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Cell Injury, Repair, Aging and Apoptosis

Up-Regulation of Cation-Independent Mannose 6-Phosphate Receptor and Endosomal-Lysosomal Markers in Surviving Neurons after 192-IgG-Saporin Administrations into the Adult Rat Brain

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The cation-independent mannose 6-phosphate receptor (CI-MPR) is a single transmembrane domain glycoprotein that plays a major role in the trafficking of lysosomal enzymes from the *trans*-Golgi network to the endosomal-lysosomal (EL) system. Because dysfunction of EL system is associated with a variety of neurodegenerative disorders, it is possible that the CI-MPR may have a role in regulating neuronal viability after toxicity/injury. In the present study, we report that 192-IgG-saporin-induced loss of basal forebrain cholinergic neurons causes a transient up-regulation of CI-MPR protein levels in surviving neurons of the basal forebrain and frontal cortex but not in the brainstem region, which was relatively spared by the immunotoxin. This was accompanied by a parallel time-dependent increase in other EL markers, ie, cathepsin D, Rab5, and LAMP2 in the basal forebrain region, whereas in the frontal cortex the levels of cathepsin D, and to some extent Rab5, were increased. Given the critical role of the EL system in the clearance of abnormal proteins in response to changing conditions, it is likely that the observed increase in the CI-MPR and components of the EL system in surviving neurons after 192-IgG-saporin treatment represents an adaptive mechanism to restore the metabolic/structural abnormalities induced by the loss of cholinergic neurons. ***(Am J Pathol 2006, 169:1140–1154; DOI: 10.2353/ajpath.2006.051208)***

The insulin-like growth factor-II (IGF-II) receptor, which is identical to the cation-independent mannose 6-phosphate receptor (CI-MPR), is a 250-kd multifunctional glycoprotein containing a large extracellular domain, a single transmembrane region, and a small cytoplasmic tail.[1–6] The receptor is widely expressed in various tissues including the brain and recognizes, via distinct sites, two different classes of ligands: M6P-containing molecules such as lysosomal enzymes, and IGF-II, a mitogenic polypeptide with structural homology to IGF-I and insulin.[2,7,8] A subpopulation of the CI-MPR is located at the plasma membrane, where it regulates internalization of IGF-II and various exogenous M6P-containing ligands for subsequent clearance or activation. However, the majority of the receptors are expressed in the *trans*-Golgi network/endosomal compartments and are involved in the intracellular trafficking of a battery of lysosomal enzymes including cathepsins B and D.[1,2,5,9,10] Given the evidence that defects in the synthesis/targeting of lysosomal enzymes or dysfunction of the endosomal-lysosomal (EL) system are associated with a variety of neurodegenerative disorders, often with progressive cognitive decline,[11–14] it is possible that the CI-MPR may have a role in regulating neuronal viability. In fact, a number of studies have shown that loss of CI-MPR function can induce cell proliferation in a variety of cancers.[15,16] Conversely, a protective role for the receptor has been suggested by two lines of evidence: cultured PC12 cells that are resistant to β-amyloid-mediated toxicity show an up-regulation of the CI-MPR[17] and overexpression or activation of the CI-MPR can block cell death induced by the mutant herpes simplex virus 1 or retinoic acid.[18,19] Nevertheless, very little is cur-

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rently known about the role of the CI-MPR in regulating neuronal viability after toxicity/injury or in any of the neurodegenerative disorders associated with dysfunction of the EL system.

Assimilated evidence suggests that CI-MPR protein and mRNA are widely distributed in the adult rat brain including cortex, striatum, and hippocampus.[20–25] At a cellular level, the receptor is localized primarily in neurons and their processes, although its presence on glial cells under normal condition has not been excluded.[24,25] A variety of experimental approaches, such as electrolytic lesioning of the entorhinal cortex[26] or intradentate injection of colchicine[27] have been shown to increase CI-MPR mRNA and/or its binding sites in selective layers of the hippocampal formation, whereas penetrating cortical injury[28] and cerebral ischemia[29,30] elevate receptor expression in neurons and/or glial cells only in the affected areas. Although these results underscore a role for the CI-MPR in lesion-induced plasticity, its association to the EL system, the major site of receptor action, remains to be defined. In addition, it is not clear whether increased levels of the receptor are associated with degenerating neurons and/or surviving neurons that undergo structural reorganization as a compensatory adjustments after surgical/pharmacological lesion.

We have recently reported that a subset of the CI-MP receptors are located in cholinergic as well as noncholinergic neurons in the basal forebrain region of the adult rat brain.[24] The majority of these forebrain cholinergic neurons express low-affinity neurotrophin receptors (p^{75NTR}), which are known to be selectively vulnerable to 192-IgG-saporin, a ribosomal toxin coupled to a monoclonal antibody against the rat p^{75NTR}. Noncholinergic cell groups of the basal forebrain and p^{75NTR}-negative cholinergic neurons remain unaffected by 192-IgG-saporin treatment.[31–33] This immunotoxin has been used extensively to study the behavioral and neurochemical sequelae of cholinergic hypofunction, but its influence on surviving neurons remains unclear.[32–36] In the present study, we report that 192-IgG-saporin-induced loss of basal forebrain cholinergic neurons is accompanied by a time-dependent increase in the levels of the CI-MPR as well as other markers of the EL system in neurons of the affected areas that survive the immunotoxin treatment. These results provide the very first evidence that upregulation of the CI-MPR and EL system may act as an adaptive mechanism to restore metabolic and structural abnormalities in neurons that survive toxicity/injury.

Materials and Methods

Materials

Adult male Sprague-Dawley rats (225 to 275 g; Charles River Canada, Montreal, QC, Canada) were used in all studies and handled in accordance with the University of Alberta policy on the handling and treatment of laboratory animals. 192-IgG-saporin was obtained from Advanced Targeting Systems (San Diego, CA). Polyacrylamide electrophoresis gels (4 to 20%) were purchased from Invitrogen (Burlington, ON, Canada), and the enhanced chemiluminescence kit was obtained from Amersham (Mississauga, ON, Canada). Polyclonal anti-choline acetyltransferase (ChAT) antiserum was from Chemicon Int. (Temecula, CA), and anti-cathepsin D, anti-lysosomal associated membrane protein 2 (LAMP2), and anti-Rab5 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Donkey anti-goat Texas Red, donkey anti-rabbit fluorescein isothiocyanate, and donkey anti-mouse fluorescein isothiocyanate-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Anti-actin, anti-vesicular acetylcholine transporter (VAChT), anti-microtubule-associated protein-2 (MAP2), and Hoechst 33258 were from Sigma (Mississauga, ON, Canada), and anti-ED1 was from Serotec (Raleigh, NC); anti-p^{75NTR} was from Promega (Madison, WI), and anti-GFAP was from Zymed (San Francisco, CA). Antiserum against the CI-MPR was a generous gift from Dr. R.G. MacDonald (University of Nebraska Medical Centre, Omaha, NE). All other reagents were from Sigma Chemical or Fisher Scientific (Montreal, QC, Canada).

Surgery

Rats were anesthetized by sodium pentobarbital (65 mg/kg i.p.) and mounted on a stereotaxic frame. Each animal received a bilateral injection of either 192-IgG saporin (0.4 μg/ml; 5 μl/ventricle) or an equivalent volume of saline through a 26-gauge Hamilton syringe into the lateral ventricles at coordinates anteroposterior -1.4 mm, mediolateral $+1.8$ mm, and dorsoventral -3.5 mm, relative to bregma. The cannula was left in place for 3 minutes after injection to allow for diffusion of the substrate. Animals were sacrificed at 4, 7, 14, 28, 60, and 90 days (10 to 12 animals per group) after surgery, and brain tissues were collected for Western blotting or immunohistochemistry as described earlier.[36]

Immunohistochemistry

Saline-treated control and 192-IgG-saporin-injected adult rats (four to six animals per group) were deeply anesthetized with 4% chloral hydrate and then perfused intracardially with phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.4), followed by 4% paraformaldehyde or Bouin's solution. Brains were sectioned (20 μm) on a cryostat and collected in a free-floating manner. Sections from the basal forebrain, frontal cortex, and brainstem areas were incubated overnight with anti-ChAT (1:250), anti-VAChT (1:250), or anti-CI-MPR (1:750), rinsed with PBS, exposed for 1 hour with anti-goat or anti-rabbit secondary antibody and developed using the enhanced glucose-oxidase method.[24] Sections were dehydrated, mounted with Permount, and then examined under bright field using a Zeiss Axioskop-2 microscope. For double-immunofluorescence labeling, tissue sections were incubated overnight with anti-CI-MPR (1:500) in combination with anti-ChAT (1:100), anti-cathepsin D (1:250), anti-GFAP (1:500), anti-ED1 (1:100), anti-Rab5 (1:250), anti-LAMP2 (1:250), or anti-MAP2 (1:500) antibodies. Other brain sections were incubated with anti-cathepsin

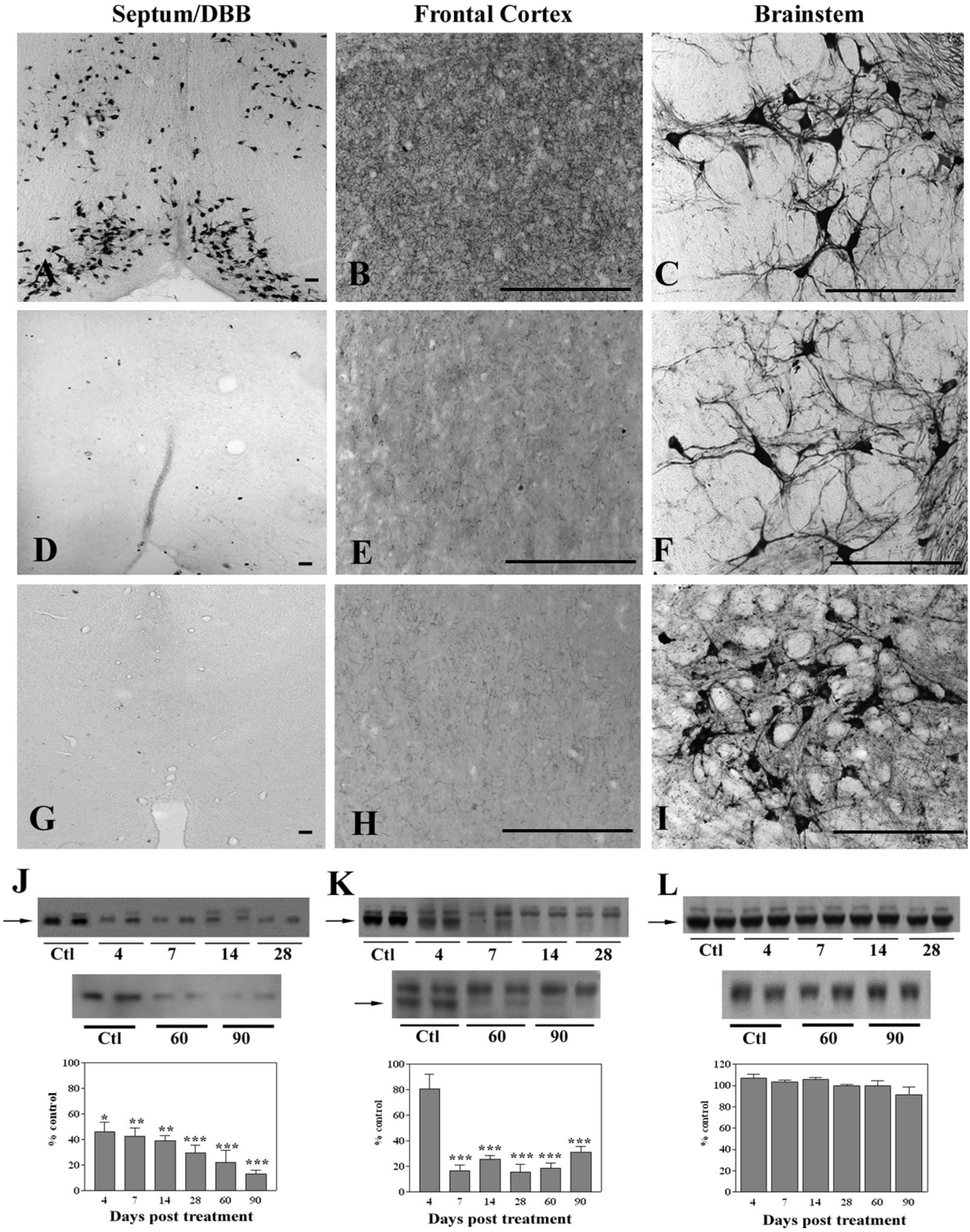


Figure 1. A–I: Photomicrographs showing the distribution profile of ChAT immunoreactivity in the septum/DBB (**A**, **D**, **G**), frontal cortex (**B**, **E**, **H**), and brainstem (**C**, **F**, **I**) of control animals (**A–C**), 14 days (**D–F**) and 60 days (**G–I**) after treatment with 192-IgG-saporin (**D–I**). A single bilateral intracerebroventricular injection of 192-IgG-saporin induced an almost complete loss of cholinergic neurons in the medial septum/DBB complex (**A**, **D**, **G**) and their fiber projections to the frontal cortex (**B**, **E**, **H**), whereas p^{75NTR}-negative cholinergic motoneurons in the brainstem remained unaffected (**C**, **F**, **I**). **J–L:** Western blots and histograms of the time-dependent decrease in ChAT levels at 4, 7, 14, 28, 60, and 90 days in the septum/DBB complex (**J**), frontal cortex (**K**), and brainstem (**L**) after administration of 192-IgG-saporin compared with saline-treated control (Ctl) rats. Western blot band used for quantification is marked with an **arrow**. Note the significant decrease in ChAT levels in the septum/DBB complex and frontal cortex but not in the brainstem of 192-IgG-saporin-treated animals. Histograms represent quantification of ChAT levels from at least three separate experiments, each of which was replicated three to four times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars = 10 μm.

Table 1. Summary of Changes in Various EL Markers at Different Time Points Following 192 IgG-Saporin Treatment

Days post-treatment	ChAT		CI-MPR		Cathepsin D		Rab5		LAMP2	
	Sep/DBB	Cortex	Sep/DBB	Cortex	Sep/DBB	Cortex	Sep/DBB	Cortex	Sep/DBB	Cortex
	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC	WB – IHC
4	↓ ↓	– ↓	↑↑ ↑↑	– ↑	– –	– –	↑↑ ↑↑	– –	↑↑ ↑↑	– –
7	↓↓ ↓↓	↓↓ ↓↓	↑↑ ↑↑	↑↑ ↑↑	– –	– –	↑↑ ↑↑	– –	↑↑ ↑↑	– –
14	↓↓ ↓↓	↓↓ ↓↓	↑↑ ↑↑	↑↑ ↑↑	↑↑ ↑↑	– –	↑↑ ↑↑	– –	↑↑ ↑↑	– –
28	↓↓ ↓↓	↓↓ ↓↓	↑↑ ↑↑	↑↑ ↑↑	↑↑ ↑↑	↑↑ ↑↑	↑↑ ↑↑	– –	↑↑ ↑↑	– –
60	↓↓ ↓↓	↓↓ ↓↓	– –	– –	– –	– –	– –	– –	– –	– –
90	↓↓ ↓↓	↓↓ ↓↓	– – –	– –	– –	– –	– –	– –	– –	– –

ChAT, choline acetyltransferase; CI-MPR, cation-independent mannose 6-phosphate receptor; IHC, immunohistochemistry; LAMP2, lysosomal associated membrane protein 2; Sep/DBB, setum/diagonal band of Broca; WB, western blotting; ↓ moderate decrease; ↓↓ significant decrease; ↑ moderate increase; ↑↑ significant increase; – no alteration.

D (1:250), anti-Rab5 (1:250), or anti-LAMP2 (1:250) in combination with anti-GFAP (1:500) or anti-ED1 (1:100). In addition, some brain sections were exposed to a combination of anti-ChAT (1:1000) or anti-VAChT (1:250) with anti-cathepsin D (1:250), anti-p^{75NTR} (1:250), anti-Rab5 (1:250), or anti-LAMP2 (1:250) antibodies. After incubation in primary antibody, sections were rinsed three times with PBS and then exposed to the appropriate fluorescent secondary antibodies for 2 hours. To determine whether CI-MPR was expressed in surviving and/or dying neurons, some brain sections from control and 192-IgG-saporin-treated animals were incubated with anti-CI-MPR antiserum and then stained with 500 ng/ml Hoechst 33258 for 20 minutes. Sections were then coverslipped and examined under a Zeiss Axioskop-2 fluorescence microscope.

Western Blotting

Control and treated animals from different groups (six animals per group) were decapitated, their brains rapidly removed, and areas of interest [ie, septum/diagonal band of Broca (DBB), cortex, and brainstem] were dissected out and homogenized in RIPA lysis buffer [20 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 1 mmol/L ethylenediaminetetraacetic acid, 1% Igepal CA-630, 50 mmol/L NaF, 1 mmol/L $NaVO_3$, 10 μg/ml leupeptin, and 10 μg/ml aprotinin]. Proteins from the brain homogenates were separated by 4 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, blocked with 8% nonfat milk, and incubated overnight at 4°C with anti-ChAT (1:500), anti-CI-MPR (1:10 000), anti-cathepsin D (1:500), anti-Rab5 (1:5000), or anti-LAMP2 (1:500) antibodies. Membranes were then incubated for 1 hour at 22°C with the appropriate secondary antibody and visualized using an enhanced chemiluminescence detection kit. Blots were stripped and reprobed with anti-actin (1:1000) to ensure equal protein loading. All blots were quantified using an MCID image analysis system as described earlier,[24] and the data presented as mean ± SEM were analyzed using one-way analysis of variance followed by Newman-Keuls posthoc analysis with significance set at $P < 0.05$.

Results

192-IgG-Saporin and Cholinergic Neurons

The immunotoxin 192-IgG-saporin was well tolerated by adult male rats with no fatalities or significant weight loss throughout the 90-day experimental paradigm. As expected, the toxin induced an extensive bilateral loss of ChAT-immunoreactive cell bodies in the basal forebrain areas [ie, septum, vertical and horizontal limbs of DBB, and nucleus basalis magnocellularis] from day 4 after injection onwards (Figure 1, A, D, and G; Table 1). A few

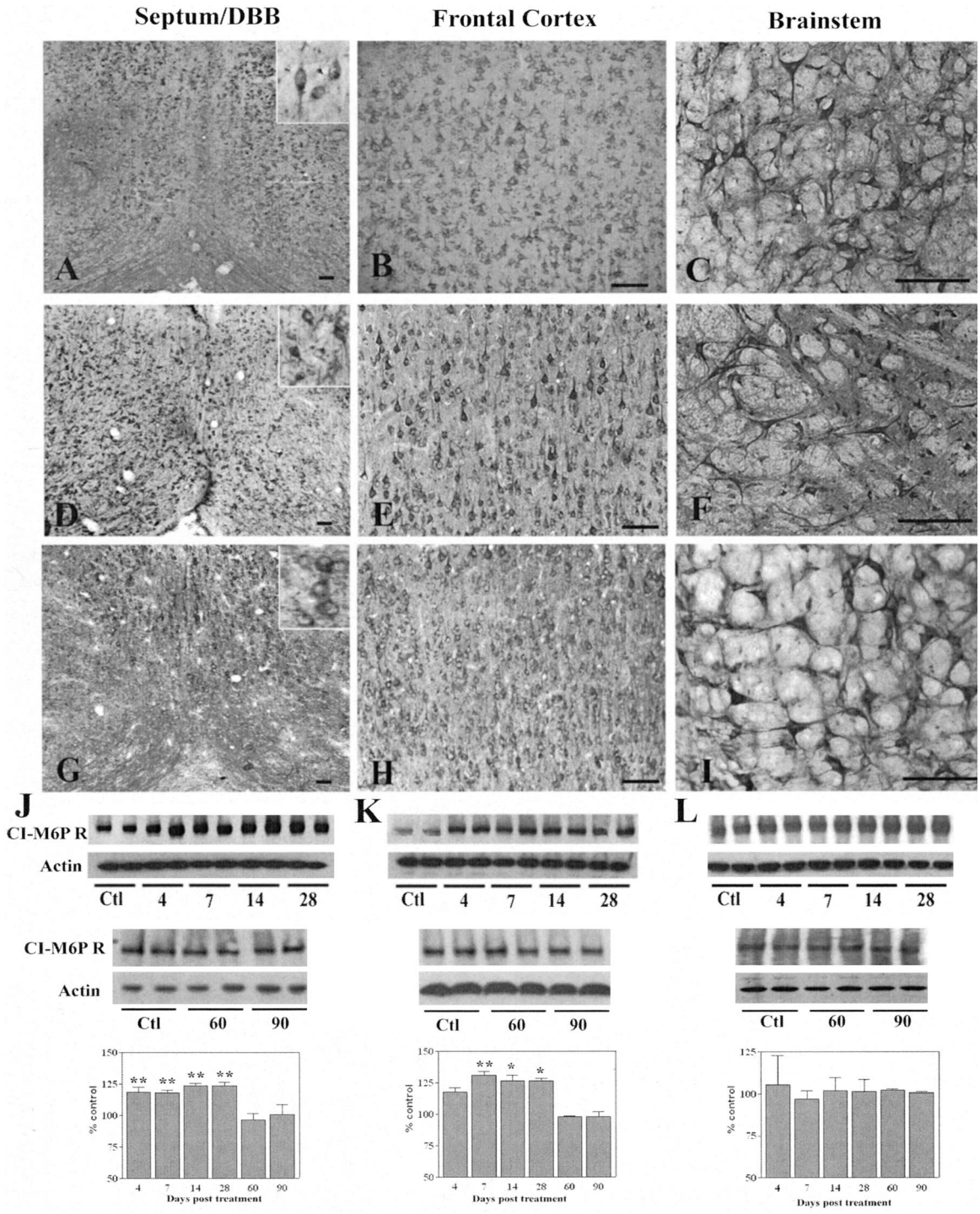


Figure 2. A–I: Photomicrographs of cation-independent mannose 6-phosphate receptor (CI-MPR) immunoreactivity in the septum/DBB (**A**, **D**, **G**), frontal cortex (**B**, **E**, **H**), and brainstem (**C**, **F**, **I**) of control animals (**A–C**), 14 days (**D–F**) and 60 days (**G–I**) after 192-IgG-saporin-treatment (**D–I**). **Insets** in **A**, **D**, and **G** show basal forebrain neuronal labeling at higher magnification. **J–L:** Western blots and histograms showing the increase in CI-MPR levels in the septum/DBB complex (**J**) and frontal cortex (**K**) throughout 4 to 28 days after injection, after which receptor levels returned to normal by 60 and 90 days after treatment compared with saline-treated control (Ctl) rats. CI-M6P receptor levels in the brainstem (**L**) were not altered at any time after 192-IgG-saporin administration. Histograms represent quantification of CI-MPR levels from at least three separate experiments, each of which was replicated three to four times. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Scale bars = 10 μm.

residual ChAT-positive cholinergic neurons were evident in the nucleus basalis magnocellularis but not in other areas of the basal forebrain. The degeneration of cholinergic neurons in the basal forebrain region was accompanied by a concomitant loss of ChAT-positive fibers in the frontal cortex throughout the 90-day experimental paradigm (Figure 1, B, E, and H; Table 1). However, the cholinergic motoneurons of the brainstem, which do not

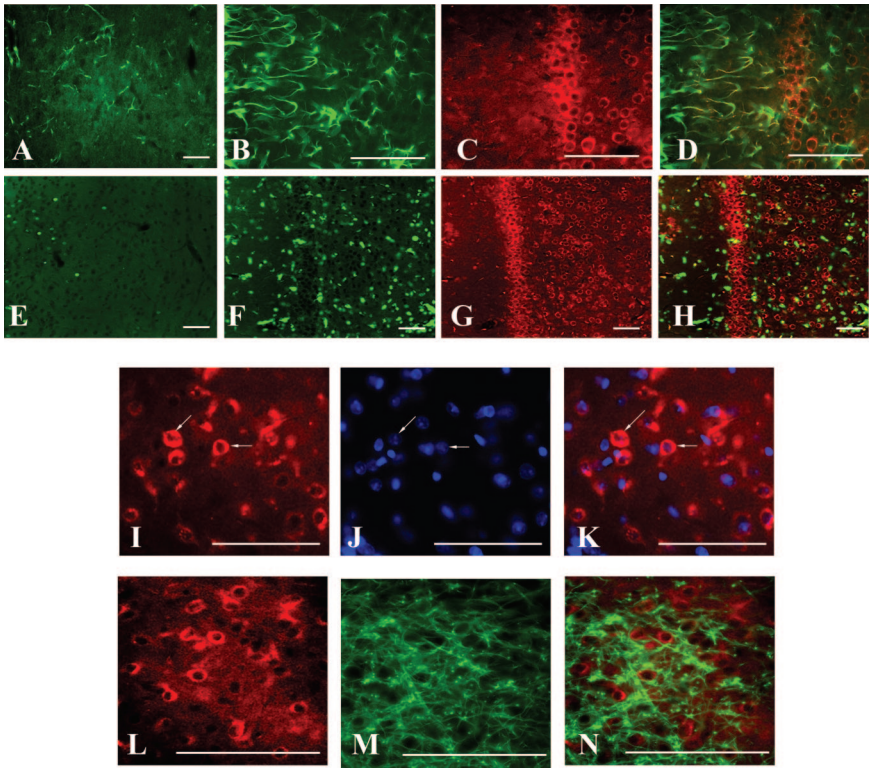


Figure 3. A–H: Photomicrographs of the basal forebrain region showing GFAP (**A**, **B**) and ED1 (**E**, **F**) immunoreactivity in the control rat (**A**, **E**) and their association with immunoreactive CI-MPR (**C**, **G**) at 14 days after treatment with 192-IgG-saporin. Note that CI-MPR-immunoreactivity (**C**, **D**, **G**, **H**) is not expressed on GFAP-positive astrocytes (**B**, **D**) or ED1-positive microglia (**F**, **H**) in the basal forebrain after administration of 192-IgG-saporin. **I–N:** Photomicrographs of CI-MPR immunoreactivity (**I**, **L**), in conjunction with Hoechst 33258 staining (**J**, **K**) and MAP2 immunoreactivity (**M**, **N**) in the basal forebrain region of 192-IgG-saporin-treated animals, 14 days after injection. Note that neurons that express high levels of CI-M6PR (**I**, **K**, **L**, **N**) do not show nuclear fragmentation (**J**, **K**) or degeneration of dendritic processes (**M**, **N**). Scale bars = 10 μm.

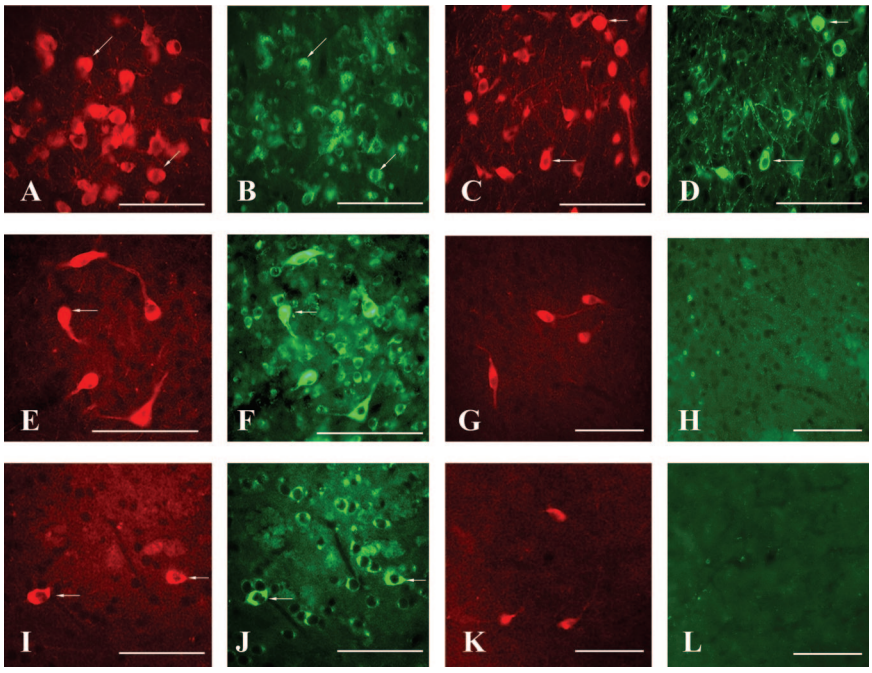
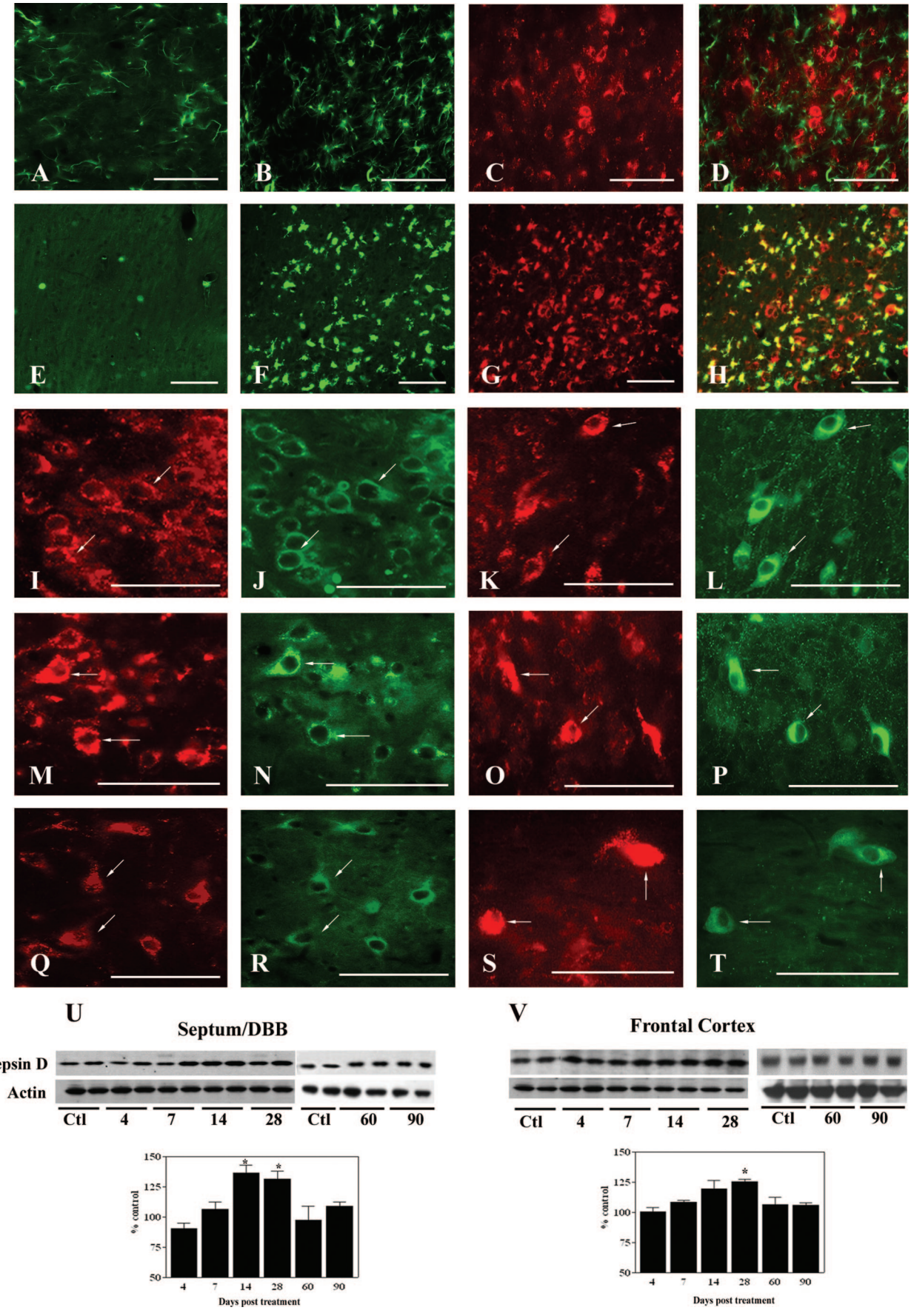


Figure 4. A, **B**, **E**, **F**, **I**, **J:** Immunofluorescence photomicrographs showing the co-localization of ChAT (**A**, **E**, **I**) and the CI-MPR (**B**, **F**, **J**) in the basal forebrain region of control (**A**, **B**) animals at 14 (**E**, **F**) and 60 days (**I**, **J**) after treatment with 192-IgG-saporin. Note the relative increase in the CI-MPR expression in ChAT-positive neurons after 14 days of treatment with 192-IgG-saporin (**E**, **F**), which returns to control levels by 60 days (**I**, **J**) after treatment. **C**, **D**, **G**, **H**, **K**, **L:** Photomicrographs depicting the distribution of ChAT-positive (**C**, **G**, **K**) neurons and those expressing the low-affinity neurotrophin receptor (p^{75NTR}) (**D**, **H**, **L**) in the basal forebrain of control rat brains (**C**, **D**), 14 days (**G**, **H**) and 60 days (**K**, **L**) after 192-IgG-saporin treatment. Note the co-localization of ChAT and p^{75NTR} immunoreactivities (**arrows**) in the basal forebrain region of the control brain (**C**, **D**). The few ChAT-positive cholinergic neurons that survive immunotoxin treatment do not express p^{75NTR} immunoreactivity (**G**, **H**, **K**, **L**).

express the p^{75NTR}, were unaffected by 192-IgG-saporin treatment, as reported in other studies (Figure 1, C, F, and I).[31,32] These immunohistochemical results were supplemented by Western blot data showing a significant reduction in ChAT enzyme levels in the septum/DBB (Figure 1J) and frontal cortex (Figure 1K) but not in the brainstem (Figure 1L) from 7 days onwards after administration of 192-IgG-saporin (Table 1).

192-IgG-Saporin and CI-MPR

To determine the possible alterations in CI-MPR levels after administration of 192-IgG-saporin, we first established the localization of the receptor in the basal forebrain, frontal cortex, and brainstem regions of saline-treated control rats. Our immunohistochemical experiments revealed that CI-MPR, as reported earlier,[24,25] ex-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hibits a widespread distribution in the aforesaid brain regions, with relatively high immunoreactivity in the medial septum, DBB, nucleus basalis magnocellularis, deep cortical layers, and the brainstem nuclei (Figure 2, A–C). In keeping with our earlier study,[24] receptor labeling in the cortex was evident in most layers with varying degrees of intensity, ie, high in layers IV to VI, moderate in layers II to III, and almost absent in layer I. To evaluate the influence of 192-IgG-saporin treatment on CI-MPR receptor levels, we performed immunohistochemical staining and Western blot analysis using a specific CI-MPR antiserum.[24] Our results clearly show that CI-MPR immunoreactivity was enhanced in both neuronal cell bodies, dendrites, and axons, in the medial septum/DBB (Figure 2D), in nucleus basalis magnocellularis, and throughout the frontal cortex (Figure 2E) from days 4 to 28 after injection and then returned to levels similar to saline-treated control rats by day 60 of 192-IgG-saporin administration (Figure 2, G and H; Table 1). The CI-MPR staining in the brainstem, however, remained unchanged throughout the 90-day experimental period (Figure 2, C, F, and I). These findings were supported by our Western blot analysis, which revealed a significant increase in receptor levels from 4 to 28 days in the septum/DBB (Figure 2J) and from 7 to 28 days in the frontal cortex (Figure 2K) of 192-IgG-saporin-treated rats compared with saline-treated control rats (Table 1). By contrast, receptor levels were not significantly altered in the brainstem region of the immunotoxin-treated rats at any time during the experimental paradigm (Figure 2L).

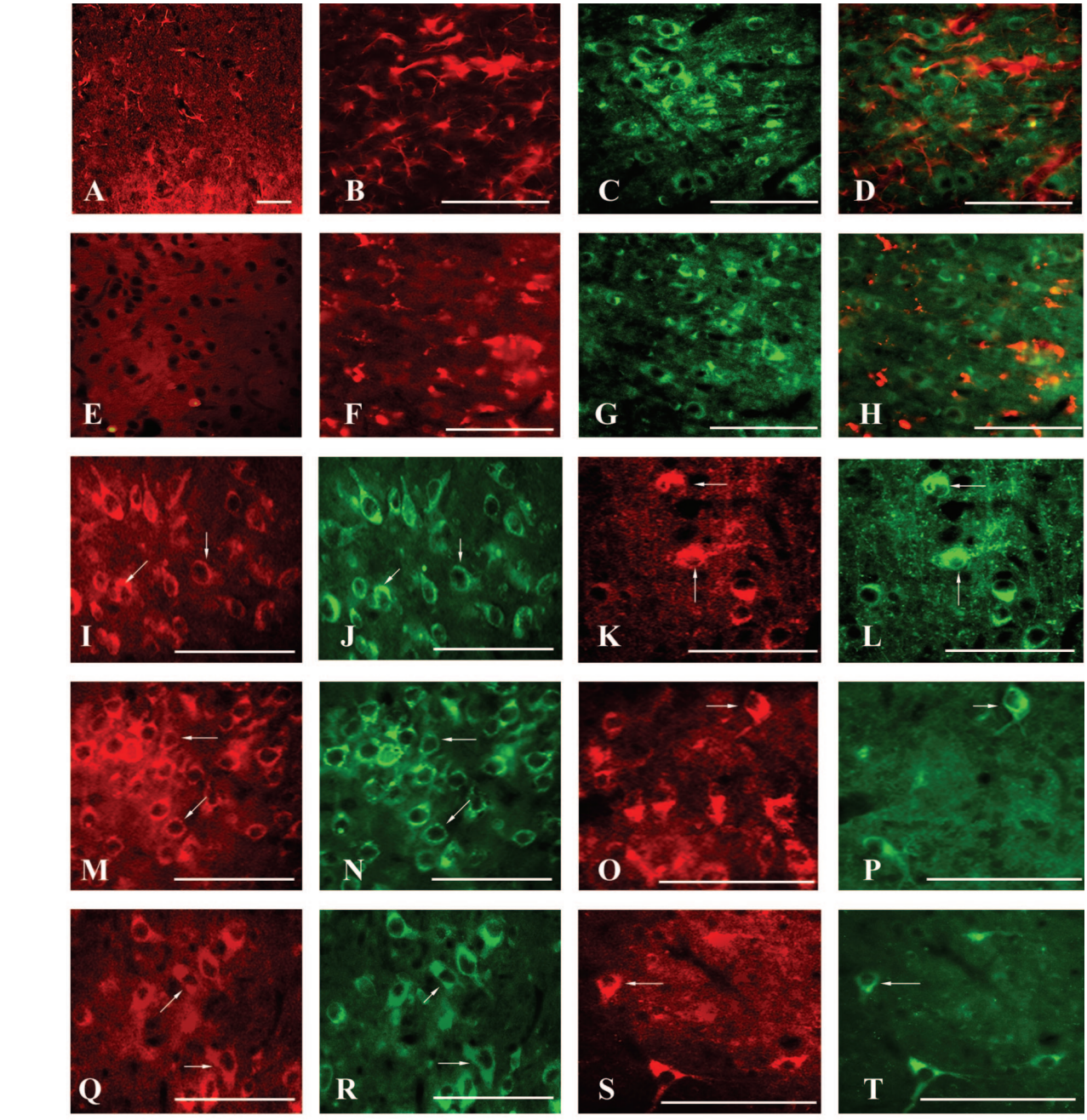
Given the evidence that glial cells are activated after 192-IgG-saporin-induced death of the basal forebrain cholinergic neurons,[37,38] we sought to determine whether the increase in CI-MPR levels is associated with either reactive astrocytes or microglia in 14-day post-treated rats. Our results clearly showed that both GFAP-positive reactive astrocytes (Figure 3, A and B) and ED1-positive activated microglia (Figure 3, E and F) were evident in the basal forebrain, but not in the cortical region (data not shown), of the immunotoxin-treated rats. Additionally, double-labeling experiments revealed that neither reactive astrocytes (Figure 3, B–D) nor microglia (Figure 3, F–H) expressed CI-MPR immunoreactivity in the basal forebrain region of the treated rats. In subsequent experiments, using nuclear marker for apoptosis Hoechst 33258[39] (Figure 3, I–K) and the neuronal marker MAP2 (Figure 3, L–N), we found that increased CI-MPR expression is associated with surviving neurons.

To evaluate CI-MPR alteration in relation to the cholinergic system, we first determined receptor expression on cholinergic neurons in the saline-treated animals. As previously reported,[24] virtually all ChAT-positive cholinergic neurons and fibers of the basal forebrain (Figure 4, A and B) and brainstem region expressed CI-MPR immunoreactivity. In the frontal cortex, co-localization was evident primarily in the fibers, whereas many CI-MPR-positive neurons were located throughout the cortex without any apparent ChAT immunoreactivity (data not shown). Interestingly, after treatment with 192-IgG-saporin, a few basal forebrain cholinergic neurons located in the ventral pallidum and ventral to the lenticular nucleus, which survived throughout the experimental paradigm, demonstrated a strong increase in receptor immunoreactivity until 28 days after treatment (Figure 4, E and F) and then returned to levels similar to that of the saline-treated control rat by day 60 (Figure 4, I and J) of the immunotoxin administration. To determine whether these ChAT-positive cholinergic neurons express p^{75NTR}, we subsequently performed double-labeling experiments in both control and 192-IgG-saporin-treated rats. Our results showed that most of the cholinergic neurons in control rat brain, as reported earlier,[31–33] express p^{75NTR} (Figure 4, C and D). However, the cholinergic neurons that survived the immunotoxin treatment were found not to express the p^{75NTR} (Figure 4, G, H, K, and L). Additionally, CI-MPR-positive cholinergic neurons in the brainstem, which are also p^{75NTR}-negative,[31,34] did not show any significant alterations in receptor expression in the treated rats.

192-IgG-Saporin and Endosomal-Lysosomal Markers

To determine whether increases in CI-MPR levels after 192-IgG-saporin treatment is associated with a parallel change in other EL proteins, we evaluated the expression and levels of cathepsin D—an enzyme that is transported primarily by the CI-MPR (Figure 5).[5,9] At the cellular level, cathepsin D immunoreactivity was evident in a wide spectrum of neurons, but not in glial cells, in both the basal forebrain and frontal cortex of saline-treated animals. Double-immunolabeling experiments also demonstrated the co-localization of cathepsin D with CI-MPR- and ChAT-positive neurons within the basal forebrain (Figure 5, I–L) and frontal cortex of the control rats. After 192-IgG-saporin treatment, the apparent intensity of immunoreactive cathepsin D was found to be transiently enhanced in cortical neurons as well as surviving non-cholinergic and p^{75NTR}-negative cholinergic neurons in the basal forebrain region between 14 to 28 days after treatment (Figure 5, M–P) and then returned to control levels by day 60 of treatment (Figure 5, Q–T; Table 1). In addition, cathepsin D immunoreactivity was found to be expressed in all ED1-positive microglia (Figure 5, F–H), but not astrocytes (Figure 5, B–D), in the basal forebrain region of the immunotoxin-treated animals. The observed

Figure 5. A–H: Double-labeling immunofluorescence photomicrographs showing GFAP (**A**, **B**) and ED1 (**E**, **F**) immunoreactivity in the control rat (**A**, **E**) and their possible association with immunoreactive cathepsin D (**C**, **G**) at 14 days after treatment with 192-IgG-saporin. Note that although there is no cathepsin D immunoreactivity (**C**, **D**) in GFAP-positive astrocytes (**B**, **D**), all ED1-positive microglia (**F**, **H**) express cathepsin D (**G**, **H**). **I–T:** Photomicrographs of cathepsin D co-localization (**I**, **M**, **Q**, **K**, **O**, **S**) with the CI-MPR-positive (**J**, **N**, **R**) and vesicular acetylcholine transporter (VAChT)-positive neurons (**L**, **P**, **T**) in the basal forebrain region of control animals (**I–L**) at day 14 (**M–P**) and day 60 (**Q–T**) after administration of 192-IgG-saporin. Cathepsin D expression is transiently up-regulated in the CI-MPR- and VAChT-positive neurons after 192-IgG-saporin treatment. **U** and **V:** Western blots and histograms showing the time-dependent increase in cathepsin D levels in the septum/DBB complex (**U**) and frontal cortex (**V**) at 4, 7, 14, 28, 60, and 90 days after treatment with 192-IgG-saporin compared with saline-treated control (Ctl) rats. $^*P < 0.05$. Scale bars = 10 μm.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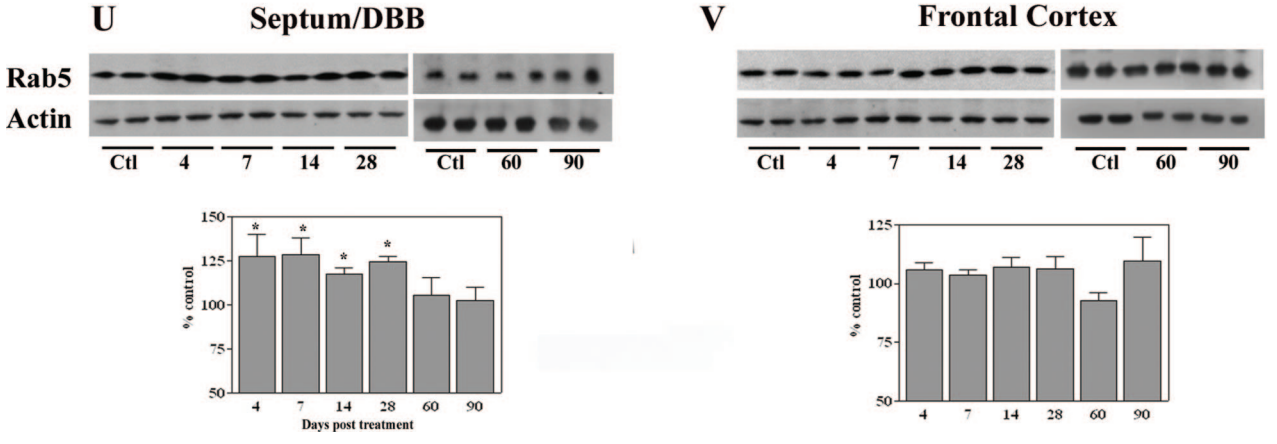

U
Septum/DBB
V
Frontal Cortex
Rab5
Actin
Ctl
4
7
14
28
Ctl
60
90
% control
Days post treatment

alteration in the enzyme level was supported by our Western blot analysis, which showed only a transient increase in cathepsin D levels in the septum/DBB (ie, 14 and 28 days) and frontal cortex (ie, 28 days) after 192-IgG-saporin treatment (Figure 5, U and V; Table 1).

To establish whether increased CI-MPR/cathepsin D levels in 192-IgG-saporin-treated rats reflect altered activity of the EL system, we subsequently measured the expression/levels of the early endosomal marker Rab5 (Figure 6, A–V; Table 1) and the lysosomal marker LAMP2 (Figure 7, A–V; Table 1). Rab5 immunoreactivity was not apparent in glial cells under normal conditions (Figure 6, A and E). However, the expression of Rab5 was widespread throughout the frontal cortex and basal forebrain in saline-treated rats, co-localizing with both CI-MPR- and ChAT-positive neurons (Figure 6, I–L). 192-IgG-saporin administration did not induce Rab5 expression in the reactive astrocytes (Figure 6, C and D) or microglia (Figure 6, G and H) in the septum/DBB complex of the treated rats. Interestingly, the intensity of Rab5 immunoreactivity was found to be enhanced transiently in the septum/DBB complex from 4 to 28 days after treatment with 192-IgG-saporin in surviving p^{75NTR}-negative cholinergic neurons and noncholinergic neurons (Figure 6, M–T; Table 1). In the frontal cortex, Rab5 immunoreactivity was found to be slightly increased only at 14 and 28 days after treatment with the immunotoxin. This was substantiated by Western blot data showing a significant increase in Rab5 levels in the septum/DBB and only a slight increase in the frontal cortex until 28 days after treatment, which then returned to control levels by day 60 after 192-IgG-saporin administration (Figure 6, U and V; Table 1). The expression of LAMP2 in the control rat brain was not apparent in the glial cells (Figure 7, A and E) but was located in neurons within the frontal cortex and basal forebrain area. Our double-immunolabeling results further indicate that LAMP2 was localized in CI-MPR- and ChAT-positive neurons in all brain areas examined (Figure 7, I–L). Administration of 192-IgG-saporin did not induce LAMP2 expression in either reactive astrocytes (Figure 7, C and D) or microglia (Figure 7, G and H) in the septum/DBB complex of the treated rats. However, the intensity of LAMP2 staining was found to be increased in neurons throughout the basal forebrain (Figure 7, M–P), but not in the frontal cortex, from days 4 to 28 after treatment and then returned to levels similar to saline-treated control rats by day 60 after immunotoxin administration (Figure 7, Q–T; Table 1). This is supplemented by Western blot data showing a significant increase in LAMP2 levels in the septum/DBB complex from 4 to 28 days after treatment, which subsequently returned to control levels by 60 days after 192-IgG-saporin administration (Figure 7U). No significant alteration in LAMP2 was apparent in the cortex of the treated animals at any time throughout the 90-day experimental paradigm (Figure 7V; Table 1).

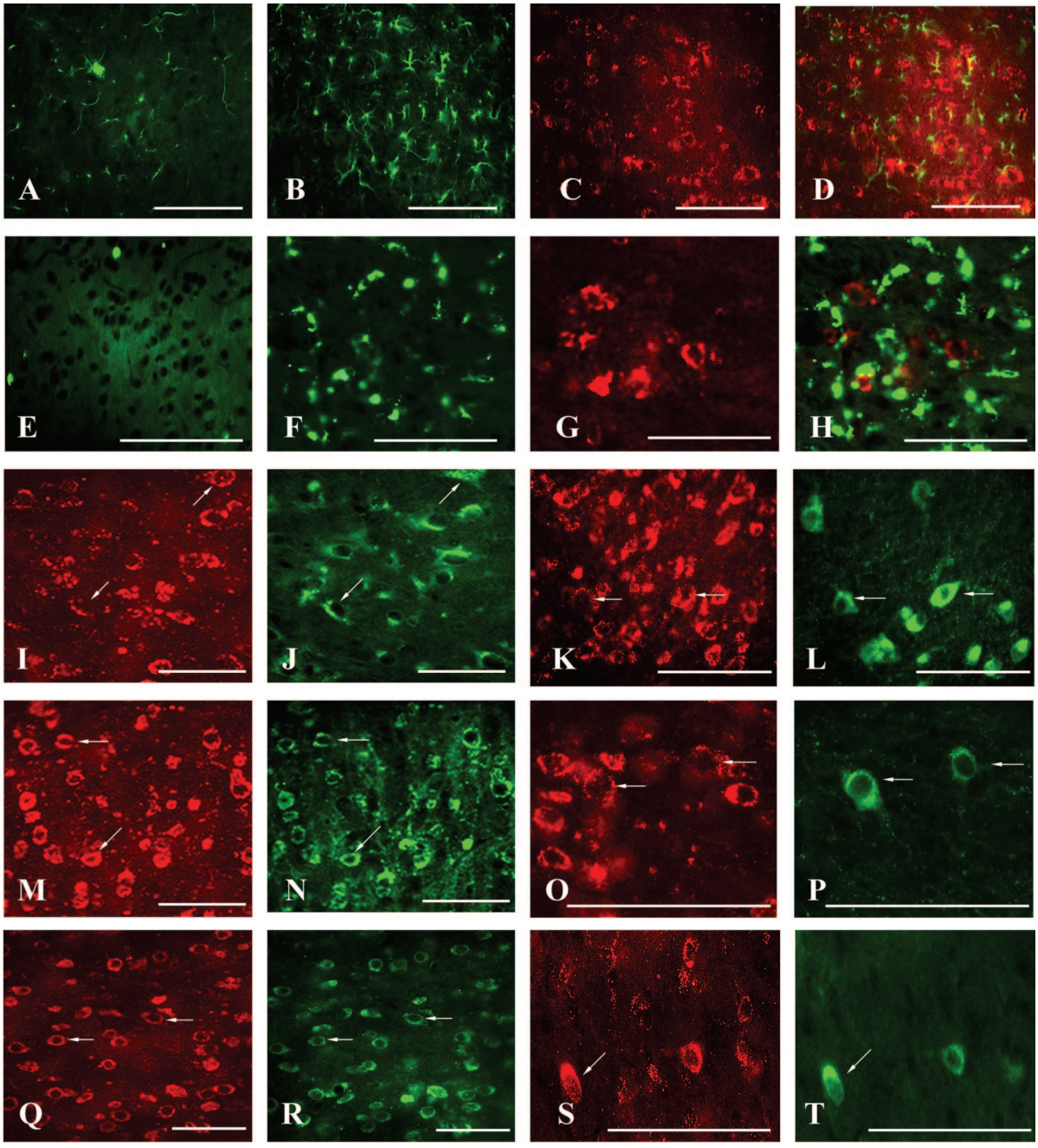
Discussion

The present study reveals that administration of 192-IgG-saporin induced a transient up-regulation of the CI-MPR in surviving neurons of the basal forebrain and frontal cortex—regions known to be affected by the immunotoxin. This was accompanied by a selective time-dependent increase in EL markers, ie, cathepsin D, Rab5, and LAMP2 in the affected regions of treated rats. Because CI-MPR plays a critical role in delivering lysosomal enzymes to the EL system, which is involved in the turnover of damaged proteins and structural reorganization in response to changing conditions,[5,6,9,11,13] it is likely that the observed increases in CI-MPR levels and that of other EL components in surviving neurons represent an adaptive mechanisms to restore metabolic and structural abnormalities that follow 192-IgG-saporin-induced loss of the forebrain cholinergic neurons.

In keeping with earlier studies,[31–33,35,36] we observed an almost complete degeneration of the forebrain cholinergic neurons by 7 days after a single intracerebroventricular administration of the immunotoxin. This was accompanied by a substantial depletion in the levels/expression of ChAT in the cortex, which receives projection from basal forebrain cholinergic neurons.[40–43] The brainstem cholinergic neurons that do not express p^{75NTR} were relatively spared. Additionally, a few p^{75NTR}-negative basal forebrain cholinergic neurons located in the ventral pallidum and ventral to the lenticular nucleus were found to be spared by the immunotoxin. This is consistent with earlier findings in rat[31] and mouse[44] and is believed to account for intact cholinergic innervations in the amygdala after 192-IgG-saporin administration.

The selective degeneration of forebrain cholinergic neurons by 192-IgG-saporin has been shown to induce transient alteration in various factors including neurotrophins and cytokines in the affected regions, which may participate either in protecting the surviving neurons and/or eliminating the neuronal debris from the site of injury.[38,45–48] In the present study, we showed that 192-IgG-saporin can transiently enhance the expression/levels of the CI-MPR throughout the 90-day experimental paradigm. This change was evident only in the affected areas, ie, basal forebrain (ie, 4 to 28 days after treatment) and frontal cortex (7 to 28 days after treatment), but not in the brainstem region. Our dual-immunolabeling experiments in the basal forebrain region revealed that increased receptor levels are associated with noncholinergic and p^{75NTR}-negative cholinergic neurons because

Figure 6. A–H: Immunofluorescence photomicrographs showing GFAP (**A**, **B**) and ED1 (**E**, **F**) immunoreactivity in the control rat (**A**, **E**) and their possible association with Rab5-positive cells (**C**, **G**) at 14 days after treatment with 192-IgG-saporin. Note that there is no Rab5 immunoreactivity (**C**, **D**, **G**, **H**) in GFAP-positive astrocytes (**B**, **D**), nor in ED1-positive microglia (**F**, **H**). **I–T:** Photomicrographs of Rab5 co-localization (**I**, **K**, **M**, **O**, **Q**, **S**) with the CI-MPR-positive (**J**, **N**, **R**) and VAChT-positive neurons (**L**, **P**, **T**) in the basal forebrain region of control animals (**I–L**), at 14 days (**M–P**) and 60 days (**Q–T**) after 192-IgG-saporin administration (**M–T**). **U** and **V:** Western blots and histograms showing the time-dependent increase in Rab5 levels in the septum/DBB complex (**U**) and frontal cortex (**V**) at 4, 7, 14, and 28 days after treatment and their return to control (Ctl) levels by 60 and 90 days after treatment with 192-IgG-saporin. $^*P < 0.05$. Scale bars = 10 μm.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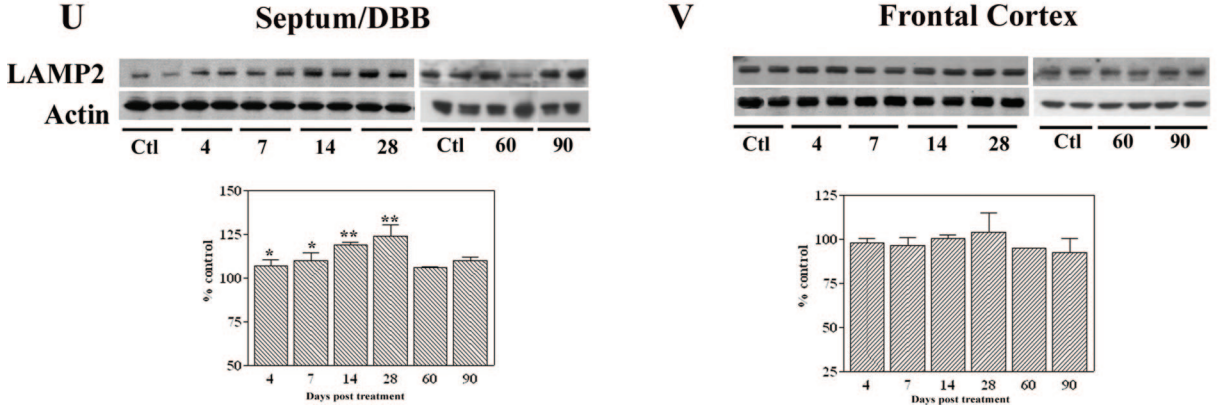
U
Septum/DBB
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Ctl 4 7 14 28 Ctl 60 90
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Days post treatment

neither activated astrocytes nor microglia exhibited CI-MPR immunoreactivity. Additionally, using an apoptotic nuclear marker, Hoechst 33258,[39] and the neuronal marker MAP2, we showed that neurons surviving the immunotoxin treatment express high levels of the receptor. Some earlier studies have reported an up-regulation of the CI-MPR in the brain after pharmacological/surgical lesions,[26–30] which may be associated with the subsequent degenerative/regenerative events that follow the lesion. Because the majority of CI-MPR are expressed within the EL system,[1,2,5,9] we investigated the possible alterations of the EL system in the affected brain regions of the 192-IgG-saporin-treated rats. Interestingly, all markers of the EL system, ie, Rab5, LAMP2, and cathepsin D were found to be increased in a time-dependent manner in the basal forebrain region until 28 days after treatment and then returned to control levels by day 60 after administration of the immunotoxin. By contrast, only cathepsin D and to some extent Rab5 showed enhanced levels for a brief period of time in the cortex of the treated rats. The regional variation in the alterations of the EL system markers may possibly relate to the severity of the immunotoxin's effect within the basal forebrain region, which harbors cholinergic cell bodies directly targeted for death, and the cortex, which receives projections from the forebrain cholinergic neurons.

Because CI-MPR are involved in the transport of lysosomal enzymes to the EL system,[1,2,5,6] which plays a critical role in protein turnover as well as cell viability, it is likely that altered levels of the receptor can influence the function/survival of neurons. Supporting the notion, it has been shown that CI-MPR overexpression in SK-N-SH cells can block apoptosis induced by the mutant herpes simplex virus 1,[18] whereas PC12 cells that are resistant to β-amyloid mediated toxicity showed an up-regulation of the CI-MPR.[17] However, given the evidence that CI-MPR levels and those of other markers of the EL system are increased in neurons that are not directly sensitive to 192-IgG-saporin, but are affected as a consequence of the loss of cholinergic input, it is likely that receptor up-regulation represents an adaptive response to restore lesion-induced metabolic/structural abnormalities by activating the EL system. This is substantiated, in part, by the experimental data showing that 1) the EL system can be up-regulated in response to the repair mechanisms resulting from cumulative aging, genetic, oxidative, and chemical factors[11,49,50]; 2) activation of the lysosomal system can restore chloroquine-induced abnormal protein deposits and synaptic decline in hippocampal slice preparation[51]; and 3) neurons that are not susceptible to death in animal models of neurodegeneration and Alzheimer's disease pathology exhibit increased activation of the EL system.[50,52,53] However, it is of interest to note that the frontal cortex, a region less severely affected by 192-IgG-saporin treatment, showed an up-regulation of the CI-MPR but no significant alterations in Rab5 or LAMP2 as observed in the septum/DBB. Because CI-MPR can mediate multiple functions including cell signaling and internalization leading to degradation/activation of various molecules (ie, IGF-II, leukemia inhibitory factor, and latent transforming growth factor-β),[1,2,5,6,9,10] it is likely that cortical receptors after the immunotoxin treatment may participate in other functions in addition to regulation of the EL system.

Although inferences about the dynamics of the endocytic process are difficult to make from fixed tissues, earlier reports have shown that neuronal increases in the endosomal marker Rab5 reflect enhanced endosomal activity.[11,54,55] Thus, it is likely that enhanced CI-MPR levels observed in the basal forebrain region reflect an increased demand for the transfer of lysosomal enzymes from the *trans*-Golgi network to endosomes to ensure efficient processing of substrates in surviving neurons of the 192-IgG saporin-treated rats. Earlier studies have shown that degeneration of cholinergic neurons by the immunotoxin can induce a severe reduction in dendritic branches and spine density resulting in altered synaptic transmission by the surviving neurons.[56–58] Because endocytosis plays an important role in the structural maintenance of axons, dendrites, and synapses to regulate intercellular communication,[59–61] the enhanced endosomal activity observed in 192-IgG-saporin-treated rats may represent part of a compensatory mechanism(s) to promote sequestration and degradation of membrane proteins and other materials. Such an effect can influence synaptic reorganization through elimination or remodeling of the established synapses and increase the ability of affected neurons to survive in the absence of cholinergic inputs.

In addition to the endocytic pathway, lysosomal abnormalities, represented by increased LAMP2 and/or cathepsin D levels, developed in the surviving neurons located in the basal forebrain and frontal cortex of 192-IgG-treated rats. This may account, at least in part, for the up-regulation of the CI-MPR and endosomal markers observed in affected regions of the treated rats. Given the role of lysosomes in degenerative phenomena, overexpression of lysosomal enzymes such as the cathepsins has long been implicated in cell death mechanism associated with lesion-induced brain injury and neurodegenerative diseases.[11,50,62–66] One mechanism by which cathepsins are postulated to directly contribute to cell death is by inducing lysosomal destabilization and enzyme leakage into cell cytoplasm.[67–70] This phenomenon has been described during oxidative stress in nonneuronal cells[71] and experimental brain ischemia in primates.[72] However, some recent studies

Figure 7. A–H: Double-labeling immunofluorescence photomicrographs showing GFAP (**A**, **B**) and ED1 (**E**, **F**) immunoreactivity in the control rat (**A**, **E**) and their possible association with LAMP2-immunoreacitve cells (**C**, **G**) at 14 days after treatment with 192-IgG-saporin. No LAMP2 immunoreactivity (**C**, **D**, **G**, **H**) was noted in either GFAP-positive astrocytes (**B**, **D**) or in ED1-positive microglia (**F**, **H**). **I–T:** Photomicrographs of LAMP2 co-localization (**I**, **K**, **M**, **O**, **Q**, **S**) with the CI-MPR-positive (**J**, **N**, **R**) and VAChT-positive neurons (**L**, **P**, **T**) in the basal forebrain region of control animals (**I–L**), at 14 days (**M–P**) and 60 days (**Q–T**) after 192-IgG-saporin administration. **U** and **V:** Western blots and histograms showing the relative increase in LAMP2 levels in the septum/DBB complex (**U**) and frontal cortex (**V**) at 4, 7, 14, and 28 days after treatment, which returned to control (Ctl) levels by 60 and 90 days after treatment with 192-IgG-saporin. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars = 10 μm.

have shown that activation of the lysosomes and/or lysosomal enzymes is also observed in the absence of cell death in animal models of neurodegenerative disorders.[50,52,53,73] This change may reflect an up-regulation of enzymes within the lysosomes, rather than in the cytoplasm, to counteract cellular abnormalities resulting from aging, toxins, or other chemical factors. This is supported, in part, by the evidence that chloroquine-induced abnormal protein deposits and synaptic decline in cultured hippocampal slices can be restored by activation of the lysosomal system.[51] The enhanced levels of the lysosomal marker and cathepsin D observed in surviving neurons of 192-IgG-saporin-treated rats, may therefore represent a compensatory response to the metabolic dysfunction and/or synaptic reorganization that follows the degeneration of the forebrain cholinergic neurons. This may also lead to the clearance of any excess abnormal proteins accumulated during synaptic remodeling.

In keeping with earlier reports,[37,38,45] we showed that loss of basal forebrain cholinergic neurons by 192-IgG-saporin can induce activation of both astrocytes and microglia in the septum/DBB but not in the cortical region of the treated rats. Our double-labeling experiments further reveal that reactive astrocytes did not exhibit either CI-MPR or other EL markers used in the study. By contrast, all activated microglia were found to express only cathepsin D in the septum/DBB complex of the treated rats. Because microglia can play an active role in removing neuronal debris generated by the immunotoxin treatment,[74,75] it is possible that enhanced cathepsin D levels may participate in the dendritic degeneration and axonal demyelination because this enzyme is capable of degrading microtubule-associated proteins[76] and myelin basic proteins.[77] These results, taken together, suggest that 192-IgG-saporin treatment can induce transient alterations in the CI-MPR and other components of the EL system in the affected regions, as a potential compensatory signaling mechanism to restore metabolic and structural abnormalities in neurons that survive toxicity/injury.

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