Restorative Neurology and Neuroscience 22 (2004) 43–54
IOS Press

Abstracts of the 18th Annual Meeting of the Japanese Society for Neural Growth, Regeneration and Transplantation

1. The combination of BDNF and GDNF gene transfer prevents motoneuron loss in the nucleus ambiguus

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Unsuccessful reinnervation after recurrent laryngeal nerve injury may attribute to the loss of motoneurons in the nucleus ambiguus. To assess the possibility of gene therapy for injury of these motoneurons, we examined the neuroprotective effect of brain-derived neurotrophic factor (BDNF) encoded by the adenoviral vector (AxCAmBDNF) on the motoneuron loss in the nucleus ambiguus after adult rat vagal nerve avulsion. We also investigated the enhancement of neuroprotective effect by addition of glial cell line-derived neurotrophic factor (GDNF) encoded by the adenoviral vector AxCAhGDNF to AxCAmBDNF. Using microhemostat forceps, the left vagal nerve of 12 weeks old Sprague-Dawley male rats was avulsed and removed at the level of jugular foramen. Immediately following avulsion, an adenoviral vector encoding the BDNF gene was inoculated at the avulsed site (BDNF group). In some animals, an adenoviral vector encoding GDNF was also inoculated in addition to AxCAmBDNF following avulsion (BDNF and GDNF group). Transgene expression in the nucleus ambiguus was examined by RT-PCR analysis. After Nissl staining, the number of surviving motoneurons in the nucleus ambiguus was counted 4 weeks postoperation to evaluate the neuroprotective effect of AxCAmBDNF alone and AxCAmBDNF with AxCAhGDNF. Four weeks after the left vagus nerve avulsion, in the BDNF group, a significantly larger number of surviving motoneurons in the nucleus ambiguus were observed compared to the control group. In the BDNF and GDNF group, a significantly larger number of surviving motoneurons were observed compared to the BDNF and control groups. Adenoviral BDNF gene transfer after vagal nerve avulsion prevented motoneuron loss in the nucleus ambiguus. The survival of motoneurons was more improved by the combination of BDNF and GDNF gene transfer compared to BDNF gene transfer alone. The combination of adenoviral BDNF and GDNF gene transfer may strongly prevent the degeneration of motoneurons in the nucleus ambiguus in adult humans with recurrent laryngeal nerve injury.

2. Neurofunctional recovery through GDNF gene therapy for recurrent laryngeal nerve palsy

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The quality of life (QOL) of patients with recurrent laryngeal nerve (RLN) palsy is degraded due to aspi-

ration pneumonia, or dysphonia. There is, however, no therapy aimed at reinnervating the RLN. To assess the possibility of gene therapy for repair of injury of these motoneurons, we examined the neurofunctional recovery of glial cell line-derived neurotrophic factor (GDNF) encoded by an adenoviral vector after crush injury of the adult rat RLN. The left RLN of 12 weeks old Sprague-Dawley rats was crushed for 60 seconds with forceps. Immediately after the crush, an adenoviral vector encoding β-galactosidase gene (AxCALacZ) was directly injected at the crushed site of the RLN and transgene expression in the nucleus ambiguus was examined by X-gal histochemistry. Some of the remaining animals were injected with an adenoviral vector encoding GDNF (AxCAhGDNF) right after the crush injury and the remainder received no injection. The conduction velocity (CV) of the RLN was measured and the movement of the left vocal cord was observed respectively after 2 or 4 weeks postoperation to evaluate the neuroprotective effect of AxCAhGDNF. In the AxCALacZ-treated animals, motoneurons and their neurites in the nucleus ambiguus were labeled with X-gal staining 4 days after AxCALacZ injection, indicating successful induction of the foreign gene into the nucleus ambiguus by the retrogradely transported adenoviral vector. When comparing AxCAhGDNF-treated animals, AxCALacZ-treated and the untreated controls, a significantly better CV of the RLN was measured in the AxCAhGDNF-treated animals 2 to 4 weeks postoperation. Compared with the number of rats in whom vocal cord movement was observed in each group, in the AxCAhGDNF-treated animals, movement was significantly larger than in the control animals (AxCALacZ-treated and the untreated controls) 2 to 4 weeks postoperation. Adenoviral vectors successfully induced a foreign gene into lesioned motoneurons in the nucleus ambiguus after crush injury of the RLN. Adenoviral GDNF gene transfer after crush injury of the RLN improved the CV of the RLN and the movement rate of the left vocal cord. Adenoviral GDNF gene therapy may improve neurofunctional recovery in adult humans with recurrent laryngeal nerve injury.

3. Generation of motor neurons from mouse embryonic stem cells

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Embryonic stem cells (ESCs) are pluripotent stem cells which are able to differentiate into various cell types including neural cells. These neural cells are expected to be applied in regenerative medicine for the central nervous system. In this study, motor neurons, which are selectively degenerated in amyotrophic lateral sclerosis (ALS), were derived from mouse ESCs using retinoic acid (RA). First, ESCs were aggregated into embryoid bodies (EBs) in the presence of retinoic acid (RA). Next, the functional properties of these neurons from ESC-derived neurospheres were examined. In the first case, when treated with high dose RA, dissociates of EBs directly differentiated into neurons, including motor neurons expressing Isl-1 and HB9. On the other hand, when treated with low dose RA, dissociates of EBs efficiently formed neurospheres in a chemically defined medium with basic fibroblast growth factor (bFGF). These neurospheres differentiated into HB9 positive motor neurons at a higher rate. Subsequently, in the electrophysiological analysis, inward sodium currents and action potentials were recorded in these neurons. Furthermore, these motor neurons formed neuro-muscular junctions *in vitro*, when cocultured with myotubes derived from a myoblast cell line. This strategy may be applied to the analysis of motor neuron related diseases *in vitro*. In a clinical situation, it may be applied in the treatment of ALS patients, provided it is applicable to human ESCs and if these motor neuron progenitors derived from ESCs can survive and function *in vivo*.

4. The proteolipid protein gene is expressed in tangentially migrating GABAergic neurons and oligodendrocytes during development of the cerebral cortex

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The *Plp* gene encodes two proteins, proteolipid protein (PLP) and its smaller isoform DM-20, major membrane proteins of myelin in the CNS. *Plp* gene expres-

sion is detected during early embryonic development in the mouse, although it is much higher in mature oligodendrocytes. In order to characterize the embryonic *plp*-expressing cells, transgenic mice expressing enhanced green fluorescent protein (EGFP) driven by the *plp* promoter have been examined. In this study, we focused on telencephalic development. In the postnatal forebrain, PLP-EGFP is expressed throughout the tissue by premyelinating and myelinating oligodendrocytes.

On the other hand, in the embryonic telencephalon, PLP-EGFP cells initially appeared only in restricted regions of ventral telencephalon, as well as in the lateral ganglionic eminence, the medial ganglionic eminence (MGE) and the anterior entopeduncular area at E11-13, where neural precursors are generated. However, PLP-EGFP cells failed to express nestin and RC2, which are markers for neural stem cells or radial glia. Most, but not all, PLP-EGFP cells in the ventricular zone of the MGE were labeled with Olig2, which has been reported to be involved in the differentiation of motor neurons and oligodendrocytes in the spinal cord. By E16, PLP-EGFP expression expanded into the cortex, suggesting that PLP-EGFP may migrate from the ventral to the dorsal telencephalon. Interestingly, at E16.5, PLP-EGFP was expressed not only in oligodendroglial progenitor cells but also in progenitor cells of GABAergic interneurons, which are defined as the expression of GABA, TuJ1 and MAP2. At later stages, PLP-EGFP was not detected in neuronal lineage. These findings suggest that the earliest *plp* may be expressed by progenitor cells, which can give rise to GABAergic interneurons and/or oligodendrocytes in the ventral telencephalon. Furthermore, it is suggested that *plp* expression is maintained in oligodendrocyte progenitors, while it is down-regulated in neuronal progenitors as development proceeds.

5. Survival promotion of axotomized cat retinal ganglion cells by electrical stimulation

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Previously we reported that electrical stimulation (ES) of the transected optic nerve stump for 2 hours enhanced the one-week survival of axotomized rat retinal ganglion cells (RGCs) (Morimoto et al., Neuroreport, 2002). Here we addressed whether ES can also enhance the survival of axotomized RGCs of adult cats. The RGCs of both retinas in the cats were retrogradely labeled with DiI from the bilateral lateral geniculate nuclei and optic tracts. More than 14 days later, the left optic nerve was transected completely at a point 4 mm behind the eyeball. Immediately after transection 20 Hz-monophasic square pulses of 500 μA, 1 mA, 3 mA, or 5 mA with duration of 300 μsec were applied for 2 hours via bipolar cuff electrodes attached to the transected optic nerve stump. At one-week survival, the numbers of DiI-labeled RGCs on both retinas were counted at the area centralis and other areas to cover whole retinas. The survival rates at the area centralis were calculated as the ratio of the number in optic nerve-transected left retina to that in intact right retina. The survival rates of total RGCs were estimated as the ratio of the sum of the numbers of whole retinal areas in the left retina to that in right retina. ES of 1 mA significantly improved the survival rate of RGCs at the area centralis, but not the rate of total RGCs. ES of 3 mA improved the survival rates of total RGCs as well as the rate at the area centralis. It was concluded that ES of 3 mA to the sectioned optic nerve via cuff electrodes can promote the one-week survival of axotomized RGCs of adult cats, to the same extent as the effect of the ES on axotomized rat RGCs.

6. Axonal regeneration from sciatic nerves removed from their cell bodies

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Axons, which are long and thin, propagate signals from their cell soma to target neurons or cells. All substances in axons are synthesized in the neural cell bodies and supplied from cell soma by axonal transport. It is therefore commonly believed that nerve fibers cannot regenerate axons after removal from their cell bodies. However, if a transected nerve site includes essential components to support neural regeneration, axons might possibly have the ability to regenerate from the transected nerve sites of nerve fibers without the help of their cell bodies. We examined this possibility using cultured sciatic nerves from adult mice or rats. Sciatic

nerves cultured in collagen gel showed that processes grew from the peripheral transected nerve sites, but not from central ones, after 24 h in culture. All of the processes were proved to be axons from the immunohistochemical staining with anti-beta tubulin class III. These regenerated axonal tissues were promoted by cultured conditioned media with oxidized galectin-1-stimulated macrophages that had been proved to promote axonal regeneration from transected-nerve sites of cultured DRG explants. Furthermore, sciatic nerves removed from a rat at 14 days after the nerve crush/freeze also showed that the operation increased the number of the regenerating axons from peripheral transected sites. Cycloheximide inhibited Schwann cell proliferation and migration but did not block axonal regeneration. Most regenerating axons started to degenerate after 24 h in culture and disappeared by 48 h. It is concluded from these experiments that sciatic nerves can regenerate axons from peripheral transected nerve sites without the help of their cell bodies.

7. Genetically modified Schwann cells for tissue-engineered peripheral nerve

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Recently we produced a tissue-engineered peripheral nerve by three-dimensional culturing of Schwann cells using the centrifugation technique. In an *in vivo* study, we reported that this artificial nerve induced an excellent axonal bridge in the rat sciatic nerve gap model. In order to induce axonal regeneration through the longer nerve gap, the genetical modification of Schwann cells (GMSCs) was performed by encoding the neurotrophic factor gene using an adenoviral vector. The rate of transfection to Schwann cells using the adenoviral vector was almost 100%, and GMSCs showed the original spindle shape. In the Western blotting, the GMSCs encoding BDNF, CNTF or NT3 indicated higher expression of neurotrophic factors compared with the non-transferred control. The S100 expression was similarly shown on gene-transferred and non-transferred Schwann cells. In an *in vitro* axonal outgrowth assay, the explants (P3 DRG) were covered with 20–3 0 μl of matrigel. The preparationsd with 3-day or 3-week culture medium, and maintained at 37°/5% CO_2 for 2 days. The axonal lengths of GMSCs (BDNF, CNTF, NT3) culture medium were longer than those of the controls. All of these results indicate. that GMSCs (BDNF, CNTF, NT3) have the potential for axonal elongation without without any alteration of normal Schwann cell characteristics. We believe that. tissue-engineered nerves including the GMSCs (BDNF, CNTF, NT3) may have the ability to induce axonal regeneration across a longer gap.

8. Regulation of neural stem cell proliferation and differentiation by melatonin

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Melatonin (N-acetyl-5-methoxytryptamine) is one of the physiologically important hormones mainly produced in the pineal gland. Recently, it was reported that melatonin showed potential neuroprotection against ischemia-reperfusion injury, however the accurate mechanism remained to be clarified. Since ischemia was shown to elicit the activation of endogenous neural stem cells (NSCs), we examined the effects of melatonin on the proliferative and differentiative functions of the NSCs using an *in vitro* culture systems. The NSCs derived from the lateral ganglionic eminences of E16 mice were cultured by the "Neurosphere" method using an EGF-containing medium for 7 days and were used in the experiments. The proliferative activity of NSCs was examined by the WST-8 assay, neurospheres counting or the BrdU incorporation assay in the presence of EGF (20 ng/ml). Differentiation was induced by the incubation in a 1% FBS-containing medium without EGF and was evaluated by fluorescence-limmunocytochemistry and ELISA using anti-TuJ1 or anti-GFAP antibodies. We found that melatonin suppressed EGF-stimulated increment of viablel cells, DNA synthesis and neurosphere formation of NSCs in a concentration-dependent manner (1–100 μM). Furthermore, the treatment with melatonin during the proliferating period facilitated the differentiation of NSCs into neurons without affecting the astroglial differentiation. However, melatonin exposure during the differentiation period failed to increase the differentiation of NSCs into neurons, and at a higher

concentration decreased both the neuronal and the astroglial differentiation. These results suggest that melatonin has the potential to modulate actions of the NSCs functions; the suppression of the proliferation and the facilitation of neuronal differentiation.

9. The effect of 2-deoxyglucose on the proliferative activity of murine neural stem cells in vitro

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Recent studies demonstrated that transient focal cerebral ischemia activates the proliferation and differentiation of endogenous neural stem cells (NSCs) *in vivo*. Since lowered energy metabolism is known to mediate the ischemic effect, we examined the effect of hypoglycemia on the proliferation and the differentiation of NSCs *in vitro*. NSCs derived from the ganglionic eminence of E16 mice were cultured by the "Neurosphere" method using EGF containing medium for 7 days, and treated with a low glucose medium with or without 2-deoxyglucose (2-DG) after mechanical dissociation. The proliferative activity of NCSs was examined by the WST-8 assay and by counting of the number of neurospheres in the presence of EGF (20 ng/ml). Differentiation was induced by incubation in 1% FBS-containing medium lacking EGF and was evaluated by fluorescence-based immunocytochemistry and ELISA using anti-Tuj 1 or anti-GFAP antibodies. We found that transient hypoglycemia for 60 min did not roliferative activity. However, under continuous conditions, the proliferative activity of NSCs was significantly diminished and more neurospheres with a smaller diameter were observed compared with control medium conditions. Furthermore, continuous hypoglycemia with 2-DG suppressed the proliferative activity completely. On the other hand, hypoglycemia during the differentiation period drastically increased the differentiation of NSCs into neurons or astroglial cells. These results suggest that continuous hypoglycemia modulates the self-renewal and the differentiative activity of NSCs *in vitro*.

10. Three-dimensional culture of rat neural stem cells in collagen gel

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Recently we have reported the usefulness of neural stem cells (NSCs) transplantation for spinal cord injury in the adult rat. In this study we focused on type 1 collagen as a scaffold for axonal regeneration to enhance the effectiveness of transplantation of NSCs. The purpose of the present study was to determine the culture condition of NSCs in type I collagen gel prior to transplantation. NSCs derived from embryonic day 14 fetal green rat forebrains were cultured using the neurosphere method as reported previously. NSCs were then seeded in type 1 collagen (cell density: $1\times10^3-5\times10^7$, concentration of collagen gel: 0.03–0.5%) and the survival rate of NSCs was quantified. To analyze the phenotype of NSCs after culture, immunostaining with anti-Tuj1, anti-GFAP or anti-CNPase antibodies was performed and the number of each type of cell was quantified. To determine the effect of brain-derived-neurotrophic-factor (BDNF) on the neurite extension of NSCs cultured in the gel, BDNF was added to the culture medium at a concentration of 50 ng/ml. The appropriate culture conditions of NSCs in type I collagen gel were a cell density of $3\times10^6-1\times10^7$ cells/ml, and a collagen gel concentration of 0.05%. The immunostaining of NSCs after differentiation revealed that the ratios of GFAP(+)/GFP(+)cells, Tuj1(+)/GFP(+)cells and CNPase(+)/GFP(+)cells were 62%, 37% and 2% respectively. BDNF increased the number of Tuj1(+)/GFP (+) cells and the lengths of neuronal dendrites in collagen gel, compared with the NSCs cultured without BDNF. Our findings suggest that NSCs can survive and differentiate into neurons, astrocytes and oligodendrocytes under the appropriated culture condition with type I collagen gel, and that BDNF promote the neuronal differentiation and neurite extension. It is plausible that type 1 collagen may be an appropriate scaffold for NSCs transplantation with neurotrophic factors.

11. Isolation of neural stem cells from the ventricle-lining cells including tanycytes at the 3rd ventricle of adult rat brain

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It is known that the principal locations of neural stem cells (NSCs) in the adult brain are the subventricular zone of the lateral ventricle and the dentate gyrus of the hippocampus. In the present study, we showed that the ventricular wall of the 3rd ventricle is another site of location of NSCs in the adult rat brain. First we demonstrated by the standard method that numerous neurospheres could be generated in the culture of the tissue from the hypothalamic region including the wall of the 3rd ventricle. There are characteristically numerous tanycytes in the wall of the 3rd ventricle compared to the other parts of the ventricular axis. Tanycytes, a type of ependymal cell extending long cellular processes toward the brain surface, can be regarded as remnant embryonic radial glial cells. Next, we showed that ventricular lining cells isolated by FACS after staining with DiI gave rise to many neurospheres, which differentiated into neurons, astrocytes and oligodendrocytes in the differentiation medium. Injection of growth factors such as bFGF into the 3rd ventricle elicited prominent mitosis of the ventricular lining cells as demonstrated by BrdU uptake at the 3rd ventricle. Cell-labeling with a GFP-recombinant adenovirus as well as DiI staining indicated that cells generated by mitosis including new neurons at the ventricular surface which had migrated along the cellular processes of the tanycytes into the deep region of the hypothalamus. The floor tissue of the 3rd ventricle continuous with the infundibulum of the pituitary gland is totally different from other parts of the 3rd ventricle: this tissue gave rise to an extensive sheet of flat cells, with only a few neurospheres in the culture. These findings indicate that the ventricle-lining cells including tanycytes at the 3rd ventricle, except for the floor part continuous with the infundibulum, have the capacity to produce NSCs in the adult rat brain.

12. Roles of Gab1 in the maintenance and differentiation in neural stem cells

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Neural stem cells (NSCs) are defined by their ability to self-renew and generate a range of different central nervous system (CNS) cells. NSCs may play a major role in mammalian CNS development and continue to function throughout adulthood. It has been shown that a number of cytokines and growth factors regulate the maintenance, proliferation and differentiation of NSCs. However, the detailed mechanisms of these modulatory and regulatory process through complex signal transduction pathways remain to be elucidated. Recently, it was reported that gp130-mediated signaling, activated by the IL-6 family cytokines, supports the maintenance of NSCs and their differentiation into glial cells. Here, we focused on Grb2 associated binder 1 (Gab1) which is a downstream signaling mediator in gp130-mediated signaling and in epidermal growth factor (EGF) receptor-mediated signaling, known as a mitotic signal for NSCs. To investigate the role of Gab1 in the maintenance and/or differentiation of NSCs, we studied CNS development in gab1-deficient mice. Since $gab1-/-$ embryos died at mid-to-late gestation date, we first analyzed the number of NSCs in the striatum at embryonic day 14 by neurosphere formation assay. The lack of Gab1 did not affect the number of NSCs, but the loss of their responses to EGF was observed. However, the number of NSCs in the periventricular area of the lateral ventricle in the adult $gab1+/-$ mice increased. On the other hand, in the embryonic spinal cord, the number of oligodendrocyte precursor cells, assessed by immunostaining of Olig2, decreased in $gab1-/-$ embryos. These results suggest that 1) Gab1 supports the proliferation of NSCs and is essential for the proliferation or the survival of the OPC in the developmental stage, 2) Gab1 suppresses the maintenance of NSCs after birth.

13. Brain-derived neurotrophic factor alters laminar fate of early-generated neuronal progenitors by facilitating neurogenesis during cortical development

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During the development of cerebral cortex, neuroepithelial cells proliferate in the ventricular zone and newly born neuronal progenitors migrate toward their final destination according to the timing of their birth; earlier generated progenitors occupy the deeper layer of the cortex. Their laminar fate depends on environmental cues to which cells respond prior to mitosis. However, the specific molecules which regulate the laminar fate of cells have not yet been isolated and the molecular basis of cortical formation is poorly understood. Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family of neurotrophic factors, regulates the differentiation, survival, and maintenance of developing and adult neurons. Recently we observed the expression of BDNF in ventricular neuroepitheilal cells, and found that injection of BDNF into the E13 embryonic telencephalon *in utero* altered the destination of neuronal progenitors to deeper cortical layers than expected. To clarify the function of BDNF for motility of neuronal progenitors, *bdnf* and *green fluorescence protein* (GFP) expression vectors were transfected by electrophoration in utero, and location of the exogenous gene expressing cells were monitored at several time points. After co-transfection of GFP and BDNF tagged with FLAG epitope expression vectors, most of the GFP expressing cells (GFP^+ cells) possessed BDNF antigen assessed by an immunohistochemical assay using anti-BDNF, and anti-FLAG antibodies ($BDNF^+/GFP^+$ cells). As a control, only a GFP expressing vector was transfected. Many $BDNF^+/GFP^+$ cells were localized in the outer layer of the cortical plate and almost the same number of the double positive cells was also seen in the ventricular zone, although GFP^+ cells only located to the outer layer but not in the ventricular zone 4 days after gene transfer. After 3 weeks, $BDNF^+/GFP^+$ cells were distributed to II-VI layers of the cortex, although GFP^+ cells were restricted to the deeper layer, the typical position that E13 born neuronal progenitors occupy. These results suggest that BDNF may facilitate neurogenesis of ventricular progenitors, which may alter the laminar fate of neuronal progenitors.

14. Visualization and culture of mouse neural crest stem cells; a transgenic approach

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Neural crest stem cells originate from the dorsal region between the neural tube and the overlying epidermis during early vertebrate development. They extensively migrate from their birthplace to generate a number of differentiated cell types including both neuronal and non-neuronal derivatives at various sites in the embryo. To elucidate how different types of cells are produced from neural crest stem cells, we need to develop methods for identifying them prospectively *in vivo* and culturing them *in vitro*. For this, we took a transgenic approach towards marking neural crest cells with green fluorescent protein (GFP). We generated transgenic mice carrying the GFP gene under the transcriptional control of the promoter/enhancer of the mouse snail gene that is expressed in neural crest cells. Observation of transgenic embryos under the confocal microscope revealed that GFP expressing cells were distributed throughout the regions where neural crest cells are known to be located. By comparing this with the immunohistochemical expression pattern of the platelet-derived growth factor receptor α-subunit (PDGFRα), a specific marker of the non-neuronal crest cells, it was indicated that GFP-expressing cells consisted of both the neuronal and non-neuronal crest cells. When we cultured cells dissociated from the heads of the transgenic embryos under sphere-forming condition, we observed clonal propagation of a GFP expression cell. Resultant spheres appeared to include both the neuronal and non-neuronal crest cells, as judged by expression pattern of GFP and PDGFRα. Our mice line with neural crest cells marked by GFP will be useful to study neural crest development both *in vivo* and *in vitro*.

15. Directional control of neuroblast migration in the adult mouse brain by Slit proteins and ventricular cerebrospinal fluid flow

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Neuroblasts are continuously born in the subventricular zone (SVZ) of the lateral ventricles of adult mice. These cells migrate as a network of chains through the SVZ and the rostral migratory stream (RMS) into the olfactory bulb (OB). In this study, we showed that neuroblast migration in the SVZ parallels cerebrospinal fluid (CSF) flow in the ventricle. The choroid plexus (CP), the source of CSF, has a repulsive activity on SVZ cells, which is in part mediated by Slit proteins. Normal beating of ependymal cilia on the ventricular surface, an important propellant of the CSF flow, is required for control of directional cell migration. Our observations suggest that CP secretes neuroblast-repulsive factors that are distributed differentially within the ventricle by the coordinated beating of ependymal cilia.

16. Nipradilol promotes axonal regeneration of retinal ganglion cells in adult cats

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Nipradilol (NPL) is a blocker of alpha and beta adrenoreceptors, an NO-donor, and protects retinal ganglion cells (RGCs) against death after transection of the optic nerve (ON). I examined whether NPL promotes axonal regeneration of cat RGCs into a transplanted peripheral nerve (PN). A solution of NPL ($1 \times 10^{(-7)}$M), nipradilol without NO2 (DNNP, $1 \times 10^{(-7)}$M), sodium nitroprussid (SNP, 100 μM), db-cGMP (250 μM), or MK-801 (10 μM) was injected into the vitreous of anesthetized cats before ON transection. A PN segment was sutured to the cut end of left ON. RGCs extending axons to 10 mm (10R-RGC) or those to 20 mm (20R-RGC) were labeled with different fluorescent dyes injected into the graft at 10 mm or 20 mm. Numbers of labeled RGCs were estimated by counting labeled cells in 0.23 mm^2 squares separated by 1 mm. An injection of NPL increased the numbers of regenerated RGCs 4 and 6 weeks after the transplantation: average numbers of 10R-RGCs in NPL injected retinas were 8,736 ($N = 4$) at week 4 and 11,278 ($N = 4$) at week 6, while those in uninjected retinas were 2,138 ($N = 6$) at week 4 and 3,678 ($N = 7$) at 6 week, respectively. The proportions of 20R-RGCs versus 10R-RGCs were 35% (4 wk) and 80% (6 wk) in the injected retinas, higher than in the uninjected (control) retinas, 17% (4 wk) and 65% (6 wk). The first axons which were estimated to reach 20 mm did so in 2.3 wk, one week earlier than the control. An injection of DNNP (11,372, $N = 3$) or SNP (6,585, $N = 2$) increased the numbers but db-cGMP and MK-801 did not. NO may increase regenerating RGCs by surviving RGCs, and blocking the receptors by NPL may promote regeneration.

17. Transplantation of dopaminergic neurons generated from ES cells to rat models of Parkinson's disease

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Embryonic stem cells (ESCs) have the multipotentiality to differentiate into three lineages and terminally to a neural fate. Nowadays there are many methods reported concerning neural differentiation. The SDIA (stromal-cell derived inducing activity) method is an adequate method to induce dopaminergic neurons, but the culture system includes other types of cells such as PA6. To eliminate these cells, we used the reporter system *TH-EGFP*. Tyrosine Hydroxylase (TH) is a key enzyme in dopamine production so that it is a good marker for dopaminergic neurons. By using enhanced green fluorescent protein (EGFP) for the reporter gene, dopaminergic neurons could be visualized. We cultured two ESC lines (*TH-EGFP* and *CAG* (*chicken actin gene*)-*EGFP* group) on the PA6 layers. Cultured ES cells through SDIA produced dopamine. EGFP and TH production was increased during coculture in the *TH-EGFP* group. We transplanted ESC-derived cells and they survived, integrated and projected axons in a rat model of Parkinson's disease. Cells from both the *CAG-EGFP* and *TH-EGFP* groups ameliorated the symptoms of Parkinson's disease. Dopaminergic neurons generated through SDIA function *in vivo* as well as *in vitro* transplantation of whole culture-generated tumors in the brain. In order to carry out transplantation as a cure for Parkinson's disease, a purification

procedure such as the reporter system discussed herein is required.

18. Regeneration of the pyramidal tract after sharp severance in young rats

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In young rats, the pyramidal tract regenerated after spinal cord severance with a sharp blade, but regeneration failed if the severance were blunt. We used retrograde neuronal double labeling to provide convincing evidence for the occurrence of the axonal regeneration after sharp severance. Double labeled neurons that took up the first tracer from the lesion site and the second tracer from the injection site caudal to the lesion site were observed in the sensorimotor cortex. In anterograde tract tracing, various patterns of regeneration were observed. The vast majority of regenerated fibers descended in the normal tract in the most successful cases, whereas fibers descended partly normally and partly aberrantly or totally aberrantly in the less successful cases. To investigate the role of astrocytes in determining the success or failure of regeneration we compared the expression of GFAP, vimentin, neurofilament (NF) and collagen type IV immunoreactivity (IR) around the lesion between sharp and blunt severance. In both lesion types, astrocytes disappeared from the tract near the lesion within 3 hrs after severance. However, by 24 hrs after a sharp severance, immature astrocytes coexpressing GFAP- and vimentin-IR appeared in the former astrocyte-free area and regenerating axons crossed the lesion. By contrast, after a blunt severance the astrocyte-free area spread and axons never crossed the lesion. By 7 days sheet-like structures with collagen type IV-IR appeared in animals with failed regeneration and they persisted. A glial scar formed later in the lesioned tract, but not when regeneration was successful. On the basis of its spatiotemporal appearance, the glial scar is not the cause but an effect of the failure of regeneration. It appears likely that the major sign, and possibly cause of failure of axonal regeneration is the prolonged disappearance of astrocytes in the lesioned tract area. Immature astrocytes appear to guide regenerating axons.

19. Attempt to regenerate oligodendrocytes by genetic manipulation of endogenous progenitors in the injured spinal cord

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We have been exploring the possibility of repairing the damaged adult spinal cord using the latent regenerative capacity of endogenous neural progenitors. Our previous studies have demonstrated that a significant number of neural progenitors in the spinal cord proliferate in response to injury. Certain environmental cues, however, appear to inhibit their differentiation into neurons and oligodendrocytes, thus hampering regeneration of damaged tissue. In this study, we attempted to regenerate new oligodendrocytes in the spinal cord by genetic manipulation of endogenous progenitors. Transection injury was made in adult SD rats (7–8 weeks of age) at the T10 level. Subsequently, high titer recombinant retroviruses that express the oligodendrogenic bHLH factor Mash1 (pMXIG-Mash1: $2 - 6 \times 10^8$ CFU/ml $\times$ 30 μl) was administered into the damaged spinal cord together with FGF-2 and EGF, mitogens for progenitors. Under these conditions, virus infection was observed in $2 - 4 \times 10^4$ cells per animal. We found that a much higher percentage (about 50%) of Mash1 virus-infected cells became NG2$^+$ oligodendrocyte precursor cells (OPCs) compared with the control virus-infected cells (10%), which resulted in a substantial increase in the total number of OPCs within injured tissue 1 and 2 weeks after transection. A minor population (4%) of these Mash1$^+$ cells further differentiated into PLP$^+$ oligodendrocytes 4 weeks after injury, which was not seen in control virus-infected cells. These results suggest that genetic manipulation of endogenous progenitors may be a promising strategy to regenerate oligodendrocytes in the injured spinal cord.

20. Axonal regeneration after administration of semaphorin3A inhibitor into the injured spinal cord

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Axons of the adult mammalian central nervous system (CNS) exhibit little regeneration after injury. It has been suggested that several axonal growth inhibitors prevent axonal regeneration in CNS. Recent research has demonstrated that semaphorin 3A (Sema3A) is one of the inhibitors of axonal regeneration that plays central roles after CNS injury. We extracted a substance from a fungus that inhibits the functions of Sema3A *in vitro* such as the chemorepulsive effect to neurite extension and growth cone collapse. To examine the effect of the Sema3A inhibitor *in vivo*, we a made spinal cord transection model using adult rats, and administered Sema3A inhibitor into the lesion site for 4 weeks using an osmotic mini pump (treatment group). In the control animals, phosphate buffer saline was administered into the lesion site (control group). In both groups, open-field walking was assessed using the BBB scale up to 14 weeks after the injury. Immunostaining for neurofilaments, GAP43 and CGRP were performed after perfusion with 4% paraformaldehyde at 14 weeks after the injury. The immunohistochemical analysis revealed that more extensive axonal regeneration was observed in the treatment group compared with the control group. Furthermore, significantly better functional recovery was observed in the treatment group than in the control group. These findings suggest that Sema3A plays a central role in the inhibition of axonal regeneration after CNS injury, and the Sema3A inhibitor is a possible therapeutic reagent for the treatment of spinal cord injury in human subjects.

21. Promotion of functional recovery by grafted human neural stem cells after spinal cord injury in primates

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Recent studies have shown that delayed transplantation of neural stem/progenitor cells (NSPCs) into the injured spinal cord can promote functional recovery in adult rats. However, a pre-clinical study using non-human primates is necessary before NSPCs can be used in clinical trials to treat human patients with spinal cord injury (SCI). We induced cervical contusion SCI in 10 adult common marmosets using a stereotaxic device. Nine days after injury, we performed *in vitro* expanded human NSPC transplantation in 5 randomly selected animals, and the remaining sham-operated control animals received culture medium alone. All of these animals survived for 8 weeks, during which we evaluated the recovery of motor function by measuring bar grip power and spontaneous motor activity, and monitored the magnitude of the SCI by the change in the intramedullary signals from magnetic resonance imaging. After 8 weeks survival, we sacrificed all the animals and performed a histological analysis, wevealed that the intramedullary high signal of MRI matched the formation of a neural cavity at the site of the injury. In the transplanted groups, the grafted NSPCs survived, differentiated into neurons, astrocytes, and oligodendrocytes, which made the neural cavities smaller than those in the control groups. There were significant improvements in the bar grip power as well as spontaneous motor activity of the transplanted animals compared with the control group subjects. Human neural stem/progenitor cells transplantation could be a feasible treatment for human SCI.

22. Fibrotic scar as a potent impediment to the regeneration of ascending dopaminergic axons

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Reactive astrocytes, chondroitin sulfate proteoglycans and the fibrotic scar all of which which occur in and around the lesion after injury in the adult mammalian CNS have been considered as obstacles for axonal regeneration. When ascending dopaminergic axons were transected in adult mice, reactive astrocytes and chondroitin sulfate immunoreactivity were observed around the lesion. Tyrosine hydroxylase (TH)-immunoreactive axons were confronted with the collagen IV-immunoreactive fibrotic scar and did not extend beyond the lesion. In contrast, reprojection of TH-immunoreactive axons to the striatum occurred in newborn mice transected at 7 days of age in which fibrotic scars were not formed, while reactive astrocytes and chondroitin sulfate immunoreactivity were still present around the lesion. Fibrotic scars containing dense collagen IV fibrils secreted by fibroblasts were formed in the lesion core of mice transected after 10 days of age. The temporal coincidence of the occurrence of fibrotic scar and unsuccessful regeneration in the developing brain strongly suggests that the fibrotic scar is a primary impediment to regeneration following injury in the adult CNS. To further confirm this hypothesis, 2, 2'dipyridyl, an inhibitor of collagen synthesis, was topically injected into the lesion site of both young and adult mice. In these animals, the fibrotic scar was completely eliminated and regenerated dopaminergic axons extended across the lesion to reproject in the striatum, despite the presence of reactive astrocytes and chondroitin sulfate immunoreactivity around the lesion.

23. Derivation and selective amplification of GABAergic neural progenitor cells from embryonic stem cells

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Up to 30% of the population of epileptics continue to have seizures despite treatment with antiepileptic drugs. Several groups have attempted to develop a new treatment for epilepsy. One of the trials supports the hypothesis that embryonic neural tissue implanted into previously kindled animals alters subsequent seizure susceptibility, and opens the possibility that neural grafts may be used for the therapy of medically intractable cases of epilepsy. Recent advances in the establishment and controlled differentiation of embryonic stem cells (ESCs) have been bringing us new model systems to access the biology of early mammalian development including human development, and new approaches to regenerative medicine. ESCs differentiate into multiple cell lineages including neurons and glia. Subsequent to the isolation of NSCs, they can be expanded, genetically modified and extensively characterized prior to transplantation. NSCs represent an ideal cell type for transplantation purpose in the central nervous system due to their multipotency. In the present study, we optimized the effective method to induce the deferentiation of GABAergic neurons from neurosphere that have been derived from ES cells. In order to induce neural differentiation of mouse ESCs, we chose clonal embryoid body formation on non-adhesive bacterial-dishes in the presence of 10% fetal calf serum (FCS) in the presence of Noggin (inhibitor of bone morphogenic factor). Neural progenitors derived from Noggin-exposed embryoid bodies generate mainly cholinergic and GABAergic neurons, and the ability to generate both types of neurons decreases over time after an extended period of culture period. To further characterize the ESC-derived neural progenitors, we analyzed the primary spheres (P1), the secondary spheres (P2), and the tertiary spheres (P3), with immunohistochemical techniques, RT-PCR, and amino acid analysis. P1 neurospheres generated more ChAT+neuron than P2 or P3. On the other hand, the expresion of GABA was more strongly detected in the P3 neurospheres than the P1 neurospheres. Transplantation of the neural progenitors into the hippocampus of a mouse model of epilepsy resulted in the functional recovery of the subjects. ESC-derived neurospheres after several passage might be useful for standardization of a donor cell population for cell transplantation therapy for clinically intractable epileptics.

24. Differentiation and migration of intrinsic neural precursor cells following traumatic brain injury

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The response of intrinsic neural precursor cells after traumatic brain injury (TBI) is not fully understood. In the present study, we examined the temporal pattern of existence and migration of these immature cells in a rat cerebral contusion model. Male Wistar rats were deeply anesthetized and a cortical contusion was induced to the unilateral sensorimotor cortex with a controlled cortical impact device. After injury, bromodeoxyuridine (BrdU; i.p.) was administered to the rats to detect the proliferating cells. Rats were sacrificed either immediately after injury, or at 1, 3, 7 or 14 days after injection, and immunohistochemically processed for BrdU, vimentin, nestin, GFAP, NeuN or doublecortin (DCX) which is known as a cytoskeletal protein which exists exclusively in immature migrating neuroblasts. Histological evaluation was focused on the subventricular zone (SVZ) of the lateral ventricle and the lesion sites including the dentate/hippocampal areas. The thickness of the ventricular wall was markedly increased following the injury. It became maximum at 3 days after injury, and was approximately 220% compared to the normal control. This increase of wall thickness appeared to be induced by the increased number of BrdU-positive proliferating cells, which also showed positive staining for vimentin and nestin expression. DCX-positive cells existed in the SVZ and dentate/hippocampal areas, 1 day after injury. DCX immunoreactivity was most prominent at 3 days after injury and the neuroblasts appeared to migrate extensively via the corpus callosum. In the contusion area, a few DCX, but no NeuN positive cells could be seen, however the number of the cells was unlikely to be sufficient for reconstruction of damaged neural circuits. After TBI, neural precursor cells respond immediately and initiate proliferation. Some of these cells show DCX immunoreactivity, suggesting that new neurons can generate in response to injury. DCX may be a useful marker for detection of regenerative responses after TBI.