virus-derived PTD such as TAT. Indeed, TAT has been shown to impair neuronal activity as well as learning and memory (24-26). Therefore, although some groups have used TAT to deliver proteins into an intact brain or ischemic regions of the brain (9,10,27), it is conceivable that TAT might cause unexpected side effects in the brain. In contrast to TAT, 11R is a synthetic peptide and has not been associated with any pathogenesis during in vivo administration (13).

Taken together, our results suggest that 11R may be utilized as a potential tool

to deliver therapeutic molecules to the brain for the treatment of ischemia.

5. Conclusion

In this study, we demonstrated that eleven poly-arginine (11R) could be delivered to the intact brain across the BBB upon systemic administration. Furthermore, the delivery was remarked enhanced and continued up to 8 h in the brain area affected by transient ischemic injury, suggesting the potential therapeutic application of 11R-mediated delivery.

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Figure Legends

Figure 1. Transduction of 11R-FITC into the intact mouse brain. (A) At 1h after administration of 11R-FITC, FITC signals were detected in the vessels and cells surrounding the vessels of the cortex, striatum and thalamus. Strong immunoreactivity was detected in the cells of the median eminence. Bars in a, b and c = 100 μm and d = 200 μm. (B) FITC signals 4 h after 11R-FITC injection in the cortex. Weak signals were observed in the cells. Bar = 50 μm. (C) Transduction of 11E-FITC. 11E-FITC was systemically administrated to mice. After 4 h, 11E-FITC was detected by immunohistochemistry using anti-FITC antibody. No FITC signals were observed in cells of the brain. Bars in a, b and c = 100 μm and d = 200 μm.

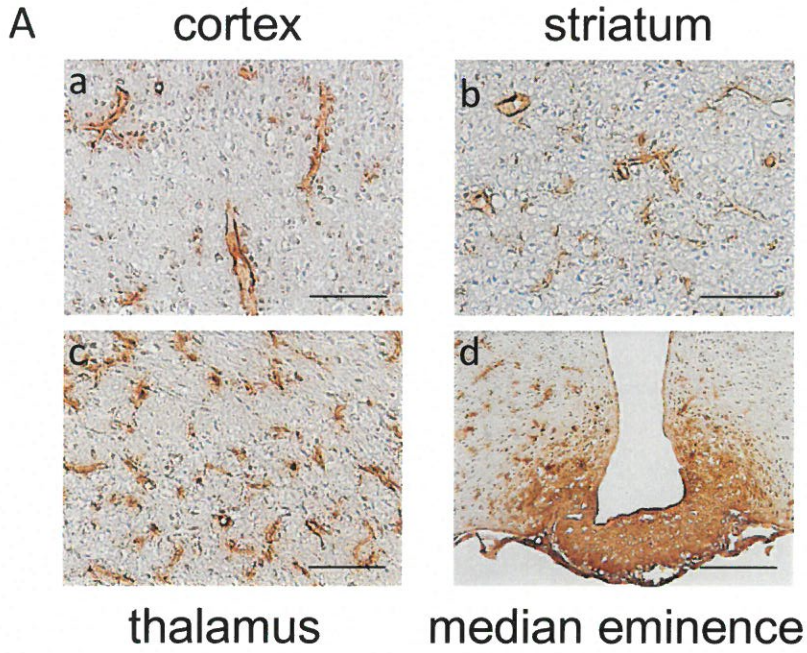
Figure 2. Transduction of 11R-FITC in neuronal and non-neuronal cells. Mice were systemically administrated with 11R-FITC. After 4h, mice were sacrificed and double-immunohistochemical analyses were performed. (A-C) Delivery of 11R-FITC to neurons of the cortex was examined using anti-FITC and anti-NeuN antibodies. Arrows indicate localization of 11R-FITC in NeuN-positive cells. Bars

= 50 μm. (D-F) Delivery of 11R-FITC to astrocytes of the striatum was examined using anti-FITC and -GFAP antibodies. Arrows indicate localization of 11R-FITC in GFAP-positive cells. Bars = 50 μm. (G-I) Delivery of 11R-FITC to microglia/macrophage of the cortex was examined using anti-FITC and anti-Iba1 antibodies. 11R-FITC was not co-localized with Iba1 positive cells. Bars = 50 μm.

Figure 3. Transduction of 11R-FITC into an ischemic lesion of the mouse brain. (A) Focal cerebral ischemia was induced by occlusion of MCA using the intraluminal filament technique. Areas a and b indicate the cortex and striatum on the contralateral side, respectively. Areas c and indicate the cortex and striatum on the ipsilateral side, respectively. (B) Localization of 11R-FITC in the cortex and striatum of contralateral and ipsilateral sides was examined 1, 2, 4 and 8 h after systemic administration. a and b, cortex and striatum on the contralateral side; c and d, cortex and striatum on the ipsilateral side. Bars = 200 μm. (C) After transient ischemic injury, mice were systemically administrated 11E-FITC. Four hours after injection, 11E-FITC in the cortex was examined by immunostaining. Bars = 200 μm.

Figure 4. Double-labeled immunofluorescence staining after ischemic injury. In an ischemic lesion of the brain, delivery of 11R-FITC to microglia/macrophage at 4h after ischemic injury was examined using anti-FITC and anti-Iba1 antibodies. 11R-FITC was not co-localized with Iba1 positive cells. Bars = 50 μm.

Figure 5. Statistical analysis of transduction of 11R in the cortex and striatum after ischemic injury. Optical density of 11R-FITC was measured at each time point shown in Fig. 3B. Significance was examined by two-way ANOVA. "B" means the baseline OD. n = 4-6.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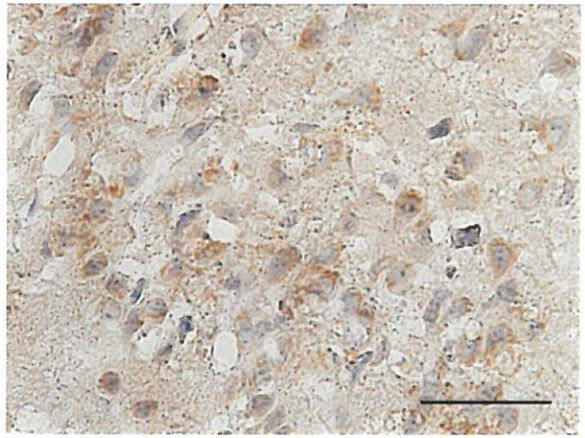
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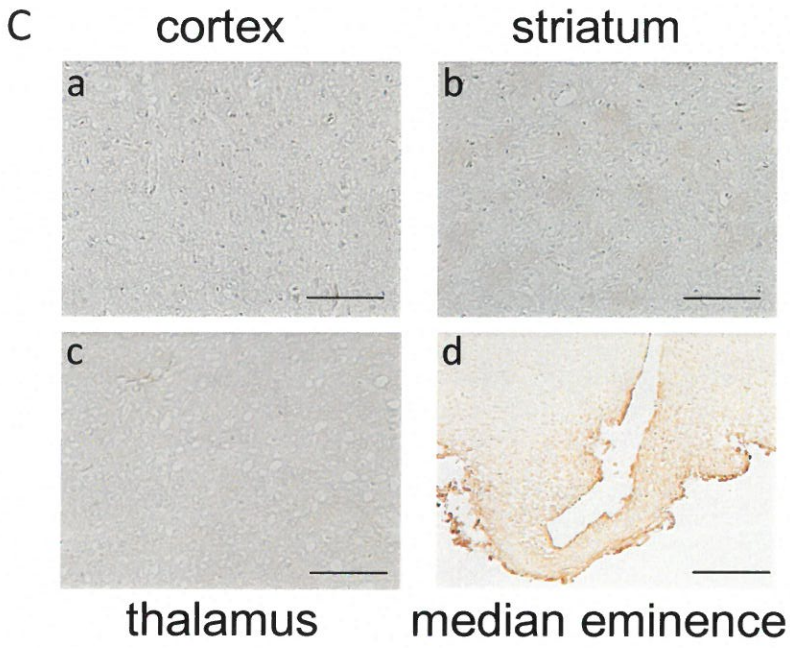
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Fig.1

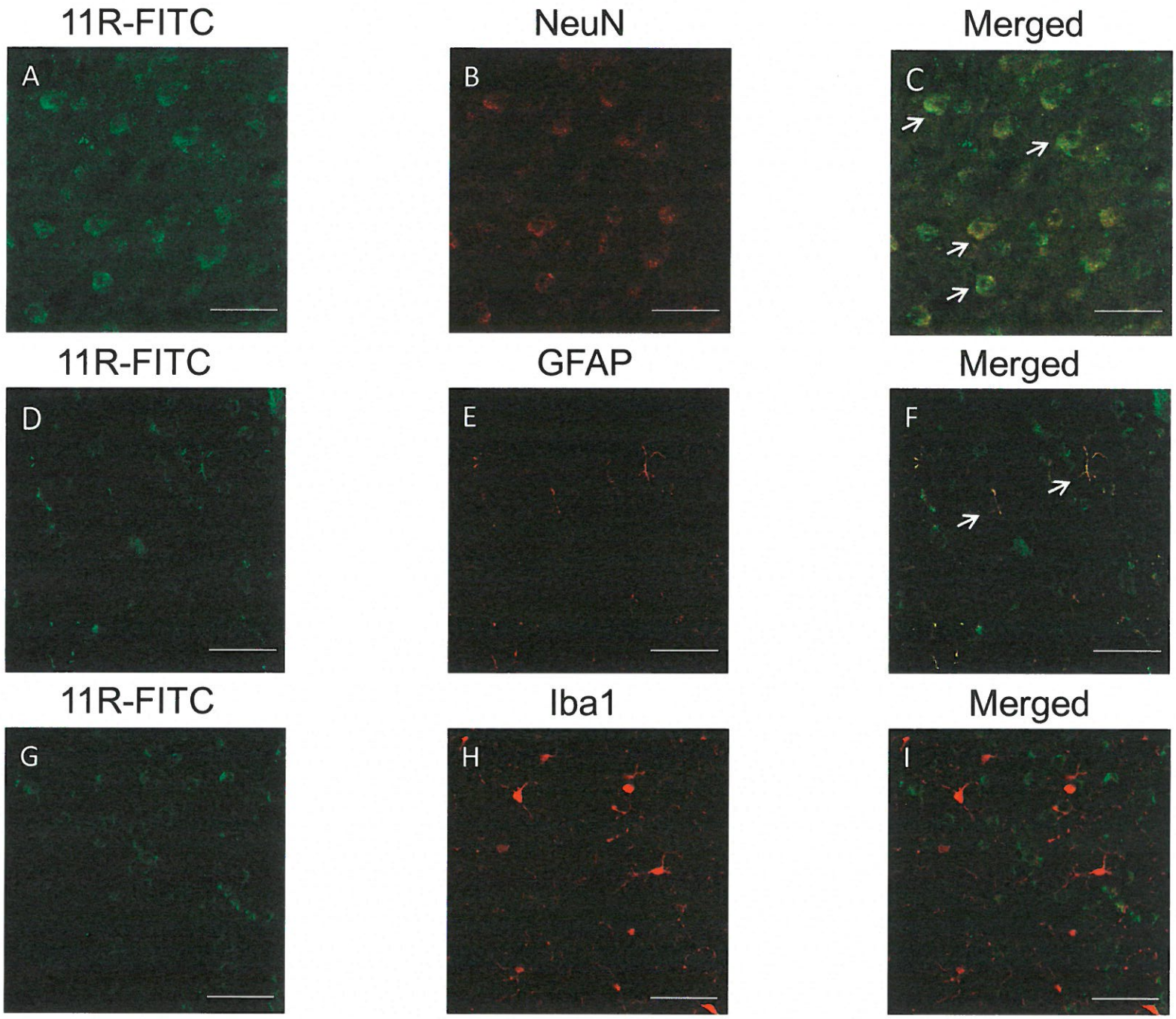
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Fig.2

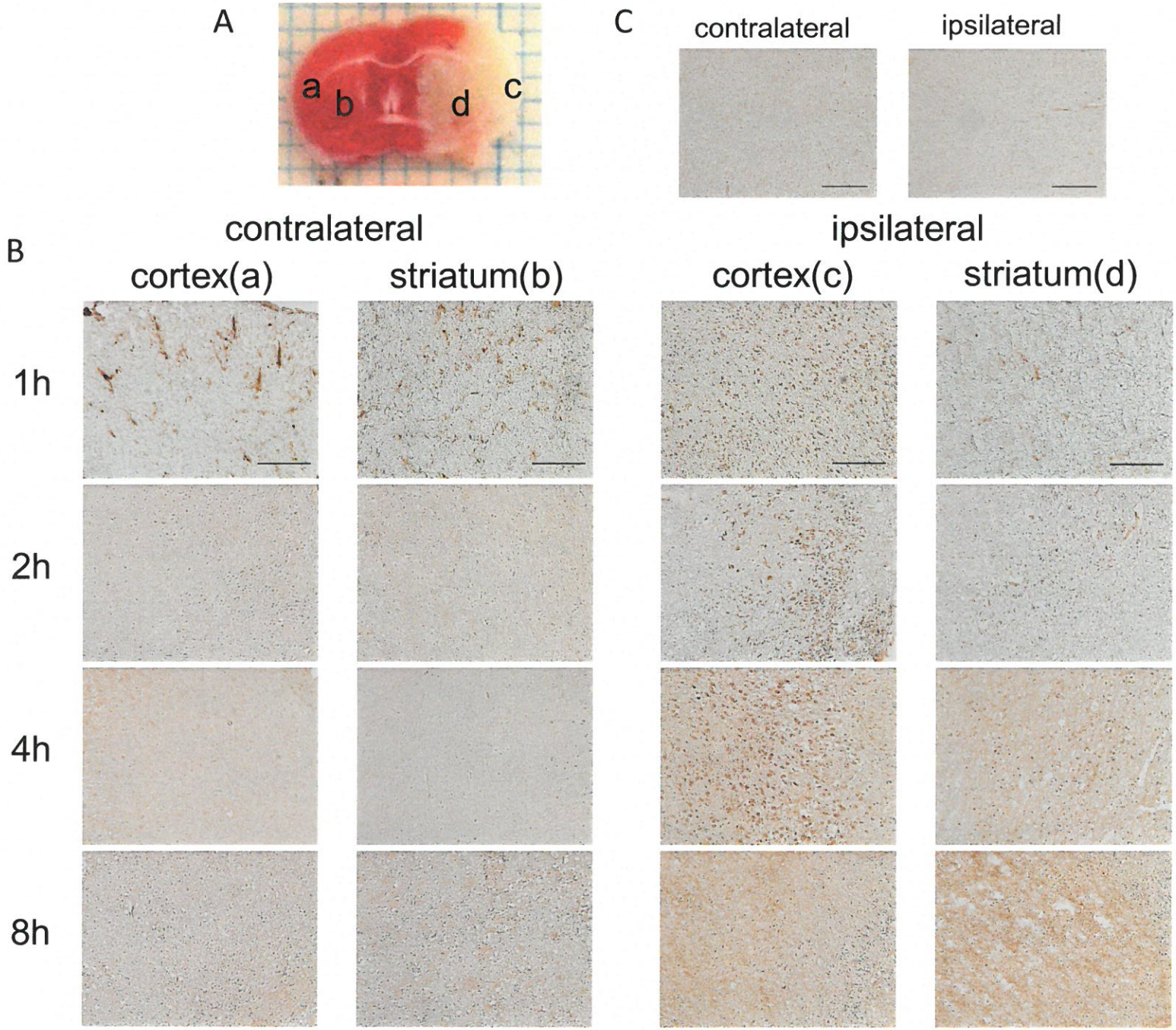
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Fig.3

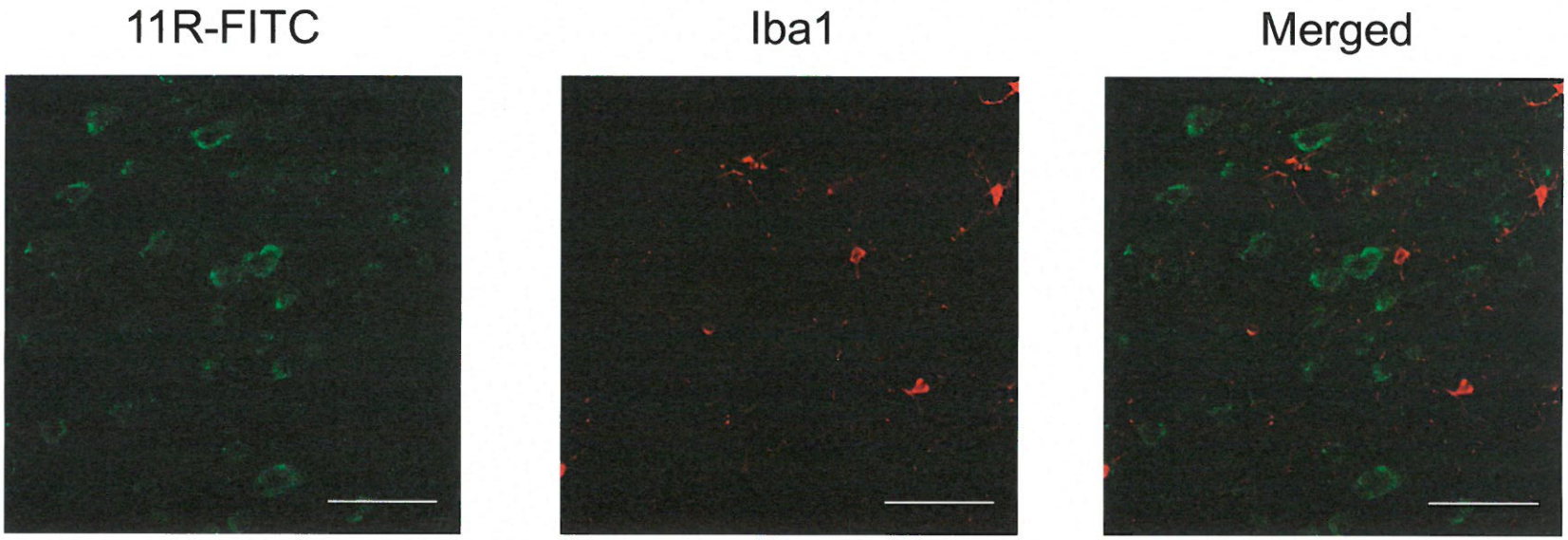

Fig.4

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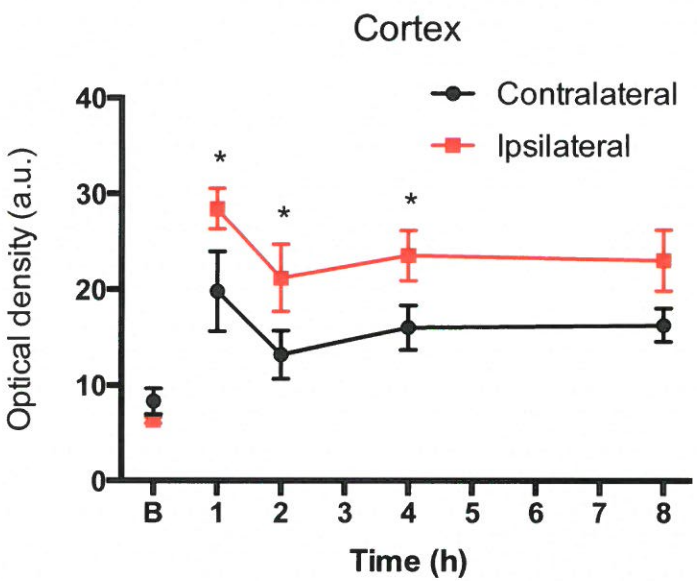

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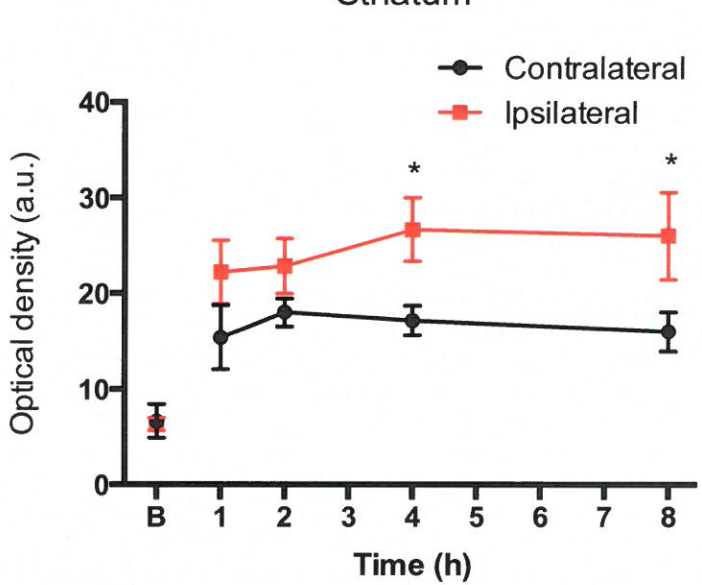
Time effect: P = 0.1

B



Region effect: P = 0.0006
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