FULL PAPER *Anatomy*

Survival Change of Ventral Mesencephalon-Derived Progenitor Cells after Grafting into Unilateral Intact Adult Rat Striatum

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ABSTRACT. Neural transplantation is one of the most promising treatments for neurodegenerative disorders. Survival rates of embryonic dopamine (DA) neurons following transplantation are low, between 2% and 20% in a number of animal models. To further establish survival changes of the transplanted gestational day 13.5 ventral mesencephalic (VM) cells into left intact adult rat striata so that design strategies of increasing survival of DA neurons, the tyrosine hydroxylase (TH) expression of VM-derived progenitor cells has been examined using immunohistochemistry and Western blot analysis. TH immunostaining revealed that the grafted VM cells developed to mature TH-positive neurons strongly at 3 weeks, peaked at 4 weeks, thereafter, gradually dropped following the degenerative expression of the grafted cells at both 5 and 6 weeks after transplantation. Western blot analysis also showed that the TH proteins were maximally expressed at 4 weeks post-grafting. Our finding suggested that the peak of surviving VM-derived TH positive cells occurred approximately 4 weeks after transplantation.

KEY WORDS: dopamine, rat brain, transplantation, tyrosine hydroxylase, ventral mesencephalon cells.

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Neural transplantation is one of the most promising treatments for neurodegenerative disorders, especially Parkinson's disease (PD). Cell replacement strategy in PD has been based on the idea that dopamine (DA) neurotransmission can be restored in the striatum by neural grafts [12]. However, survival rates of embryonic DA neurons following transplantation are low, between 2% and 20% in a number of animal models [24]. Earlier studies have shown that 80–95% of grafted DA neurons die following transplantation [6, 14]. In a pioneering study employing a marker for cell death in neuronal grafts, Mahalik *et al.* revealed a progressively decreasing number of stained cells at 15 days, as compared to 21 and 28 days post-transplantation [13]. Barker *et al.* suggested that most of the DA neurons die within the first week after transplantation by counting tyrosine hydroxylase (TH) immunopositive neurons in the graft at various time points post-implantation [2]. Zawada *et al.* demonstrated that the majority of apoptotic neurons appeared in mesencephalic grafts as early as 24 hr after transplantation, and their numbers decreased by 75% after 7 days [27]. However, Emgård *et al.* demonstrated that neither the total number of TH-positive cells in the graft nor the total graft volume changed significantly, using more accurate stereological estimates between 6 and 42 days after transplantation [5].

Because the midbrain DA neurons are generated from precursor cells in the ventricular zone of the developing ventral mesencephalon (VM), they are interesting candidate sources for donor cells that could be used in transplantation studies of DA degenerative disorders [9]. However, there is a narrow time-window during which ventral mesencephalic tissue may most effectively be harvested; in rats, this occurs at gestational day (GD) 13.5-GD 14.5 [20]. It has often been reported that the optimum donor age corresponds to the time of maximal neurogenesis of the population of neurons being transplanted; in the case of the GD 14 VM, a high proportion of DA neurons have already differentiated or are about to do so and still have little or no neuritis [3, 4]. The most commonly stated reasons for this critical time window are that neurons must be of sufficient age to be committed to a DA phenotype but prior to the stage of extensive neurite plexus development that would render them susceptible to trauma during graft tissue dissection and implantation preparation [22].

To further establish survival changes of the transplanted cells, in order to design increasing DA neuron survival strategies, we have therefore undertaken the present investigation to evaluate the TH expression of VM-derived progenitor cells at 3, 4, 5, and 6 weeks after transplantation into the unilateral intact adult rat striatum.

MATERIALS AND METHODS

Animals: The experiments were conducted in accordance with the guidelines of Gyeongsang National University. Forty-eight adult female Sprague-Dawley rats (weighing 200–250 g at the start of the experiment) were used in this study. They were divided into control group (n=4) and transplantation group (n=44) (immunohistochemistry group

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(n=24) and Western blot group (n=20)), and housed in a room 25°C under 12 hr light/12 hr dark conditions with *ad libitum* access to food and water.

Preparation of donor tissue: Pregnancies were dated by inspection for the vaginal plug, and the day of the plug was defined as GD 0.5. VM tissue was obtained from 3 pregnant Sprague-Dawley rats of GD 13.5 and then digested in 0.1% trypsin/0.04% DNase/HBSS (Hanks Balanced Salt Solution) at 37°C for 30 min. The tissues were washed with 0.04% DNase in HBSS and then triturated with a fire polished Pasteur pipette to form a dissociated cell suspension. The cells were centrifuged and re-suspended and then were plated at a density of 200,000 cells/m*l* into poly-L-lysine (50 μg/m*l* in PBS; Sigma, St. Louis, MO, U.S.A.)-coated 25-cm flasks and cultured in DMEM (Sigma) containing 5 μg/m*l* insulin, 55 μg/m*l* transferring, 5 μg/m*l* sodium selenite, 33 mM glucose, 2 mM glutamine, 12.5 μM ascorbic acid, 26 mM $NaHCO_3$, 20 mM HEPES, 20% fetal calf serum, and 1% Penicillin/Streptomycin, and Fungizone. The cells were maintained in a humidified CO_2 incubator (5% CO_2, 95% air; 37°C) for 2 days. The harvested cells with 0.1% trypsin were spun and resuspended. The cell viability, assessed with a trypan blue dye exclusion test, was over 95% just before grafting, and the cell concentration was 40,000 per microliter.

Transplantation surgery: Recipient rats were anesthetized with ketalar (7 mg/100 g, i.p.) and rompun (1 mg/100 g, i.p.) and mounted in a stereotaxic frame. Using a 20 μl Hamilton syringe (22-gauge), one stereotaxic deposit of 10 μl cell suspension ($4 \times 10^4/\mu l$) was injected over a 10 min period into the left striatum at the following coordinates: A= +0.5 mm (anterior) to bregma; L=3.0 mm lateral to the midline; V=5.5 mm below dura [17]. After implantation, the needle of the Hamilton syringe was left in place for an additional 5 min and then slowly withdrawn. A piece of bone wax was applied to the skull defect to prevent leakage of the solution. In control groups, each rat received an injection of 10 μl of vehicle (HBSS) at the same coordinates.

Tissue processing and immunohistochemistry: The rats were sacrificed at 3, 4, 5 or 6 weeks after transplantation surgery. The animals were anesthetized with lethal doses of anhydrous ethyl ether, and perfused transcardically with physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. After a 3 days post-fixation incubation in the same fixative, the brains were immersed in 20% sucrose in PB at 4°C for 3 days, embedded in O.C.T. compound, and frozen in liquid nitrogen. Coronal sections were cut at 14 μm in a cryostat.

Frozen sections were dried in slide warmer (37°C) for 3 hr. The sections were washed twice in phosphate buffer saline (PBS) and preincubated with normal goat serum 1:20 in PBS for 1.5 hr at room temperature to block the non-specific staining, and then incubated with a rabbit anti-TH antibody (Pel-Freez Biologicals, Rogers, AR, U.S.A.) at a dilution of 1:500 overnight at 4°C. After rinsing twice in PBS, the sections were treated with a biotinylated anti-rabbit IgG (1:200, Sigma) for 1.5 hr, rinsed and reincubated with avidin-biotin-conjugated horseradish peroxidase for 1.5 hr. Following two 15-min rinses in PBS, the sections were visualized by incubation in 0.05% diaminobenzidine in PBS containing 0.015% hydrogen peroxide for 10 min. Thereafter, they were dehydrated in increasing concentrations of ethanol, cleared in xylene, and coverslipped with mounting medium. Adjacent sections were processed for routine histology using cresyl violet staining.

Western blot analysis: For Western blot analysis, rats were decapitated at corresponding time point after operation. Brains were rapidly removed. The left and right corpora striata were dissected out and homogenized with lysis buffer containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl (pH7.5), 1 mM phenylmethyl sulfonyl fluoride, and 0.2 U/m*l* aprotinin. Lysates were clarified by centrifugation at 20,000 g for 10 min at 4°C, frozen in liquid nitrogen, and stored at –80°C until use. Protein concentrations in supernatants were determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Supernatants from different specimens, after minor volume adjustments to provide equal amounts of total proteins (70 μg), were added directly into SDS-sample buffer, boiled, and then applied to a 12.5% SDS- polyacrylamide gel electrophoresis under reducing conditions. After electrophoresis, proteins were transferred to PVDF membrane using the transfer-buffer system (39 mM glycine, 48 mM Tris, 20% MeOH and 0.037% SDS electrotransfer) and semidry electrophoretic transfer cell (15 V, 90 min). The membrane transferred was blocked in Tris buffer saline containing 0.1% Tween 20, 5% non-fat dry milk to reduce nonspecific binding. They were incubated in primary antibody (rabbit-derived anti-TH polyclonal antibodies, 1:500) for 24 hr at 4°C, followed by secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Bio-Rad 1:1,000) for 2 hr at room temperature. The proteins were detected by the ECL Western blotting detection system (Amersham Pharmacia Biotech, Little Chalfont, England).

Quantification and statistical analysis: Counting of TH-positive neurons and neuron-like cells in cresyl violet staining were performed using an OLYMPUS CX 31 light microscope at × 100 magnification. TH-positive cells and neuron-like cells were quantified by counting the total number of cells in each other interval section of a 1-in-12 series through the striatum containing grafted cells of every animal. Total number of six sections was combined with the area of a rat graft and used to calculate TH (+) cells as a percentage of neuron-like cells of six sections. The mean ± S.D. was obtained by averaging values from every group six animals for each time point.

The object band from Western blot was scanned and analyzed by densitometry using a computer based on the Sigma Gel System (SPSS Inc., Chicago, IL, U.S.A.). Density values were expressed as the mean ± S.D. of five experiments. The statistical analysis was performed using student's t test.

RESULTS

Histological and immunohistochemical examinations of the grafts: Cresyl violet staining revealed that the grafted VM cells survived and localized in the left striatum in twenty-four recipient rats, but they were not distributed diffusely or homogeneously in the graft (Figs. 1A, 2A, 3A, 3C). They revealed bipolar, tripolar, or multipolar neuron-like morphology (Fig. 1C). At three weeks and four weeks post-implantation, there was little infiltration of microglia-like or apoptotic nucleus around the implanted site. Only some hemosiderin-containing macrophages of the host tissue were observed at the graft site.

TH immunostaining revealed that the grafted VM cells developed to mature TH-positive neurons strongly three weeks after transplantation (Fig. 1B), peaking four weeks after grafting (Fig. 2B), and appeared to have undergone little degeneration (Figs. 3B, 3D). The cell bodies were smaller in comparison to the endogenous DA neurons and exhibited ovoid or multipolar mature neuronal morphological characteristics (Fig. 1D). The majority of grafted TH (+) cells were distributed at the periphery of the transplants, in contrast with the endogenous TH positive cell. The core of the grafts lacked relatively VM tissue-derived TH immunoreactive neurons. The neurite extension and arborization were much stronger at post-grafting four weeks. In addition, the TH-positive cells were detected along the injection tract in the neocortex and corpus callosum at this time point. The degenerative expression of the grafted cells was viewed at both five and six weeks. These neurons were round with few immunoreactive processes, and the TH staining appeared to be clumped, rather than evenly distributed, in the cell bodies. In some cases, cells were outlined by immunoreactive membrane. As expected, the four control animals did not exhibit any TH immunoreactivity in the vicinity of the injection track following vehicle injection (data not shown).

Quantification of cell survival: Counting of TH-positive neurons and neuron-like cells were performed with six, every other sections of a 1-in-12 series of the VM grafts at three, four, five, and six weeks post-implantation, respectively. Approximately 194 TH (+) cells were counted four weeks after transplantation, with significant differences compared to three weeks post-grafting. The latter was also significantly higher when compared to five and six weeks post-grafting. A percentage of the number of survival TH (+) cells against neuron-like cells inside the grafts is also shown in Fig. 4. The percentage of TH-positive cells ranged from 3.33 ± 0.55% to 6.21 ± 0.42% at various time points. At four weeks post-implantation, the percentage of implanted cells surviving was indicated significantly effective of the statistical condition with respect to the three weeks time point (6.21 ± 0.42% vs. 5.29 ± 0.55%). And the latter was statistically effective related to five and six weeks (5.29 ± 0.55% vs. 3.45 ± 0.52% and 3.33 ± 0.55%). It could indicate that the peak of surviving graft-derived TH (+) cells occurred approximately four weeks after transplantation.

Western blot analysis: Western blot analysis was per-

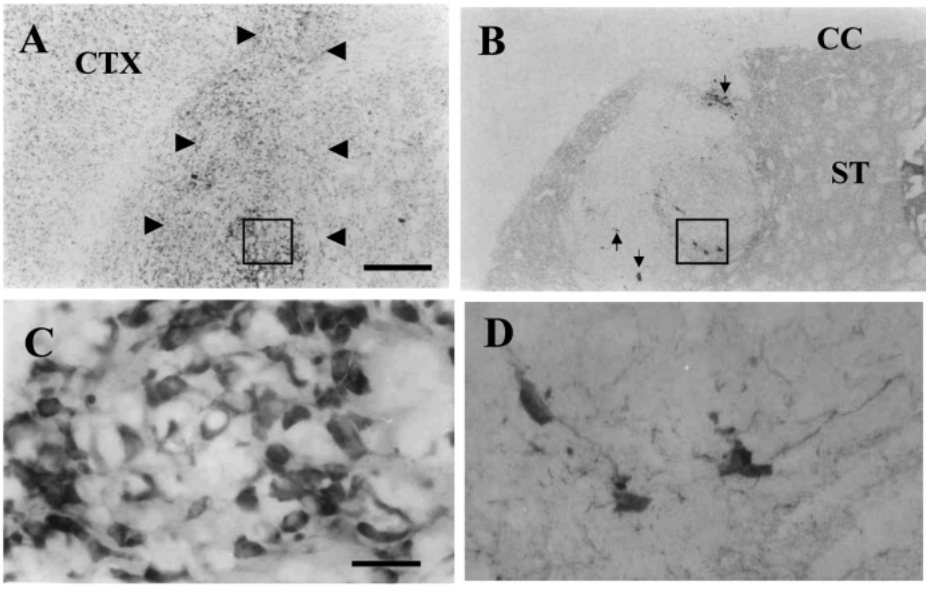

Fig. 1. Bright-field photomicrographs showing cresyl violet staining (A and C) and TH (B and D) immunohistochemistry of a graft 3 weeks after implantation of VM cells into left intact adult rat striatum. Arrowheads delineate a transplanted region in A; arrows indicate that the grafted VM cells develop to mature TH-positive neurons strongly, and the majority is distributed at the periphery of the transplants in B. Panels C and D are higher-magnification images from boxes in panels A and B, respectively. Neuron-like cells of cresyl violet staining reveal bipolar, tripolar, or multipolar in shape in C. ST, striatum; CC, corpus callosum; CTX, cortex. Scale bars: in A, 200 μm for A and B; in C, 25 μm for C and D.

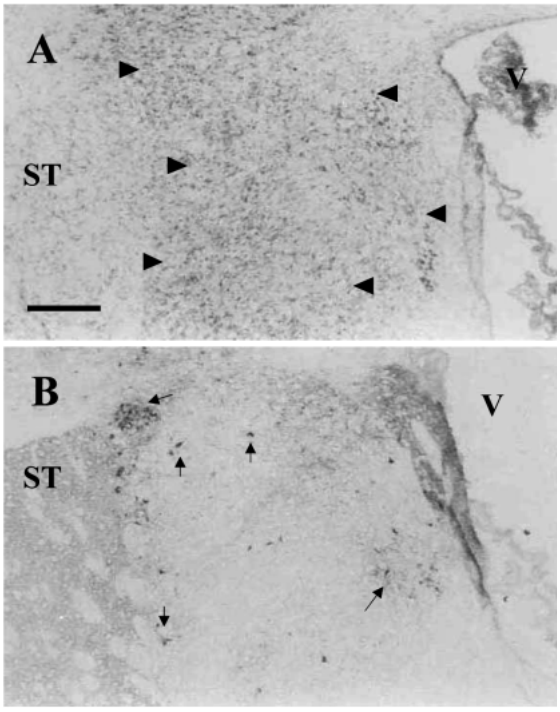

Fig. 2. Bright-field photomicrographs showing cresyl violet staining (A) and TH (B) immunohistochemistry of a graft 4 weeks after implantation of VM cells into left intact adult rat striatum. Arrowheads delineate areas of transplant in A; arrows indicate that the number of the differentiated TH-positive neurons peaks, and the most are present at the periphery of the grafts in B. ST, striatum; V, lateral ventricle. Scale bars: in A, 200 μm for A and B.

formed to determine whether over time had significant difference on TH proteins within the grafted striatum among the various groups. Representative immunoblot bands for the TH proteins are shown in Fig. 5A. At 4 weeks post-implantation, the groups showed a maximal signal on striatal TH tissue levels (Fig. 5B). The 3-week groups revealed higher TH expression, too. However, in both 5- and 6- week groups, it decreased significantly (Fig. 5B).

DISCUSSION

In the present study, we have investigated the survival time course and changes of VM-derived progenitor cells following transplantation into the intact adult rat striatum. Previous research has established the time course of the glia reaction and its relation to cell division [1]. Also, selective sorting of mesencephalic precursor cells was performed to generate dopaminergic neurons [20]. Our preliminary finding that the percentage of TH-positive cells against neuron-like cells ranged from 3.33 ± 0.55% to 6.21 ± 0.42% is consistent with previous reports. TH immunohistochemical examination of the grafts revealed that the proportion of TH-positive cells rose from three to four weeks post-grafting and then dropped at five and six weeks after implantation in our study. Olson and Seiger have demonstrated that DA neurons increase in number at a maximal rate at GD 12 to GD 13, although they continue to increase in number until GD 15 and GD 16 [16, 21]. Moreover, Sawamoto *et al.* have shown that embryonic VM tissue includes both mitotic and post-mitotic precursors [20]. It was implied that the embryonic tissue used in our study contained a mixture of differentiated neurons that had already undergone the final mitosis and neural progenitor cells already committed to a DA neuron fate but were still at a precursor stage of development. Thus, the DA neuron precursors from embryonic VM-derived cells could continue to proliferation and differentiation at three weeks, and peaking four weeks after transplantation. Beginning five weeks post-grafting, a lack of neuronal precursors together with depleting trophic factors [15, 18] and poor vascularization [11, 15] in sites of transplantation resulted in increasing apoptotic dopaminergic neurons, and the percentage of TH-positive cells gradually dropped with time.

The existence of specific positional cues, sonic hedgehog (Shh) and fibroblast growth factor 8 (FGF-8) have been shown to determine the location and phenotype of ventral DA neurons [8, 25]. Kim *et al.* suggest that Shh and FGF-8 might be the sources of signals required for the induction of both cellular and morphological differentiation of neural stem cells into DA phenotypes [9]. Moreover, Rafuse *et al.* propose that neuroprotective properties of cultured neural progenitor cells are associated with the production of Shh [19, 26]. In the present study, the lower proportion of differentiation into dopaminergic neurons might be related to a lack of Shh and FGF-8 signals in transplantational regions.

The need for immunosuppression after transplantation remains a subject of debate. It is well known that the brain is relatively immunoprivileged. In our experiment, an immunosuppressant drug was not used. Some macrophages and lymphocytes were seen within the grafts, while survival of embryonic DA neuronal cells was observed in grafted rats without immunosuppression. This result is consistent with that obtained by Sladek *et al.* [23]. In one patient, numerous macrophages, T-cells and B-cells were seen within the surviving graft site despite six months of cyclosporine treatment [10], while cell survival was observed in an autopsied patient who was never immunosuppressed [7]. It is suggested at least that chronic immunosuppression is not necessary [25].

In summary, the graft-derived VM precursor cells could survive and differentiate in the striatum, neocortex and corpus callosum of adjacent graft region. The majority of grafted TH-positive cells were localized at the periphery of the transplants. The core of grafts lacked VM tissue-derived TH immunoreactivity neurons relatively. TH immunoreactive cells number of the grafted VM cells peaked at 4 weeks post-grafting, thereafter, gradually dropped over time. The post-grafting 4-week groups also showed a maximal expression on striatal TH protein. Our finding suggested that the peak of surviving VM-derived TH positive cells occurred

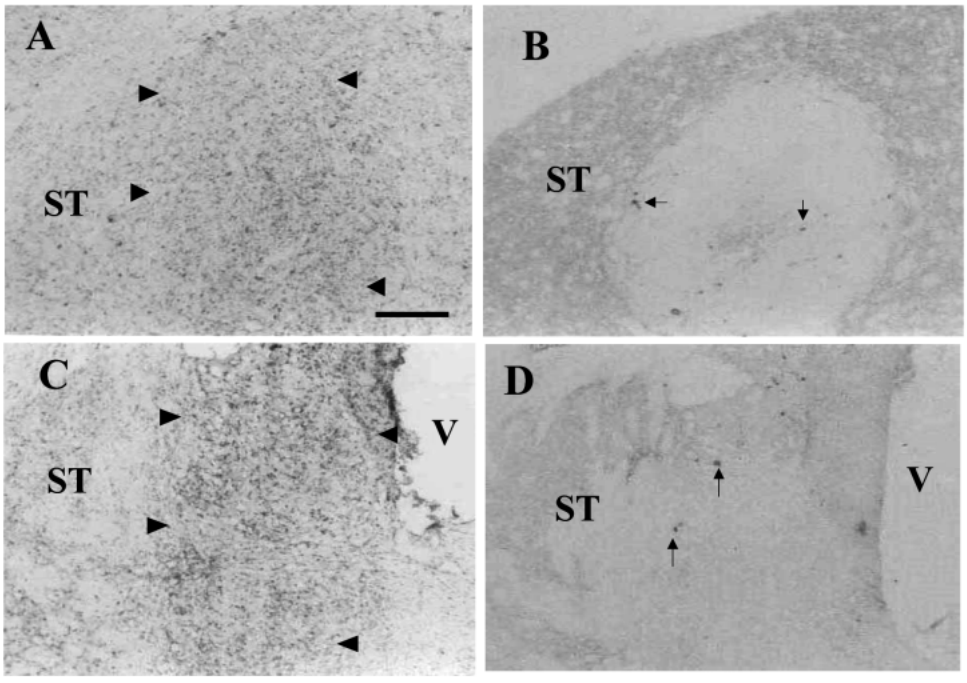

Fig. 3. Bright-field photomicrographs showing cresyl violet staining (A and C) and TH (B and D) immunohistochemistry of grafts at 5 (A and B) and 6 (C and D) weeks after implantation of VM cells into left intact adult rat striatum. Arrowheads delineate areas of transplant in A and C; arrows indicate that the most are distributed at the periphery of the grafts in B and D. The number of the differentiated TH-positive neurons gradually drops over time. ST, striatum; V, lateral ventricle. Scale bars: in A, 200 μm for A, B, C and D.

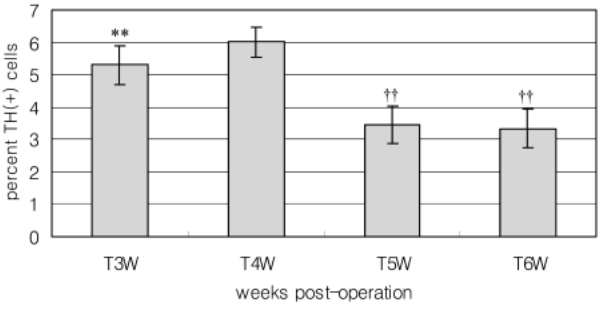

Fig. 4. Percentage histograms of the number of survival TH-positive cells against that of neuron-like cells inside the grafts at each time point. ** P<0.01 vs. T4w; †† P<0.01 vs. T3w (t-test).

approximately 4 weeks after transplantation.

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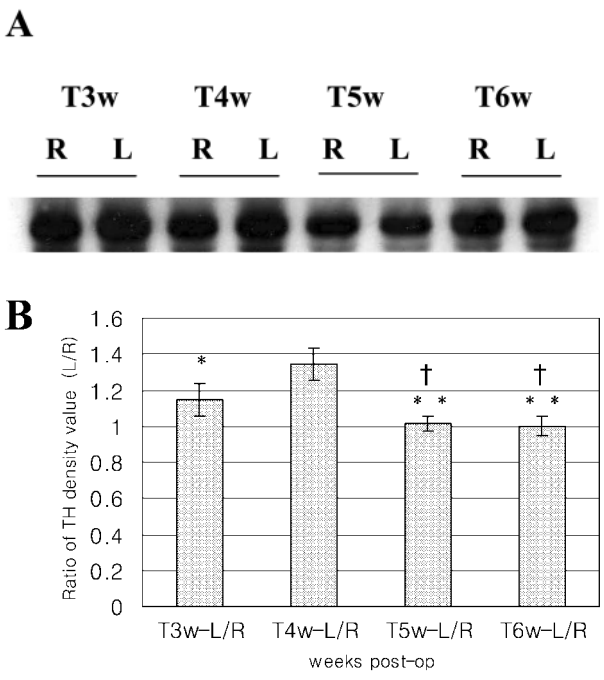

Fig. 5. Western blot analysis of TH protein at each time point expressed as ratio (mean ± S.D.) of left (grafted side) versus right striatum (ungrafted side). A: Immunoblots of TH protein expression in bilateral striata of each group. The immunoblots were labeled with an anti-TH polyclonal antibody. The TH protein is identified in the blots by a specific band at 60 kDa. For each group n=5. B: Ratio of densitometric evaluation of bands in A. * p<0.01 ** p<0.001 vs. T4w-L/R; † p<0.01 *vs.* T3w-L/R.

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