Effects of Rat Glial Cell Line-Derived Neurotrophic Factor on Kainic Acid-Induced Hippocampal Damages

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Kainic acid 에 의한 해마 손상에 대한 GDNF 의 효과

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Abstract

In order to determine the potential of a recombinant adenoviral vector, which expresses rat glial cell line-derived neurotrophic factor (Ad-GDNF), as a therapeutic modality for the treatment of excitotoxic damage, Ad-GDNF was pre-inoculated into kainic acid (KA)-treated rat hippocampi. Ad-GDNF pretreatment resulted in the suppression of KA-induced stage 6 tonic-clonic convulsions. In situ apoptosis labeling demonstrated a significant reduction in apoptotic cells in the CA3 and dentate hilus, in the Ad-GDNF-treated rats, compared to KA injected rats. Striking reductions in the density of GAD-67 neurons were observed in the CA3 and dentate hilus of the KA injected rats. On the other hand, GAD-67 positive cells recovered to control levels in the Ad-GDNF rats.

Following the administration of KA, GDNF expression increased in the CA3 and dentate hilus areas, in which apoptotic cell death had occurred at significant levels. In the Ad-GDNF rats, enhanced GDNF expression was reduced to control levels.
Immunoblot analysis further confirmed that GDNF and p53 expression had increased, while GAD-67 levels decreased following KA injection, and then returned to control levels in the Ad-GDNF rats. However, Bcl-2, anti-apoptotic factor, was opposite to p53 expression. Although the basal levels of Bcl-2 were expressed in saline and KA rats, Bcl-2 protein was up-regulated in Ad-GDNF rats.

In series of experiment, animals received the KA 3 days prior to Ad-GDNF and hippocampal neural stem cells (NSCs)-infected Ad-GDNF. Seven days after treatments of Ad-GDNF or Ad-GDNF-NSCs, hippocampus was sampled. *In situ* apoptosis labeling was only slightly reduced in Ad-GDNF and Ad-GDNF-NSCs, compared to Ad-GDNF-infected rats before KA insults in Figure 2. Moreover, immunofluorescence labeling, ELISA assay, and immunoblot analysis revealed that GDNF has no effects on the protection, alteration, and induction of GAD-67-positive interneurons. In contrast, Ad-GDNF pre-inoculation before KA injection played a protective role in GAD-67 interneurons. However, GDNF immunoreactivity (IR) and protein level were increased significantly by Ad-GDNF infection. p53 and Bcl-2 protein levels were down- and up-
regulated by Ad-GDNF, respectively.

To test the applications for cell replacement therapy, hippocampal neural stem cells (NSCs) were studied on the ability of the differentiation to neurons and glia, and the migration to each hippocampal region using BrdU immunofluorescence double-labeling and immunoblotting analysis. Bcl-2 was related to cell survival, apoptosis, and migration. In this experiment, Bcl-2 protein was not increased by NSCs only, but increased dramatically when treated with Ad-GDNF. Although Bcl-2 protein, which is related to cell survival in Ad-GDNF rats, was up-regulated, TUNEL-positive cells were not decreased significantly in hippocampus areas. In contrast, Bcl-2 protein in Ad-GDNF-NSCs was increased importantly by NSCs transplantation. Migrating cells were observed in Ad-GDNF-NSCs more than NSCs only rats. In this study, Ad-GDNF-infected NSCs were successfully migrated from the target region to a variety of hippocampal areas, and some of them were differentiated to GDNF and GAD-67 expressing cells. It suggests that GDNF and GDNF-induced factors also have an effect on the survival and migration of hippocampal neural stem cells.
Taken together, GDNF may prevent the apoptosis from excitotoxic insults, and GDNF or GDNF-induced factors, as a p53 and Bcl-2, may be related to the survival of intact GAD-67-positive interneurons, and the migration and differentiation of NSCs. Thus, Ad-GDNF and NSCs may be the useful tools for controlling the seizure-stimulated patho-physiological changes and the cell replacement therapy for seizure-induced damage by inducing KA excitotoxicity.
요 약

홍분성 신경약물에 의한 손상에 대하여, 쥐 glial cell line 에서 유래한 신경영양인자를 발현하는 제조합 아데노바이러스 (Ad-GDNF)의 치료 효율성을 검증하기 위하여 kainic acid (KA)가 복강내로 주입된 쥐의 뇌 조직인 해마에 Ad-GDNF 를 전처리하였다. Ad-GDNF 의 전처리는 KA 에 의해 유도되는 발작 단계 중 stage 6 인 강직성-간대성 발작을 억제시켰다. 세포 사멸에 대한 in situ 염색은 KA 만 주어진 쥐와 비교하여 Ad-GDNF 가 처리된 쥐에서는 CA3 와 dentate hilus 부위에서 현저한 세포 사멸 감소가 나타났다.

또한, 상당량의 GAD-67 발현 신경세포의 감소가 KA 처리된 쥐의 CA3 와 dentate hilus 에서 관찰되었다. 반면에 Ad-GDNF 를 주입한 쥐는 대조군과 비슷한 정도의 GAD-67 발현이 이루어졌다.

KA 처리에 의해, 세포 사멸이 현저하게 발생한 부위인 CA3 와 dentate hilus 에서 GDNF 발현도 증가하였다. Ad-GDNF 가 주입된 쥐에서 GDNF 의 발현은 대조군과 비슷하였다. 단백질 분석을 통해 KA 는 GDNF 와 p53 단백질의 발현을 증가시키고, GAD-67 발현을 감소시키지만, 감소된 GAD-67
단백질의 발현은 Ad-GDNF 쥐에서 대조군과 비슷하게 회복되었다. 그러나 항세포 사멸 인자인 Bcl-2 발현은 p53 발현과 반대의 결과가 관찰되었다. KA 와 생리식염수가 주어진 쥐에서 Bcl-2 의 발현은 낮았지만, Ad-GDNF 쥐에서는 증가함이 보였다.

다음 실험에서, Ad-GDNF 와 Ad-GDNF 에 감염된 해마신경줄기세포 (Ad-GDNF-NSCs)가 해마로 주입되기 3 일전에 KA 가 쥐에게 처리되었다. 그리고 7 일 후 해마는 실험에 사용되었다. 세포 사멸에 대한 염색에서, 이전 실험의 Ad-GDNF 전처리처럼 현저한 세포 사멸 감소가 나타나진 않았지만, Ad-GDNF 와 Ad-GDNF 에 감염된 해마신경줄기세포 (Ad-GDNF-NSCs) 처리된 쥐에서 의미 있는 세포 사멸 감소가 관찰되었다. 또한, 면역형광염색, ELISA, 단백질 분석의 결과는 GAD-67 발현 신경세포의 보호, 변이, 유도에 큰 효과가 없는 것으로 나타났다. 반면에 Ad-GDNF 가 전처리 되었던 앞선 실험에서는 GAD-67 발현 신경세포에 대한 보호 효과가 보여졌다. 그러나, 조직염색과 단백질의 양에서 GAD-67 이 약간의 증가, 또는 거의 변화가 없는 것과는 대조적으로 GDNF 는 Ad-GDNF 주입에 의해 현저하게 증가하였다.
p53과 Bcl-2의 단백질 발현은 Ad-GDNF에 의해 각각 감소, 증가하였다.

또한, 과발현된 GDNF는 신경진구세포의 이동과 분화를 조절하는 유전자들을 유도한다. 세포대체치료의 가능성을 실험하기 위해서, 해마신경줄기세포가 신경세포와 glia 세포로 분화할 수 있는지, 그리고 분화된 세포들이 세포 이동의 정도를 나타낼 BrdU와 결합할 수 있는지를 면역형광이중염색을 통해 검증하였고, 이들의 단백질 분석도 실시하였다. Bcl-2는 세포의 생존, 사멸, 이동과 관련되어있다. 이 실험에서 Bcl-2의 단백질은 해마신경줄기세포(NSCs)만 주입되었을 때에는 증가하지 않았지만 Ad-GDNF에 의해서 증가하는 것으로 나타났다. 세포생존과 관련된 Bcl-2의 단백질 발현이 Ad-GDNF 쥐에서 증가하였지만 세포 사멸 염색에서는 큰 효과가 없었다. 그러나 Ad-GDNF 감염된 해마신경줄기세포가 처리된 쥐에서는 Bcl-2 단백질 발현이 현저하게 증가하였다. 또한 해마신경줄기세포만 주입된 쥐에서보다는 Ad-GDNF 감염된 해마신경줄기세포가 주입된 쥐에서 더 많은 세포 이동이 관찰되었다. 본 실험에서, Ad-GDNF에 감염된 해마신경줄기세포는 비록 주입된 세포수에
비교하여 적은 양이지만 세포가 주입된 지점에서부터 다양한 해마 조직 부분들로 성공적으로 이동하였고, GDNF 와 GAD-67을 발현하는 세포들로 분화되었다. 이것은 GDNF 와 GDNF 에 의해 유도된 인자들이 해마신경세포의 생존과 이동에 효과가 있었음을 암시한다.

결론으로, GDNF 는 홍분성 신경약물로 인해 발생한 손상에 대해 세포 사멸을 억제하는 효과를 가지는 것으로 사료되며, GDNF 또는 GDNF 에 의해 유도되는 p53 과 Bcl-2 같은 인자들은 GAD-67 발현 신경세포의 생존에 영향을 미치며, 해마신경줄기세포의 이동과 분화에 효과가 있는 것으로 사료된다. 따라서, Ad-GDNF 와 해마신경줄기세포는 발작으로 유발된 병리학적-생리학적 변이와 KA 신경독성에 의해 유도되는 발작에 따르는 손상에 대한 보호와 세포대체치료에 유용할 것으로 사료된다.
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Introduction

Kainic acid (KA) is a cytotoxic agent which causes pronounced excitation of mammalian CNS neurons (Johnston et al., 1974). Intraperitoneal injection or microinjection of KA into the hippocampus has been demonstrated to cause seizure behavior and pathological damage in rodents. These effects mimic those reported in human temporal epileptic brains (Ben-Ari, 1985; Ueda et al., 1997). KA injection has also been reported to induce cellular and molecular alterations (Nadler et al., 1980b; Ben-Ari, 1985; Shetty and Turner, 2000b; Shetty and Turner, 2001) including cell loss, apoptosis, neurogenesis, and the modification of synaptic transmission systems, particularly in the hippocampal area. Such alterations in the hippocampus culminate in the reorganization of circuitry and hyperexcitability in the CA3 and dentate regions. Glia cell line-derived neurotrophic factor (GDNF) reportedly functions as a neurotrophic factor for midbrain dopaminergic neurons (Shetty and Turner, 1999a), motor neurons (Nadler et al., 1980a), and cerebellar Purkinje cells (Robert et al., 1997). In addition, GDNF exerts protective effects on axotomy-induced degeneration (Beck et al., 1995), and protects nigral dopamine neurons against 6-hydroxydopamine toxicity in vivo (Kearns and Gash, 1995). Recent research shows that intracerebroventricular and intraparenchymal administration of GDNF protects the cerebral hemispheres from damage by middle cerebral artery (MCA) occlusion (Kitagawa et al., 1998). After KA
stimulation, the expression of GDNF mRNA and protein levels in the dentate gyrus and CA1-4 regions of the hippocampus increased significantly. This increase in GDNF mRNA levels has a slow onset and is relatively prolonged, suggesting the possible involvement of GDNF in long-lasting structural and/or functional reorganization in the hippocampal formation (Humpel et al., 1994; Mikuni et al., 1999). Moreover, GDNF effectively suppresses the production of KA-induced hydroxyl free radical production in the hippocampus. Histological analysis revealed that GDNF reduced the level of damage occurring to the pyramidal neurons in the CA3 and CA4 regions of the hippocampus (Henrich et al., 2004). This implies a potential use for GDNF supplements in the treatment of KA-induced excitotoxic damage.

In this study, adenoviral vector-encoding rat GDNF (Ad-GDNF) was constructed and assessed with regard to its protective effects on chemoconvulsant-induced hippocampal damage. Among the variety of alterations induced by KA injection, neuronal cell loss, apoptosis, and neurogenesis were monitored. Furthermore, the expression levels of GAD-67, p53, Bcl-2 and GDNF were evaluated. GAD-67, a GABA synthesizing enzyme, is known to be down-regulated after KA injection (Shetty and Turner, 2000a; 2000b; 2001). GAD-positive interneuron incurs significant and permanent alterations in the adult hippocampus following degeneration of CA3 pyramidal neurons induced by an intracerebroventricular administration of KA. Therefore, strategies that restore in GAD-positive interneuron to levels observed in intact hippocampus may be beneficial for both
restoring the functional inhibition and ameliorating hyperexcitability (Shetty and Turner, 2000b; 2001). p53 expression, as a marker of apoptotic cell death, is known to be elevated in damaged neurons in acute models of injury, including ischemia and seizure (Morrison and Kinoshita, 2000; Myriam et al., 2002). The systemic injection of KA induced p53 expression in neurons exhibiting morphological damage (Morrison and Kinoshita, 2000). This suggests that p53 induction may be linked to apoptosis, specifically apoptosis as the result of seizure-associated excitotoxicity. Lack of p53 has been demonstrated to protect neurons from a wide variety of acute toxic insults, including KA, ionizing radiation, MPTP, glutamate, etc. (Morrison et al., 1996; Morrison and Kinoshita, 2000). Bcl-2, anti-apoptotic factor, plays an important role in the maintenance of the balance between the death and survival of cells under different physiological and pathological conditions. The expression of Bcl-2 is increased in the rat brain following KA-induced seizure or ischemia reperfusion. (Lee et al., 2002). Induction of Bcl-2 blocks cell death after a variety of stimuli such as glutamate and reactive oxygen species (Morrison et al., 1996). Further, Bcl-2-expressing cells show enhanced migration and invasion in Matrigel invasion as well as in fetal rat brain (Wolfgang et al., 1998).

Neural cells with stem cell properties have been isolated from the embryonic, neonatal, and adult rodent CNS (Jonathan et al., 1998). Neural stem cells (NSCs) are operationally defined by their ability to differentiate into cells of all neural lineages; to
self-renew; and to populate developing and/or degenerating CNS regions (Mckay, 1997; Morrison SJ et al., 1997). In the KA models of temporal lobe epilepsy, repair of damaged tissues is critical for the survival. However, the adult mammalian CNS has weak capabilities for both endogenous cell replacement and pattern repair (the rewiring of specifically organized long-distance connections), both of which are essential to achieve a significant functional recovery. Cell transplantation might help us to overcome the intrinsic inability of the nervous tissue to replace lost elements. In addition, grafted cells might produce some beneficial effects by providing host cells with trophic support (Ferdinando and Elena, 2002). To test the applications for cell replacement therapy, in this experiment, hippocampal neural stem cells (NSCs) were shown to have the ability of differentiation to neurons, glia, GAD-67, and GDNF expression cells.

Ad-GDNF showed the obvious protective effects against KA excitotoxicity. Both an in situ apoptosis assay and immunohistochemistry revealed the reduction of apoptotic cells in hippocampal tissues, and the protection of GAD-67-positive GABAergic interneurons rescued by Ad-GDNF. The gene susceptible to regulation by p53 is Bcl-2, which exhibit repression (Morrison et al., 1996). The relationship between p53 and Bcl-2 from immunoblotting results suggests that Bcl-2 was up-regulated by Ad-GDNF may be the protective role in the GAD-67-positive GABAergic interneurons through the anti-apoptotic action and related with successful migration of NSCs from target region.
Materials and Methods

Animals and injection of kainic acid

Male Sprague-Dawley rats, weighing 200-220 g each, were used in this study. The animals were housed in groups of 2-3 per cage, in a room with a controlled light-dark cycle (12 h light/12 h dark) and temperature (23°C); they were given free access to laboratory feed and tapwater. Kainic acid (KA; 10 mg/kg, ICN Biomedicals, Ohio, U.S.A) was dissolved in saline (adjusted to pH 7.4) and injected intraperitoneally into rats. Experimental rats were infected with Ad-GDNF 7 days prior to KA injection, and were sampled 3 days after injection of KA. Control animals received an equal volume of saline intraperitoneally. In series, rats were received the KA prior to 3 days the transplantation of only NSCs or NSCs that were infected the Ad-GDNF into the rat hippocampus.

Behavioral assessment

KA-injected animals were observed during the seizure period to determine the duration and severity of seizure activity. The onset of seizures occurred within 30 minutes of injection, and persisted for 2-4 hours. Behavioral observations during the first 1-2 hour after KA injection revealed a progression of increasingly severe seizure-related behaviors. These behaviors were scored on a scale from 0 to 7, with 0
representing normal behavior, and 7 representing death. Within a few minutes after KA injection, typical activities such as walking, exploring, sniffing, and grooming ceased, and the rat became motionless (stage 1). This immobility was frequently followed by a period of forelimb and/or tail extension, giving the appearance of a rigid posture, and/or running fits (stage 2). Automatisms, such as repetitive scratching, circling, or head bobbing (stage 3) were followed, in most animals, by seizure behaviors comprised of forelimb clonus, as well as rearing and falling (stage 4). This pattern sometimes repeated continuously (stage 5). Surviving rats exhibited more severe tonic-clonic seizures, characterized by barrel rolling and the inability to rise (stage 6) (Morrison et al., 1996).

**Hippocampal neural stem cells (NSCs) and Differentiation**

Cells obtained from the embryonic rat hippocampus during active neurogenesis (E16) have been immortalized (Shetty and Turner, 2000b). Hippocampus was removed from rat embryos (E16) and mechanically dissociated with a fire-polished Pasteur pipette in serum free medium composed of a 1:1 mixture of Dulbeccos modified Eagles medium (DMEM) and F-12 nutrient (Gibco, Grand Island, NY, USA). Cells were plated 2.6 × 10^6 cells into 25 cm² tissue culture flasks (Falcon, Franklin Lakes, NJ, USA) with no substrate pretreatment. The culture medium was supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml). Medium was changed every 5 days.

Cells within undifferentiated spheres were immunopositive for the immature cell
marker nestin (Chemicon, Temecula, CA, U.S.A; data not shown). To assess the differentiation capacity of these cells, it was dissociated in 0.25% trypsin and re-plated on 24 wells with poly-L-ornithine coated glass coverslips at 2.4×10^5 cells/well. Individual wells were supplied with growth medium that is DMEM/F-12 containing 7% fetal bovine serum (FBS). The cultivation after 3 days, antibodies that are nestin, MAP-2, GFAP and Gal-C, detected differentiated cells.

**BrdU injection and exposure on NSCs culture**

5-bromo-2-deoxyuridine (BrdU; Sigma, U.S.A) was administered to the rats, in order to assess the proliferative activity of the hippocampal regions. All rats received BrdU (120 mg/kg, sigma) dissolved in saline, and given as an intraperitoneal (i.p.) injection, 1 day prior to KA treatment. To identify the transplanted NSCs, BrdU (10 µM/flask) was treated also in growth medium at 1 day prior to Ad-GDNF infection to NSCs.

**Construction of the recombinant GDNF adenoviral vector (Ad-GDNF)**

Primary cultures of dissociated cortical astrocytes were obtained from the brains of 1-3 day old neonatal rats. Cells were mechanically dissociated with a fire-polished Pasteur pipette in 8 ml of Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco, U.S.A) medium, supplemented with 7% heat-inactivated fetal bovine serum and 10mM Na₂HCO₃. The cells were then plated onto poly-D-lysine-
coated 75 cm³ tissue culture plates (8×10⁵ cells/plate).

After 2 weeks of astrocyte culture, total RNA was extracted by the guanidinium isothiocyanate extraction procedure, with the addition of phenol-chloroform-isoamylalcohol to increase RNA purity. The prepared total RNA sample was analyzed by RT-PCR. cDNA synthesis then was performed, using reagents from a single master mix (Bioneer, Korea).

Two sets of PCR primers were used for glial cell line-derived neurotrophic factor (GDNF, GenBank Accession No. L15305). The primer used for the PCR of GDNF (nucleotides 1-700, 700 bp) was as follows: forward, 5´-ggtctacggagaccggatcc-3´; reverse, 5´-tctctggagccagggtcaga-3´. PCR was performed with 2µl of cDNA in a 50 µl reaction mixture, containing 10 pM of the primer, 5 U of Ex Taq polymerase (TaKaRa, Japan), 2.5 mM of dNTP, and 5 µl of 10 × buffer. The reaction was performed in a 0.2 ml tube in a Biometra thermal cycler. The 30 cycles of PCR amplification progressed as follows: denaturation at 94°C for 40 seconds, annealing at 69°C for 40 seconds, and extension at 72°C for 2 minutes. PCR products were subjected to 0.8% agarose mini-gels, stained with ethidium bromide, and visualized under UV light.

The Ad-CMV5-GDNF adenoviral vector was constructed by the insertion of the 742 base pair (bp) HincII/SmaI fragment of GDNF, which had been isolated from normal astrocytes, in between the single BglII and PmeI sites of Ad-CMV5, down-stream of the CMV promoter (Quantum, U.S.A). In order to generate a recombinant adenovirus, 5 µg
of linearized Ad-CMV5-GDNF plasmid DNA was co-transfected with 5 \( \mu g \) of the large ClaI fragment of Ad-CMV5 DNA into transformed human kidney cell line 293, using FuGENE 6 (Roche, Germany). After 10 days of incubation, clumps of the 293 cells routinely detached from the monolayers, manifesting as a plaque. Virus titer was determined by the Tissue Culture Infectious Dose 50 (TCID50) method. Titers of Ad-CMV5-GDNF \((2 \times 10^5 \text{p.f.u.} / \mu l)\) were statistically calculated by TCID50, using Karber's formula.

**Adenoviral (Ad-GDNF) infection and NSCs transplantation**

In order to inoculate the viral stock 7 days prior to KA injection, rats were anesthetized with a mixture of ketamine (50 mg/ml) and zylazine (6 mg/ml), at a dose of 1.5 ml/kg body wt, and then placed in a stereotaxic apparatus. In each rat, the dorsal surface of the skull was exposed by midline incision, and a burr hole was drilled at the following coordinates: anteroposterior, -3.3 mm to bregma; and lateral, 3.5 mm right lateral to the midline. A 10-\( \mu l \) Hamilton syringe fitted with a 25-gauge needle and filled with viral stock solution (Ad-GDNF or Ad-lacZ) was placed over the burr hole and inserted 3.7 mm below the surface of the brain. 10 \( \mu l \) of viral stock solution at \( 5 \times 10^6 \) p.f.u./\( \mu l \) was then injected, at a rate of 2 \( \mu l / \text{min} \). The needle was left in place for 10 minutes, and slowly retracted. Control animals’ brains were injected with an equal volume of saline and vehicle \((5 \times 10^6 \text{p.f.u.} / \mu l)\). After 7 days of Ad-GDNF infection,
rats were injected with KA and sampled 3 days after KA injection for investigation.

In series of experiment, rats were received KA (i.p.) prior to 3 days the transplantation of only NSCs and NSCs (1.6-1.8 × 10^5 cells/rat) which was infected Ad-GDNF (3 × 10^8 p.f.u/μl), and was sampled at 7 days after for the studies. 3 days prior to KA administration, other group rats were injected also with saline and Ad-GDNF into the hippocampus.

**Nissl staining**

For Nissl staining, sections were mounted on 3-Aminopropyltriethoxysilane (AES)-coated slides, air-dried, dehydrated by immersion in ascending grades of alcohol (70, 90, 95, and 100%, 3 minutes each), and treated with xylene for 10 minutes. The sections were then treated with descending grades of alcohol (100, 95, 90, 70, and 50%, 3 minutes each), immersed in distilled water for 5 minutes, and incubated for 1 minute in a 1% solution of cresyl violet. Sections were then washed thoroughly in cold tap water, rinsed briefly in 1% acetic acid solution for 10 seconds, dehydrated by immersion in ascending grades of alcohol, cleared with xylene, and coverslipped using Permount.

**Histology**

Animals were deeply anesthetized and trans-cardially perfused with 100 ml phosphate buffer (pH 7.4) followed by fixative (4% paraformaldehyde in phosphate buffer, pH 7.4)
delivered over 30 minutes. The brain was rapidly removed via decapitation, kept in the same fixative for 24 hours at 4°C, and then incubated in 20% sucrose for 24 hours. The brain was then serially cut into 10 µm coronal sections on a cryostat. Brain sections were rinsed 3 times in PBS, and then were preincubated in 10% normal goat serum for 2 hours. Goat polyclonal anti-rabbit GAD-67 (1:200, Santa Cruz Biochemical, San Diego, CA, U.S.A), GDNF (1:200, Santa Cruz Biochemical, San Diego, CA, U.S.A), and mouse monoclonal anti-BrdU (1:150; Chemicon, Temecula, CA, U.S.A) were diluted with PBS, containing 0.3% Triton X-100 and 1.5% normal goat serum, and applied to consecutive sections, which were then incubated for at least 24-72 hours at room temperature in a humidified container. In order to determine the efficiency of the in vivo gene delivery by Ad vector, the sections receiving the Ad-lacZ were also labeled by rabbit polyclonal anti-β-galactosidase (1:1500; Chemicon, Temecula, CA, U.S.A).

For immunofluorescence double-labeling for BrdU and GDNF or GAD-67, the sections were pretreated to denature DNA by the following steps: 2 h incubation in 50% formamide/2× SSC [standard saline citrate (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7] at 65°C; 5 min rinse in 2× SSC; 30 min incubation in 2 M HCl at 37°C, and 10 min rinse in 0.1 M boric acid, pH 8.5. Sections then were blocked and incubated for 48 h at 4°C with a pooled solution of mouse monoclonal anti-BrdU (1:150; Chemicon, Temecula, CA, U.S.A) and GDNF (1: 250) or GAD-67 (1: 200).

The primary antibody was conjugated with rabbit anti-goat-Texas Red and goat anti-
mouse- or rabbit-FITC secondary antibody (Vector Laboratories, Burlingame, CA, U.S.A.) at 37°C for 1-2 hours. In brief, sections were rinsed with PBS, and mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, U.S.A.). After being mounting on coverslips, sections were directly observed under a fluorescence microscope.

**TUNEL assay**

To more accurately assess apoptotic cell death, we used terminal transferase (TdT)-mediated dUTP nick end-labeling (TUNEL). This procedure has been used extensively to monitor apoptotic cell death. The TUNEL assay procedure was performed according to the manufacturer’s protocols (Roche, Penzberg, Germany), with minor modifications.

Brain slices, overspread with OCT compound, were sectioned at 10 μm. Cryo-sectioned brain slices were placed in PBS (pH 7.3), and incubated at 80°C for 20 minutes. After the slides were washed for 5 minutes with PBS, the sections were incubated for one hour at room temperature with 10% normal goat serum, containing 0.3% Triton X-100. In brief, sections were rinsed twice with PBS for 5 minutes, and slides were incubated for 1 hour in a humidified container at 37°C, with an TUNEL reaction mixture containing the following: 5 μl enzyme solution (Terminal deoxynucleotidyl transferase (TdT) in storage buffer); 50 μl label solution (fluorescein–nucleotide mixture in reaction buffer). After the slides had been washed
three times in PBS, sections were directly observed under a fluorescence microscope.

**ELISA assay**

Analysis of GDNF concentration was performed using an enzyme-linked immunosorbent assay (ELISA). In brief, plates (Maxisorp, NUNC, US) were coated overnight at 4°C with antigen in a carbonate coating buffer, at a pH of 8.2, followed by 2 hours of blocking at room temperature. The samples and standards (concentration 1000 pg/well) were applied to the plates, and incubated for 6 hours at room temperature, with agitation. Wells were washed and rabbit polyclonal anti-GDNF antibody (1:1000; Santa Cruz Biochemical, San Diego, CA, U.S.A) was added before plates were incubated for 2 hours at room temperature. After another washing step, anti-rabbit IgG-HRP conjugate was added, and the plates were agitated at room temperature for 2 hours. Following a final wash procedure, TMB and peroxidase substrate were applied to the wells. After 15 minutes of incubation at room temperature, the reaction was stopped with 1 M H₂SO₄, and the absorbance was measured at 450nm using a spectrophotometer. The standard curve was linear in the observed detection range.

**Immunoblot analysis**

Rats were sacrificed by decapitation, and the brain was rapidly extracted from the skull. The hippocampus was excised and quickly put on ice. The tissue samples were
homogenized in homogenization buffer (10 mM Tris-HCl buffer, at a pH of 7.4, containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1% Triton X-100, and 100 mM NaCl) using a teflon-coated homogenizer. The homogenate was centrifuged 30 minutes at 10,000 x g and 4°C, at which time the supernatant was collected to be used as a sample for immunoblot analysis.

Total proteins from the hippocampus were extracted using a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. After boiling for 10 minutes, about 15 µg of crude protein lysate per well was separated by 7.5 and 10% SDS–PAGE. This was then transferred to nitrocellulose filters, where it was allowed to react with specific antibodies recognizing GDNF, GAD-67, Bcl-2 and p53 (Santa Cruz Biochemical, San Diego, CA, U.S.A.), and then visualized with an enhanced chemiluminescence (ECL) system (Amersham Biosciences, UK) using the manufacturer’s recommended procedures. The values of the detected proteins were evaluated using ImageTool (UTHSCSA, San Antonio, CA, USA), based on a gray-scale threshold.
Results

Protective effects of Ad-GDNF against KA-induced cell death

Three days after KA injection, we examined the protective effects of the recombinant adenoviral vector encoding for rat glial cell line derived neurotrophic factor (Ad-GDNF) on the hippocampal neurons. A remarkable loss of the hippocampal CA3 pyramidal cell layer and dentate hilus (indicated by asterisks in Fig. 1F and K) was detected in both the KA and vehicle group, accompanied with gliosis (glial scars) (indicated by arrowheads in Fig. 1H, I, M and N). The CA1 pyramidal cell layer and dentate granule cell layer regions of all groups proved resistant to KA injection (Fig. 1G, J, L, and O). This corroborated the previous reports that KA injection induces a selective loss of CA3 and dentate hilar regions via an excitotoxic process (Shetty and Turner, 1996; 1999a; 2000b; Nadler et al., 1980b). In the rats which had been infected by Ad-GDNF 7 days before KA injection (Ad-GDNF rats), both the CA3 pyramidal cell layer and dentate hilus were only slightly damaged (Fig. 1P), and gliosis was not detected (Fig. 1R and S). The CA1 pyramidal cell layer and dentate granule cell layer were not damaged in Ad-GDNF rats, although they were damaged in the control and KA-injected rats (Figs. 1Q and T).

In the cresyl violet-stained sections of the KA-injected rat brains, fragmented nuclei were observed in the CA3 and dentate hilus, indicating the occurrence of apoptotic cell death in these regions (Fig. 2E, F, H and I). We used the terminal transferase (TdT)-
mediated dUTP nick end-labeling (TUNEL) technique to assess apoptotic cell death more accurately. This procedure has been used extensively in the monitoring of apoptotic cell death. In this study, the TUNEL assay was used to measure the degree to which the hippocampal cells had been damaged. TUNEL-positive cells were not detected in the control rats (Fig. 2A, B, and C), while a large number of TUNEL positive cells were observed in the stratum pyramidale of the CA3 and dentate hilus region of the brains of both KA and vehicle-injected rats (Figs. 2E, F, H, and I). TUNEL positive-cells were also found, albeit sparsely, in the stratum pyramidale and stratum oriens of the CA1 and subgranular zones of the dentate gyrus, following KA injection (Fig. 2D and G). In contrast, no TUNEL-positive cells were found in the granule cell layer (data not shown). In the Ad-GDNF rats, the number of TUNEL positive-cells in all subfields was markedly reduced compared to both KA and vehicle-injected rats (Fig. 2J, K, and L).

**Suppression of KA-induced alteration of GAD-67 and GNDF expression**

Behavioral seizure results in the modification of synaptic transmission systems, the release of neurotrophic factor, and neurogenesis. In particular, the GABAergic interneurons undergo significant alteration following KA-injection, which confers hyperexcitability to the hippocampal circuitry (David, 2001; Shetty and Turner, 2001). In the next experiment, we attempted to ascertain whether Ad-GNDF injection suppressed the functional loss of GABAergic interneurons, using antibodies to the
GABA-synthesizing enzyme, glutamic acid decarboxylase-67 (GAD-67). In the control rat hippocampi, GAD-67 positive neurons were observed in all layers of the different subfields (Figs. 3A, B, and C). The density of GAD-67 neurons in KA or vehicle-injected rats appeared to have been reduced in all layers in every subfield (Figs. 3D, E, F, G, H, and I). This decrease in the number of GAD-67 positive neurons was particularly apparent in the dentate hilus, the junction of the hilus and the granule cell layer (Figs. 3F and I). Modest reductions were also observed in the stratum oriens and stratum radiatum of the CA1 and CA3 regions (Figs. 3D, E, G, and H). However, in the Ad-GDNF rat hippocampi, GAD-67 positive cells were observed in both the CA3 pyramidal cell layer and dentate hilus. In the CA1 regions of these rat brains, as many GAD-67 positive cells were observed as in the control rats (Figs. 3J, K, and L). Quantitative analysis corroborated this observation (Fig. 4). The density of GAD-67 positive neurons decreased in the CA3 and dentate gyrus of the KA injected rats by 85.8% and 67.6%, respectively, and by 41.9% in the CA1 region, compared with control values. In the Ad-GDNF rats, the reduction of density was 9.7%, 17.4%, and 16.3%, in the CA1, CA3 and dentate gyrus regions, respectively.

It has been established that GDNF expression increases as a result of KA-induced seizures (Humpel et al., 1994; Mikuni et al., 1999). Increased GDNF levels exert protective effects on a variety of neurons and tissues (Nadler et al., 1980a; Turner and Wheal, 1991; Morrison et al., 1996; Robert et al., 1997; Shetty and Turner, 1999a). In
order to characterize the relationship between cell loss and GDNF expression due to KA, GDNF immunoreactivity was labeled. Basal GDNF expression was first assessed in the control rats (Fig. 5A, B, and C). 3 days after KA injection, GDNF was expressed at high levels in the dentate hilus, and the stratum oriens and stratum pyramidale of the CA3 region (Figs. 5D, E, F, G, H, and I). The pattern of GDNF expression was similar to that seen in the TUNEL assay, suggesting that GDNF expression and TUNEL-labeled apoptosis are both related to KA-induced hippocampal damage. GDNF expression was suppressed to control levels in the stratum pyramidale and stratum oriens of the CA3 region, and also in the dentate hilus (Fig. 5J, L; arrowheads), but was increased in the CA1 region of the Ad-GDNF rats, compared to the levels observed in the KA-injected rats. The increased GDNF immunoreactivity in the CA1 regions of the Ad-GDNF rats is thought to be due to Ad-GDNF expression in this region.

The ELISA assay for GDNF expression also revealed that the concentrations of GDNF in the Ad-GDNF rats was significantly lower than that of the KA- and vehicle-injected rats, but about two times as high as control concentrations. The GDNF levels in the control, KA-, vehicle, and Ad-GDNF-injected rats were: 101.8 ± 18.48 pg/ml, 263.8 ± 32.12 pg/ml, 266.2 ± 39.04 pg/ml, and 203.6 ± 21.76 pg/ml, (n=3) respectively (Fig. 6). As GDNF expression was shown to increase in a similar pattern of cell loss following KA injection (Fig. 5), the reduced expression of GDNF in Ad-GDNF rats 7 days prior to KA injection is thought to be due to the protective effects of enhanced
GDNF expression as the result of pre-inoculation with Ad-GDNF. In fact, Ad-GDNF protected the hippocampus from KA-induced patho-physiological changes, such as CA3 degeneration or glial scars (Fig. 1).

To test the notion that increased GDNF expression in response to KA insult may be initiated via the apoptotic process, the relationship of protein expression between GDNF and p53, a tumor suppressor gene implicated in apoptotic death, was further analyzed by immunoblotting. GDNF and p53 expression appeared to progress in parallel; GDNF and p53 expression were highest in the KA-injected rats and lowest in the control rats, supporting the notion of the neurotrophic response of GNDF to excitotoxic insult. In contrast, GAD-67 and p53 expression exhibited an inverse relationship. Moreover, in this study, the levels of Bcl-2 protein also increased as the result of Ad-GDNF infection in the hippocampus (Fig. 7); GAD-67 expression was lowest in the KA-injected group and highest in the control rats. In Ad-GDNF rats, GAD-67 expression reached a similar level to that seen in control rats, and the expression of both p53 and GDNF was reduced to the level of the control rats. The values of the detected proteins were evaluated using ImageTool (UTHSCSA, San Antonio, CA, USA), based on a gray-scale threshold (mean ± SE).

**Suppression of KA-induced seizure and neurogenesis**

In addition to the excitotoxic effects of KA, it has been well documented that the
intraperitoneal injection of KA induces a complex behavioral seizure in rodents. These behaviors are usually scaled in stage, from 0 to 7, as described in the Methods and Materials section (Morrison et al., 1996). Therefore, we evaluated the effects of Ad-GDNF transfection on KA-induced seizure by comparing the time to reach each stage in the KA-injected rats and the Ad-GDNF rats. Most animals exhibited catatonic posture with staring behavior and motionlessness within 5-10 minutes after KA injection (stage 1). Subsequently, myoclonic twitching, initially restricted to the head, face, and one or both forelimbs, was observed, coupled with frequent rearing and falling, and hemorrhagic foaming from the mouth (stage 3-4). The incidence of “wet dog shakes” increased markedly; this symptom was later often masked by convulsions. Severely affected rats often exhibited other characteristic behavioral changes within 1-2 hours. These behaviors included repeated extension, hunched posture, body tremors, and tonic-clonic seizures coupled with barrel rolling (stage 6). This stage of behavior persisted for up to 3 hours after KA injection. However, in the rats who had been pretreated with Ad-GDNF before KA injection, the development of behavioral seizures was significantly delayed, with more apparent effects in stage 3-5. Significantly, stage 6 behaviors—characterized by tonic-clonic seizures with barrel rolling—did not occur (Fig. 8A). The transfection was confirmed by Ad-lacZ, which codes for β-galactosidase (Fig. 8B).

The KA models of temporal lobe epilepsy revealed that chemoconvulsant-induced seizures enhance cell proliferation (Gray and Sundstrom, 1998). Thus, we labeled the
constitutively proliferating cells by systemic BrdU administration, one day before the KA injections. 4 days later, we immunohistochemically identified the labeled cells. In the control rats, BrdU-positive cells were detected only at trace levels in all subfields of the hippocampus (Figs. 9A, B, and C). Remarkably, increased cell proliferation was detected in the dentate hilus of the hippocampi of the KA or vehicle-injected rats. A few BrdU-positive cells were also observed in the subgranular zone (Fig. 9F and I), and in the stratum pyramidale and stratum radiatum of the CA3 region (Figs. 9D, E, G, and H). BrdU-positive cells in the hippocampi of Ad-GDNF rats were greatly reduced in the dentate hilus, and in the granule cell layer of the dentate gyrus (Fig. 9L), compared to the KA- and vehicle-injected rats. BrdU-positive cells were not found in any subfields of the CA1 and CA3 regions of the hippocampi of Ad-GDNF rats (Figs. 9J and K).

**Differentiation capacity of neural stem cells and BrdU incorporation**

Further, to test the effect of GDNF and the ability of cell replacement after KA administration, in series of experiment, hippocampal neural stem cells (NSCs), Ad-GDNF, or Ad-GDNF-NSCs was injected into the hippocampus at 3 days after KA injection. To classify NSCs *in vitro*, cells with undifferentiated spheres shape were labeled with nestin antibody as the immature cell marker (Fig. 10E, F, and G). To assess the differentiating capacity of these cells, cells of undifferentiated spheres were dissociated, plated onto poly-ornithine-coated glass coverslips, and cultured in
DMEM/F-12 containing 7% fetal bovine serum (FBS). The process of differentiation was monitored through the phase-contrast inverted microscope. Undifferentiated cells were suspended in growth medium. Meanwhile, differentiated cells were attached on poly-ornithine-coated surface, and grew well toward outside from cell core (Fig. 10A, B, C, and D). NSCs appeared to possess the capacity to differentiate into neurons, astrocytes, oligodendrocytes, and GABAergic interneurons. They were also shown to incorporate BrdU, as the indicative of cell division and migration marker \textit{in vivo}. Immunofluorescence labeling using an antibody to nestin, MAP-2, GFAP, Gal C, and GAD-67 indicates the progenitor cells, neurons, astrocytes, oligodendrocytes, and GABAergic interneurons, respectively (Fig. 10H, I, J, K, and L).

To investigate the BrdU level in the differentiated cells after transplantation \textit{in vivo}, immunofluorescence double-labeling for BrdU and MAP-2, GFAP, GAD-67, and GDNF of the cells was performed. This experiment indicated that the differentiated neuron, astrocyte, GABAergic interneuron, and GDNF expressing cell clearly incorporated BrdU (Fig. 11C, D, G, H, K, L, O, and P).

\textbf{TUNEL assay for cell death}

Hippocampus was sampled 7 days after either injection or transplantation of saline, Ad-GDNF, hippocampal neural stem cells (NSCs) and Ad-GDNF infected NSCs (Ad-GDNF-NSCs). To test the level of apoptosis, TUNEL assay was carried out. TUNEL
positive cells were found in hippocampal areas of all rats. However, the level of TUNEL activity was different among saline, Ad-GDNF, NSCs, and Ad-GDNF-NSCs rats. A large number of TUNEL positive cells were shown in the stratum pyramidale of CA1 and dentate hilus of dentate gyrus area in saline and NSCs rats. Weakly labeled cells were detected in stratum pyramidale of CA3 area. TUNEL positive cells were sparsely found in the stratum radiatum and stratum oriens of CA1 and CA3 areas (Fig. 12A, B, C, G, H, and I). The number of TUNEL positive cells in Ad-GDNF-NSCs rats was markedly smaller compared to either saline or NSCs rats in all areas. A significant reduction of TUNEL positive cells were observed in stratum pyramidale of CA1 and CA3. TUNEL activity of hilus of dentate gyrus was inhibited a little (Fig. 12J, K, and L). Interestingly, the TUNEL activity was not highly reduced in stratum pyramidale of CA3 and hilus of dentate gyrus areas of in Ad-GDNF infected rats (Fig. 12D, E, F, J, K, and L), while it was largely diminished in these areas of rats that were infected by Ad-GDNF 7 days prior to KA injection.

Together, TUNEL activity in the rats that received Ad-GDNF and Ad-GDNF-NSCs was reduced compared to that of saline and NSCs rats.

**Alteration of GAD-67 positive interneurons and BrdU incorporation**

GABAergic interneuron undergoes a significant alteration following KA, which confers hyperexcitability to the hippocampal circuitry. In this experiment, it was tested
whether Ad-GDNF suppresses alteration of GABAergic interneurons and NSCs restore loss of GABAergic interneurons using antibody to glutamic acid decarboxylase-67 (GAD-67), a GABA synthesizing enzyme and immunofluorescence double-labeling to BrdU, a migration marker. In the hippocampus from saline rats, GAD-67 positive neurons were hardly observed. Only weakly labeled neurons of saline rats were found in stratum radiatum of CA3, dentate hilus of dentate gyrus, and stratum pyramidale of CA1 (Fig. 13A, B, and C). In NSCs-transplanted rats, GAD-67 neurons were found in all areas more than those in saline rats, but less than those in Ad-GDNF and Ad-GDNF-NSCs rats. GAD-67 immunoreactivity of NSCs rats increased in stratum pyramidale and stratum radiatum of CA3, stratum radiatum of CA1, and hilus and granule cell layer of dentate gyrus (Fig. 13G, H, and I). To test migrating and differentiating capacity of NSCs, NSCs which was labeled with BrdU was injected into the site between fimbria and stratum oriens of CA3 in hippocampus. A few BrdU-positive GAD-67 neurons in NSCs rats were detected in subgranular zone of dentate gyrus and stratum oriens and stratum radiatum of CA3 (Fig. 13H and I). In Ad-GDNF rats, GAD-67 neurons also increased in pyramidal layer of CA1 and CA3, and granule cell layer and subgranular zone of dentate gyrus (Fig. 13D, E, and F). The GAD-67 immunoreactivity of Ad-GDNF-NSCs rats was increased in pyramidal layer of CA1 and CA3, and granule cell layer of dentate gyrus. BrdU-positive GAD-67 neurons were observed in stratum pyramidale and stratum radiatum of CA1 and CA3, and hilus, subgranular zone, and
molecular layer of dentate gyrus (Fig. 13J, K, and L). These results suggest that NSCs migrated successfully from the injection site to each region and differentiated to GAD-expressing cells.

**GDNF and ELISA assay**

To study the relationship between GDNF expression and cell death by KA, GDNF immunoreactivity was detected. Hippocampus was sampled 7 days after injection or transplantation of saline, Ad-GDNF, NSCs, and Ad-GDNF-NSCs. The basal expression of GDNF was detected in hippocampus from saline rats (Fig. 14A, B, and C). In Ad-GDNF rats, GDNF labeling was observed in all hippocampal areas. Stratum pyramidale of CA3 and CA1, and granule cell layer of dentate gyrus elicited significant increase in GDNF immunoreactivity (Fig. 14D, E, and F). NSCs-received rats exhibited an increased GDNF immunoreactivity compared to saline rats. Furthermore, BrdU-positive cells were found in all areas, but double-labeled cells with GDNF were detected in some areas including subgranular zone of dentate gyrus, and stratum oriens and stratum radiatum of CA3 areas (Fig. 14G, H, and I). The GDNF level of Ad-GDNF-NSCs rats was similar to that of Ad-GDNF except the significant induction of dentate gyrus. GDNF expression of Ad-GDNF-NSCs rats was observed in hilus and granule cell layer of dentate gyrus and stratum pyramidale of CA1 and 3 (Fig. 14J, K, and L). TUNEL positive cells in Ad-GDNF-NSCs were also reduced the same areas (Fig. 12J, K, and L).
BrdU-positive cells were observed in stratum pyramidale of CA1 and CA3, and dentate gyrus (marked by arrowheads in Fig. 14J, K, and L). NSCs was transplanted into the target site between fimbria and stratum oriens of CA3 in hippocampus, and then migration and differentiation to the cells that express GDNF, compare to NSCs, were markedly increased in Ad-GDNF-NSCs rats. These results suggest that GDNF overexpression or GDNF-induced factors may up-regulate the migration and differentiation of NSCs from injection site to a variety of hippocampal regions.

ELISA assay for GDNF expression revealed that GDNF concentration of saline and NSCs rats was significantly lower than that of Ad-GDNF rats. GDNF expression was also higher at 3 days, and 7 days after Ad-GDFN-NSCs transplantation, but similar to that of NSCs. GDNF expression levels of saline, Ad-GDNF, and NSCs rats were 108.8 ± 13.9 pg/ml, 258.6 ± 21.5 pg/ml, and 128.9 ± 16.5 pg/ml. At 3 days, 7 days, and 14 days after Ad-GDNF-NSCs transplantation, GDNF expression levels were 381.9 ± 44.61 pg/ml, 233.5 ± 25.96 pg/ml, and 200.7 ± 29.66 pg/ml, respectively (Fig. 15).

**Immunoblot analysis**

To test the relationship of GDNF, GAD-67, p53, a tumor suppressor gene, and Bcl-2, an anti-apoptotic gene, in response to KA insult their expression was further analyzed by immunoblot method. GDNF and Bcl-2 expression appeared to be parallel, but p53 was the opposite; GDNF and Bcl-2 expression were highest in Ad-GDNF and Ad-GDNF-
NSCs rats, and lowest in saline rats, suggesting the relationship between GDNF overexpression and its anti-apoptotic action.

GAD-67 protein level was not increased in Ad-GDNF rats that treated to the KA 3 days prior to Ad-GDNF infection (Fig. 16), while GAD-67 expression in figure 3 was highest in Ad-GDNF rats, which was infected 7 days prior to KA injection (Fig. 7). Although GDNF protein level in figure 16 was increased by Ad-GDNF, GAD-67 expression was not up-regulated. In figure 7, GDNF and GAD-67 expression was parallel. In contrast, Bcl-2 that has an anti-apoptotic function is increased both Ad-GDNF and Ad-GDNF-NSCs rats. In TUNEL assay, the TUNEL positive cells of Ad-GDNF and Ad-GDNF-NSCs decreased in hippocampus (Fig. 12D, E, F, J, K, and L). Although GAD-67-positive interneuron was not rescued from KA insults by the increase of GDNF and Bcl-2, the reduction of TUNEL activity in Ad-GDNF and Ad-GDNF-NSCs rats may be due to transplantation of the fresh NSCs cells into the hippocampus after insults and the effect of increased GDNF and Bcl-2 protein levels. A values of the detected proteins are evaluated using ImageJ (NIH, Bethesda, MD, USA) based on a gray value (mean ± SE).
Discussion

Glial cell line-derived neurotrophic factor (GDNF) is a distantly-related member of the transforming growth factor-beta superfamily. Previous studies have indicated that GDNF is widely expressed, and promotes the survival and maturation of central dopaminergic and noradrenergic neurons, as well as various subpopulations of peripheral sensory and sympathetic neurons (Arenas et al., 1995; Buj-Bello et al., 1995; Nadler, 1980a; Shetty and Turner, 1999a; Robert et al., 1997; Takahashi, 2001).

Furthermore, the possibility that exogenous recombinant human (rh) GDNF may have anticonvulsant properties was investigated using a model of temporal lobe epilepsy in the rat. When rhGDNF was injected intracerebroventricularly 1 h before peripheral administration of kainic acid (KA), it suppressed KA-induced tonic-clonic convulsions and prevented the associated neuronal cell loss in hippocampal, thalamic and amygdaloid regions. These results suggest that GDNF was related in the circuitry alteration and cellular damage that was associated with excitotoxic processes (Martin et al., 1995).

In this study, the effects of enhanced GDNF expression, induced by Ad-GDNF injection into the hippocampus, explored on KA-induced pathological and behavioral changes. KA-injected rats exhibited seizure behaviors, which progressed from stage 1 to stage 7. 3 days after KA injection, the significant neurodegeneration, apoptotic cell
death, and the proliferation of newly born cells in the CA3 and dentate hilar regions was observed. Furthermore, the increased expression of GDNF and p53, a marker for apoptotic cell death also was detected. GAD-67 levels were reduced following KA injection. In the Ad-GDNF injected rats, apoptotic cell death and the proliferation of newly born cells were detected only at trace levels, and p53 and GAD-67 level were comparable to those observed in the control rats. These effects were accompanied by the suppression of seizure progression. To my knowledge, this study is the first to demonstrate that the injection of adenoviral vector encoding for GDNF into the hippocampus results in a suppression of seizure behavior, and protects the hippocampus from KA-induced cellular damage and alteration.

In series of experiment, animals received the KA 3 days prior to Ad-GDNF and hippocampal neural stem cells (NSCs)-infected Ad-GDNF (Ad-GDNF-NSCs). TUNEL activity was reduced in Ad-GDNF-NSCs (Fig. 12D, E, F, J, K and L). Moreover, immunofluorescence labeling, ELISA assay, and immunoblot analysis revealed that GDNF has no effects on the protection, alteration, and induction of GAD-67-positive interneurons. In contrast, Ad-GDNF pre-inoculation before KA injection played a protective role in GAD-67 interneurons (Fig. 3J, K, and L and Fig. 7). p53 and Bcl-2 protein level were down and up-regulated by Ad-GDNF, respectively, but GAD-67 protein expression showed no changes (Fig. 13D, E, F, J, K, and L and Fig. 16A and B). However, in this study, Ad-GDNF-NSCs successfully migrated from the target region to
a variety of hippocampal areas than the NSCs only, and differentiated to GDNF and GAD-67 expressing cells (Fig 13G, H, I, J, K, L and Fig. 14G, H, I, J, K, L).

**GDNF expression in response to seizure**

It has been established that GDNF mRNA is induced following KA-induced seizures in the hippocampal area. This induction of GDNF mRNA occurs in a predictable and distinctive temporal and spatial pattern. It appears in scattered neurons of the dentate granule cell layer as early as 3 hours after KA injection, and exhibits its highest levels of expression at 6 hours, in all dentate granule cells and individual neurons in the hilus/CA4 area. Finally, GDNF mRNA levels return to control levels, 24 hours after KA injection. In contrast, 24 hours after KA injection, GDNF mRNA was detected at significant levels in neurons in the hilus/CA4, CA1, and CA2/3 areas. Similarly, GDNF protein increased dramatically in the dentate granule cells within 3 hours of KA injection into the hippocampus, maintaining significant expression levels for a subsequent 4 days (Humpel et al., 1994; Mikuni et al., 1999). Thus, it has been suggested that the early expression of GDNF plays a role in protecting the dentate granule cell layer from seizure insults. This hypothesis is further supported by the fact that acute seizures occur 1-2 h after KA injection into the hippocampus, and proceed to the silent period within several hours. In contrast, GDNF mRNA is expressed in the CA3 and hilus areas long after GDNF expression occurs in the dentate granule cells.
(Humpel et al., 1994), corresponding to the above mentioned silent period. During the silent period, a long-lasting structural change occurs, which culminates in the reorganization of the hippocampal circuitry. After KA injection, early effects, including the shrinkage and pyknosis of neurons, were followed by irreversible damage, such as tissue apoptosis and loss of nerve cells. These irreversible deleterious effects appeared to take place at least 24 hours after KA injection (Sperk et al., 1983). However, these late irreversible alterations appear to be restricted mainly to the pyramidal cell layer of the CA3 region, and do not occur in the dentate granule cell layer, in which increased concentrations of GDNF mRNA were primarily observed during the early 24-hour period after injection. Indeed, the dentate granule cells proved to be relatively resistant to KA-induced pathological changes (Humpel et al., 1994). This interpretation is consistent with my observation that, at 3 days after KA injection, cresyl violet staining and TUNEL assay indicated selective damage occurring in the CA3 and hilus areas, and relative resistance to damage in the dentate granule cell layer.

**Protective role of enhanced GDNF expression**

It has been shown that recombinant human GDNF (rhGDNF) suppresses KA-induced tonic-clonic convulsions, and prevents associated neuronal cell loss in the hippocampal, thalamic, and amygdaloid regions (Martin et al., 1995). Intraventricular GDNF injection prevents the behavioral progression of kindling-induced seizures and mossy fiber
sprouting in the CA3 area (Li et al., 2002).

In this study, Ad-GDNF-injected rats exhibited a reduced degree of cell loss, as evidenced by Nissl staining and TUNEL assay, than did the KA or vehicle-injected rats. This indicates that the enhanced GDNF expression due to Ad-GDNF pretreatment ameliorated the patho-physiological changes induced by KA. The GDNF family ligands send signals via a unique multicomponent receptor complex, consisting of glycosyl-phosphatidylinositol (GPI)-anchored coreceptors (GFRα1-4) as a ligand binding component, and the Ret receptor, tyrosine kinase, as a signaling component. GFRα-2-deficient mice have been demonstrated to be resistant to kindling stimulus (Nanobashvili et al., 2003). It has also been demonstrated that seizures initiated by electroconvulsive shock result in the increased expression of GFRα-1 and GFRα-2 (Chen et al., 2001). GFRα-1, then, appears to be partially responsible for the generation of epileptic seizures. Meanwhile, one possible mechanism for the cell survival effect of enhanced GDNF is the neurotrophic signal sent through the GDNF-Ret system. Ret is a main receptor tyrosine kinase for GDNF. Retinoic acid (RA) has been shown to induce apoptosis in cultured human neuroblastoma, with an associated expression of Ret, and the activation of the p53-mediated pathway (Hishiki et al., 1998). The subsequent addition of GDNF resulted in a suppression of RA-induced apoptosis, as well as the nuclear accumulation of p53 in the cells, supporting the notion that neurotrophic signals sent through the GDNF-Ret system may prevent neuronal cell death (Naoyuki et al.,
Furthermore, the expression of the Ret (rearranged during transfection) proto-oncogene encoding for the tyrosine kinase receptor (Ret receptor) induced apoptosis, which was inhibited in the presence of its ligand, GDNF (Marie-Claire et al., 2000). The present interpretation of this experiment is supported by previous studies, which reported that GDNF mRNA and its receptor components, including receptor tyrosine kinase c-ret, were induced by KA (Humpel et al., 1994; Reeben et al., 1998).

Alternatively, KA injection enhanced p53 immunoreactivity, predominantly in the nuclei of apoptotic neurons (Sakhi et al., 1996). p53 promotes apoptosis via the down-regulation of the expression of target genes, including Bcl-2, a microtubule-associated protein (MAP4), and presenilin-1 (Miyashita et al., 1994). In various studies, GDNF activated MAP kinase and Bcl-2 signaling that may contribute to neuronal survival after spinal cord contusion, and up-regulated the mRNA of bcl-2 gene family members, and exerted protection against apoptosis by activation of phosphatidylinositol 3-kinase and the subsequent up-regulation of Bcl-2 and Bcl-x (Sawada et al., 2000; Lenhard et al., 2002). In this study, Bcl-2 protein levels were also enhanced upon Ad-GDNF hippocampal infection (Fig. 7). These results suggest that GDNF may contribute to neuronal survival via the activation of Bcl-2 signaling after apoptosis-induced injury or stimuli.

However, in series of experiment, Bcl-2 was not increased by NSCs only, but was induced by Ad-GDNF infection after KA (Fig. 16). Although Bcl-2 protein in Ad-GDNF
rats was up-regulated, TUNEL-positive cells were not decreased significantly in the hippocampal areas (Fig. 12). Additionally, Bel-2 protein in Ad-GDNF-NSCs was increased by NSCs transplantation.

**Suppression of seizure-induced alterations**

In addition to its protective role against cell death, it is also significant that enhanced GDNF expression mitigates seizure-related alterations. Several experiments have reported a sustained loss of GAD expression in a major fraction of interneurons after KA injection, culminating in the loss of functional inhibition (Shetty and Turner, 2000b). Therefore, the restoration of GAD-synthesizing interneurons to levels observed in the intact hippocampus may be crucial in the treatment of hyperexcitability. In these results, the reduction of GAD-67-positive interneurons due to KA injection was prevented by inoculation with Ad-GDNF (Fig. 3). In contrast, in series of experiment, GDNF immunoreactivity and protein level was increased significantly by Ad-GDNF infection after KA, while GAD-67-positive interneurons were marginally restored (Fig. 13 and 16). It is not clear from results of this experiment whether the protective effects of GDNF with regard to the GAD-67-positive interneurons are due to the prevention of the death of GAD interneurons, or rather to the inhibition of the apoptosis in the CA3 pyramidal cell layer, through the GDNF-Ret neurotrophic signal pathway. In this regard, a recent study revealed persistent reductions in the numbers of hippocampal GAD-
positive interneurons, without a comparable diminution in Nissl-stained interneuron numbers. This result indicates the selective and indirect loss of GAD expression in a majority of interneurons, 1-6 months after KA exposure (Robert et al., 1997; Shetty and Turner, 1999b; 2000a; Xiaohua et al., 2002).

Recently, increased levels of dentate granule cell neurogenesis have been reported in pilocarpine and kainic acid models of temporal lobe epilepsy. Several lines of evidence implicate newly-generated neurons in the development of structural and functional network abnormalities in the epileptic hippocampal formations of adult rodents. These abnormalities include aberrant mossy fiber reorganization, persistence of immature dentate granule cell structure, and the abnormal migration of newborn neurons to ectopic sites in the dentate gyrus (Parent and Lowenstein, 2002). In the course of this study, 3 days after KA injection, the BrdU-positive cells were located densely in the dentate hilus, and sparsely in the subgranular zone of the DG. The proliferation of new neurons due to chemoconvulsant shock is known to begin in the subgranular zone of the adult hippocampus. Thus, the BrdU-labeled cells in the CA3 region may indicate abnormal proliferation and/or migration. The density of KA-induced newly generated cells was suppressed by Ad-GDNF infection in the hippocampus. In addition to its suppressive effects on GAD expression alteration and neurogenesis, GDNF prevents the alteration of hippocampal circuitry.

Additionally, overexpression of GDNF induces genes regulating migration and
differentiation of neuronal progenitor cells. Axonal guidance depends on cell-surface molecules and extracellular matrix proteins (Jens et al., 2004). N-CAM has now been identified as a receptor for GDNF (Feng-Quan et al., 2003). The neural cell adhesion molecule N-CAM can function as a signaling receptor for members of the GDNF ligand family. GDNF stimulates Schwann cell migration in newborn mouse sciatic nerve explants and axonal growth in hippocampal and cortical neurons via binding to NCAM and activation of Fyn, but independently of Ret (Gustavo et al., 2003). GDNF may prevent neuronal death through the up-regulation of Bcl-2, an anti-apoptotic factor. Recently, it was reported that GDNF has been reported to up-regulate the expression of Bcl-2 and Bcl-XL in spinal cord injury, and the bleomycin sulfate (BLM) and L-buthionine-[S,R]-sulfoximine (BSO)-induced apoptosis of mesencephalic neurons (Sawada et al., 2000; Ghribi et al., 2001; Cheng et al., 2002). Up-regulated Bcl-2 also promoted migration and invasiveness in human glioma cells (Wolfgang et al., 1998). These results suggest that Bcl-2 was related to cell survival, apoptosis, and migration. In previous study, GDNF is also involved in the development and maintenance of neural tissues. In addition, mutations in components of its signaling pathway lead to severe migration deficits of neuronal crest stem cells, tumor formation, or ablation of the urinary system (Jens et al., 2004). GDNF-deficient mice completely lack the enteric nervous system (ENS), urethras, and kidneys, suggesting a key role of GDNF in organogenesis (Tuszynski and Kordower, 1999). Thus, GDNF and GDNF-induced
factors may be related to stem cell migration and organic formation.

To test the applications for stem cell replacement therapy, in this experiment, hippocampal neural stem cells (NSCs) were shown to have the ability of differentiation to neurons, glia, GAD-67, and GDNF expressing differentiated cells (Fig. 10 and Fig. 11). NSCs have a capacity that can be differentiated to the various types of cells including neurons and glia cells, and migrate to the other regions. In this study, Ad-GDNF-infected NSCs successfully migrated from the target region to a variety of hippocampal areas, and, even if its numbers was a few, differentiated to GDNF and GAD-67 expressing cells (Fig 13G, H, I, J, K, L and Fig. 14G, H, I, J, K, L).

Taken together, GDNF may prevent the apoptosis from excitotoxic insults, and GDNF or GDNF-induced factors, as a p53 and Bcl-2, may be related to the survival of GAD-67-positive interneurons and the migration of transplanted NSCs. However, the protective roles of GDNF and GDNF-induced factors may have no effect on the lesioned hippocampus.
Conclusions

Although GDNF in lesioned cells or areas have no significant effect, GDNF and GDNF-induced factors may be protective against the excitotoxic damages and cellular alteration. Moreover, NSCs differentiates to GDNF and GAD-67-positive cells. However, it is not clear whether GDNF has the ability to induce NSCs to the specific type of cells and differentiation for cell replacement therapy.
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Fig. 1. Hippocampal lesions after intraperitoneal (i. p.) KA administration is stained using Nissl staining. The classical loss of neurons is demonstrated in the hippocampal CA3 subfield. 3 days after the i. p. administration of KA, the loss of hippocampal CA3 pyramidal cell layer and dentate hilus (asterisks in F, K) is observed. Cell loss and gliosis (arrowheads in H-I, M-N) are exhibited in both KA and vehicle-injected rats. Note that both the CA1 pyramidal cell layer and dentate granule cell layer in CA3-lesioned hippocampus are intact. In the rats infected by Ad-GDNF, both the CA1 pyramidal cell layer and dentate hilus are not significantly damaged following i. p. KA administration (R, S). Like normal and KA-injected rats, CA1 pyramidal cell layer and dentate granule cell layer also are intact (Q, T). SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL, stratum lucidum; DH, dentate hilus; GCL, granule cell layer; ML, molecular layer. Scale bar is 100 μm.
Fig. 2. TUNEL assay following KA injection and Ad-GDNF infection. Note that TUNEL positive cells in normal rats were not detected (A-C), while both KA and vehicle received groups exhibited large numbers of TUNEL positive cells in the CA3 pyramidal cell layer and dentate hilus regions (E-F, H-I). In addition, the CA1 region is slightly damaged following KA administration (D, G). However, the dentate granule cell layer (GCL) is completely intact. TUNEL staining cells are markedly decreased in the Ad-GDNF infected rats (J-L). Scale bar represents 100 μm.
Fig. 3. Glutamic acid decarboxylase-67 (GAD-67) immunoreactivity after KA injection and Ad-GDNF infection. In the normal hippocampus, GAD-67 positive neurons are observed in all layers of the different subfields. The decreased density of GAD-67 neurons is observed primarily in the dentate hilus, the junction of the hilus, and the granule cell layer (F, I) and stratum oriens and radiatum of CA1 and CA3 (D-E, G-H) in KA and vehicle-injected rats. However, the severely declining regions, namely the CA3 pyramidal cell layer and dentate hilus, survived due to infection with Ad-GDNF (J-L). All layers of the CA3 region are remarkably preserved against KA injection. The reduction of GAD-67 positive neurons of CA1 subfields is quite small in all groups. Scale bar represents 100 μm.
Fig. 4. Histogram shows comparison of GAD-67 positive neuron density in different hippocampal regions. Note that the density of the GAD-67 positive neurons in the KA-injected rats was very dramatically reduced in all hippocampal regions. Reductions in the density of GAD-67 neurons are striking in the CA3 region. However, in the Ad-GDNF infected groups, all subfields of the regions, including CA1, CA3 and dentate gyrus, survived. Filled bar: control (n = 6), open bar: KA-injected rats (n = 4), Scratched bar: Ad-GDNF infected rats 7 days before KA injection (n = 6). At the $P < 0.01$ and $P < 0.001$, * and ** are significantly different from the KA-injected rats and Ad-GNDF infected rats, respectively.
Fig. 5. The labeling of GDNF expression following KA and Ad-GDNF administration. Basal GDNF expression was first assessed in the control rats (A-C). 3 days after KA injection, GDNF was profoundly expressed in the dentate hilus and stratum oriens and stratum pyramidale of CA3 region (D-I). GDNF expression was suppressed to control levels in the stratum pyramidale and stratum oriens of the CA3 region, and also in the dentate hilus (J, L; arrowheads), but was increased in the CA1 region of the Ad-GDNF rats, compared to the levels observed in the KA-injected rats. Scale bar represents 100 μm.
Fig. 6. Levels of GDNF in the hippocampus from animals injected with Ad-GDNF. In Ad-GDNF received rats, 10 μl of viral stock solution at 5×10^6 p.f.u./μl was injected at a rate of 2μl/min. The normal groups received no treatment. 7 days later, rats received the administration of KA, and then 3 days later, hippocampal tissues were processed and the extracts were analyzed by ELISA assay for GDNF concentrations, as described under Materials and Methods (mean ± SE). At the $P < 0.05$ level, * and ** are significantly different from normal and KA, respectively.
Fig. 7. Expressed protein levels of GDNF, GAD-67, Bcl-2, and p53 are analyzed by immunoblot method for each group. Interestingly, the pattern of GDNF (■) and p53 (▲) expression is parallel, while GAD-67 (●) and p53 exhibit an inverse relation. This result may suggest that chemoconvulsant-induced p53 expressed- or induced-apoptosis is related to the reduction in GAD-67 expression in the GABAergic interneurons. On the other hand, we suggest that the increased GDNF, which resembles p53 induction in terms of proportion, may play a role in protection against KA-induced hippocampal damage, and/or p53-induced apoptosis. In addition, Bcl-2 protein levels (▼) also increased as the result of Ad-GDNF infection. The values of the detected proteins were evaluated using ImageTool based on a gray-scale threshold (mean ± SE).
Fig. 8. Changes in behavioral patterns after KA injection (10 mg/kg) and adenoviral vector (Ad-GDNF) infection. The rats that received Ad-GDNF exhibited different behavioral patterns from the KA-injected rats. Particularly, Ad-GDNF-infected rats do not exhibit severe, stage 6, behavioral characteristics, and the progression of each stage is slower than in the typical KA rats (A). At the $P < 0.001$ level, KA-injected rats and Ad-GDNF-infected rats are significantly different (*). To examine the efficiency of *in vivo* gene delivery by Ad vector, the section which received the Ad-lacZ was also labeled by polyclonal anti-β-galactosidase (B). Scale bar represents 100 μm.
Fig. 9. Alterations in the newly generated BrdU-positive cells after KA injection and Ad-GDNF infection. In normal rats, nuclear BrdU-labeled cells were only sparsely detected in all subfields (A-C). Remarkably, increased cell proliferation in the adult hippocampus after KA administration was detected in the subgranular zone and dentate hilus (F, I). Note the greater cell proliferation in the subgranular zone of the KA-injected rats, compared to the Ad-GDNF infected rats. Although BrdU-positive cells in the hippocampus of Ad-GDNF infected rat was only lightly labeled in the dentate hilus and granule cell layer of dentate gyrus (L), it was nearly undetectable in all subfields of the CA1 and CA3 region (J, K). Scale bar represents 100 μm.
Fig. 10. Differentiation and verification of hippocampal neural stem cells. Cells obtained from the embryonic rat hippocampus during active neurogenesis (E16) have been immortalized. Cells were plated $2.6 \times 10^6$ cells into 25 $cm^2$ tissue culture flasks for bulk culture (A). To assess the differentiation capacity of these cells, they were dissociated in 0.25% trypsin and re-plated on 24 wells with poly-L-ornithine coated glass coverslips. Differentiated cells following the time course were observed (B-D). Cells within undifferentiated spheres were labeled with nestin (E-G). On differentiation, immunofluorescence labeling using antibodies to nestin, MAP-2, GFAP, Gal C, and GAD-67 revealed the progenitor cells, neurons, astrocytes, oligodendrocytes, and GABAergic interneurons, respectively (H-L). Scale bar represents 100 $\mu$m.
Fig. 11. The differentiated cells incorporated with BrdU. Immunofluorescence double-labeling for BrdU and MAP-2, GFAP, GAD-67 and GDNF of the cells was performed to investigate the level of corporation with BrdU. BrdU was utilized also to show the migrate capacity in vivo. L: light field. Scale bar represents 100 μm.
Fig. 12. TUNEL activity was labeled in saline, Ad-GDNF, NSCs, and Ad-GDNF infected NSCs. To test the level of apoptotic process, TUNEL assay was carried out. TUNEL positive cells were found in hippocampal areas of all rats. A large number of TUNEL positive cells in all groups were shown in the stratum pyramidale of CA1 and dentate hilus of dentate gyrus area (A, D, G, J and C, F, I, L). Otherwise, TUNEL positive cells of stratum pyramidal of CA3 were reduced slightly in Ad-GDNF and Ad-GDNF-NSCs (E, K), and slightly decreased in NSCs (H). Scale bar represents 100 μm.
Fig. 13. Both GAD-67 and BrdU immunopositive cells were labeled in hippocampal areas. The alteration and restoration of GABAergic interneurons was evaluated using antibody to glutamic acid decarboxylase-67 (GAD-67), a GABA synthesizing enzyme and immunofluorescence double-labeling to BrdU, as a migration marker. Only weakly labeled neurons of saline rats were found in stratum radiatum of CA3, dentate hilus of dentate gyrus, and stratum pyramidale of CA1 (A-C). GAD-67 immunoreactivity of NSCs rats increased in stratum pyramidale and stratum radiatum of CA3, stratum radiatum of CA1, and hilus and granule cell layer of dentate gyrus (G-I). A few BrdU-positive GAD-67 neurons in NSCs rats were detected in subgranular zone of dentate gyrus and stratum oriens and stratum radiatum of CA3 (H, I). In Ad-GDNF rats, GAD-67 neurons also increased in pyramidal layer of CA1 and CA3, and granule cell layer and subgranular zone of dentate gyrus (D-F). The GAD-67 immunoreactivity of Ad-GDNF-NSCs rats was increased in pyramidal layer of CA1 and CA3, and granule cell layer of dentate gyrus. BrdU-positive GAD-67 neurons were observed in stratum pyramidale and stratum radiatum of CA1 and CA3, and hilus, subgranular zone, and molecular layer of dentate gyrus (J-L). NSCs and Ad-GDNF-NSCs rats were migrated successfully in all areas (G-L) and differentiated to GAD-67 expressing interneurons (marked by arrowheads). Scale bar represents 100 μm.
GDNF and BrdU immunofluorescence double-labeling. GDNF was detected mainly in Ad-GDNF received rats (D-F and J-L). The basal expression of GDNF was detected in hippocampus from saline rats (A-C). In Ad-GDNF rats, GDNF labeling was observed in all hippocampal areas. Stratum pyramidale of CA3 and CA1, and granule cell layer of dentate gyrus elicited significant increase in GDNF immunoreactivity (D-F). NSCs-received rats exhibited an increased GDNF immunoreactivity compared to saline rats. Furthermore, BrdU-positive cells were found in all areas, but double-labeled cells with GDNF were detected in some areas including subgranular zone of dentate gyrus, and stratum oriens and stratum radiatum of CA3 areas (G-I). The GDNF level of Ad-GDNF-NSCs rats was similar to that of Ad-GDNF except the significant induction of dentate gyrus. GDNF expression of Ad-GDNF-NSCs rats was observed in hilus and granule cell layer of dentate gyrus and stratum pyramidale of CA1 and 3 (J-L). BrdU-positive cells were observed in stratum pyramidale of CA1 and CA3, and dentate gyrus (J-L; marked by arrowheads). Scale bar represents 100 μm.
GDNF (pg/ml)

Saline  Ad-GDNF  NSC  3D  7D  14D

*
Fig. 15. ELISA assay for GDNF level. Rats were received KA (i. p.) prior to 3 days the transplantation of only NSCs and NSCs (1.6-1.8 × 10^5 cells/rat) which was infected Ad-GDNF (3 × 10^8 p.f.u/µl), and was sampled at 7 days after for the studies. 3 days after KA administration, other group rats were injected also with saline and Ad-GDNF into the hippocampus, and then 7 days later, hippocampal tissues were processed and the extracts were analyzed by ELISA assay for the GDNF concentration, as described under Materials and Methods (mean ± SE). At the P < 0.05 level, * is significantly different from both Ad-GDNF and NSCs.
Fig. 16. Immunoblot assay for GDNF, GAD-67, Bcl-2, and p53. To investigate the relationship of GDNF, GAD-67, p53 (▲), a tumor suppressor gene, and Bcl-2, an anti-apoptotic gene, proteins in response to KA administration, the protein expression was analyzed by immunoblot method. GDNF (■) and Bcl-2 expression (▼) appeared to be parallel, but p53 was the opposite. Bcl-2 that has an anti-apoptotic function in cell is increased in both Ad-GDNF and Ad-GDNF-NSCs rats. GAD-67 expression (●) was not changed significantly in all rats. The values of the detected proteins are evaluated using ImageJ based on a gray-scale threshold (mean ± SE).
감사의 글

여러 번 계절이 변하는 동안 많은 고마운 분들의 도움과 믿음으로 여기에 부족하지만 작은 결실을 맺게 되었습니다. 부족한 논문이기에 시작부터 교정에 많은 어려움을 느껴셨을 김정호 교수님, 이창중 교수님, 김정호 교수님께 고마운 마음을 전합니다. 여러 번의 귀찮은 부탁에도 전혀 싫으신 내색 없이 많은 도움을 주신 가천의과대학교 신경외과 이언 선생님, 그리고 한양대학교 의과대학 해부세포생물학교실 백두진 선생님께는 어떤 말씀으로 감사의 마음을 표현해도 부족할 듯 합니다. 고맙습니다. 또 생명과학과 여러 교수님께도 많은 감사의 마음을 드립니다.

 언제나 걸에 있어주신 가족처럼 놓고 편안했던 우리 신경과학실험실 사람들. 은진, 호영, 완식, 령희… 그리고 서로 다른 곳에서 많은 일들을 하고 있는 우리 실험실 선배, 동기, 후배들… 몇 년 후 한번은 다같이 모여 얘기할 날만을 기쁘게 기다립니다.

나의 졸업에 많은 의심을 갖고 있던 여느 친구들… 많은 기억들을 함께한 나희들이 있어서 힘든 시간도 잘 견디어온 듯하다. 정말 고맙다.

어떤 말로 마음을 표현해야 할까? 떠돌아오는 사람… 선배이자 선생님이 었고 또한 친구이며 나의 부족한 부분을 많이 채워준 사랑하는 아내 영미… 여기에 몇 글자 적는 것만으로 고마움을 표현한다는 것이 부끄럽게 느껴집니다. 정말 정말 고맙고 사랑합니다. 그리고 내가 사랑하는 나의 아내 민희. 널 생각하면 늘 웃음이 입가에서 떠나지 않는단다. 사랑한다.

항상 저에 대한 믿음을 잃지 않고 배려와 격려를 아끼지 않았던 나의 가족들. 아버지, 어머니, 그리고 또 한 분의 아버지, 어머니인 장인, 장모님께도 감사의 말씀을 드립니다. 나를 걱정해준 모든 가족들 여기에 다 말할 수는 없지만 그 분들의 작은 격려가 저를 여기까지 올 수 있게 했다고 믿고 있습니다. 모두 감사 드립니다.

마지막으로 마음 속에서 언제나 함께 해주신 지금은 멀리 계신 어머니에게도 부끄럽지만 여기까지 했노라고 말씀 드리고 싶습니다. 고맙습니다.