Involvement of phospholipase C-γ in the pro-survival role of glial cell line-derived neurotrophic factor in developing motoneurons in rat spinal cords

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Abstract. The glial cell line-derived neurotrophic factor (GDNF) has been proven to be the most powerful neurotrophic factor in neuronal development. However, it remains uncertain as to which intracellular signaling pathway interacting with GDNF is invovlved in motoneuron (MN) development. In this study, we investigated whether phosphoinositide phospholipase C-γ (PLC-γ) is involved in GDNF-promoted MN development. The primary spinal MNs from 12- to 14-day-old embryos of Sprague-Dawley rats were cultured and survival was sustained by GDNF. A specific inhibitor of PLC-γ, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino) hexyl]-1H-pyrrole-2,5-dione (U73122), was used to block the pro-survival effect of GDNF. Our results showed that MN-like cells appeared at 72 h after initial implantation and were sustained for a period of up to seven days under GDNF treatment. These cultured MNs expressed neuron-specific enolase, SMI-32, 75-kDa low-affinity neurotrophic receptor and choline acetyltransferase. The survival rate of the cultured MNs at 24 h was significantly lower in the GDNF + U73122-treated group (31.87±2.17%), compared either with that of the GDNF- (81.38±1.13%) or GDNF + DMSO (79.39±1.22%)-treated groups. The present data suggest that PLC-γ may be one of the intracellular signals that play a role in the survival-promoting effects of GDNF in developing spinal MNs.

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Abbrevations: 6-OHDA, 6-hydroxydopamine; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; ChAT, choline acetyltransferase; CNS, central nervous system; DAB, diaminobenzidine; DMSO, dimethyl sulfoxide; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFL, GDNF family of ligands; GFRα1-4, glycosylphosphatidylinositol-anchored co-receptor; MAPK, mitogen-activated protein kinase; MN, motoneuron; NSE, neuron-specific enolase; p75NTR, 75-kDa low-affinity neurotrophin receptor; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PLC-γ, phosphoinositide phospholipase C-γ; RGC, retinal ganglion cell; TGF, transforming growth factor; U73122, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl]-1H-pyrrole-2,5-dione

Key words: glial cell line-derived neurotrophic factor, phosphoinositide phospholipase C-γ, motoneuron, development

Introduction

The glial cell line-derived neurotrophic factor (GDNF) has been recognized as one of the most powerful neurotrophic factors for developing and injured dopaminergic neurons and motoneurons (MNs) (1,2). Moreover, GDNF has been proven to play a pro-survival role in developing and injured MNs (1,3-8). However, it remains uncertain as to which intracellular signaling pathway interacting with GDNF is involved in MN development. Previous studies have found that GDNF signals through a unique multicomponent receptor system consisting of RET tyrosine kinase and glycosylphosphatidylinositol-anchored co-receptor (GFRα1-4) in the cell membrane (9). Notably, the GDNF-TrkB signaling pathway has been proven to be involved in the transcription, translation and trafficking of proteins during neuronal and synaptic plasticity of the nervous system (10,11), and these functions are carried out by a combination of the three signaling cascades triggered when GDNF binds TrkB: the mitogen-activated protein kinase (MAPK), phosphoinositide phospholipase C-γ (PLCγ) and phosphatidylinositol 3-kinase (PI3K) pathways (10-13). The brain-derived neurotrophic factor (BDNF)-PLC-γ pathway has been found to widely participate in many neuronal activities such as neurite outgrowth (14,15) and differentiation (10,16). In addition, PLC-γ is involved in the survival and growth of the developing nervous system of rats in response to neurotrophin (17). The GDNF-PLC-γ signaling pathway also plays a neuroprotective role in Parkinson's disease and in cognitive processes of the hippocampus (18,19). Previous studies, including our own, have proven that GDNF is capable of rescuing avulsion-injured spinal MNs in adult rats (20-22). However, GDNF is not capable of penetrating the brain-blood-barrier due to its high molecular weight, and this creates an

obstacle in clinical practice (23). Therefore, in this study, we aimed to investigate the GDNF-related intracellular signals in the spinal MNs in order to find a more useful molecular target for the treatment of avulsion-induced spinal MN death. In the present study, we investigated whether the GDNF-PLC-γ signaling pathway is involved in the survival of rat spinal MNs.

Materials and methods

Primary spinal MN culture. The spinal MN cultures were established from 12- to 14-day-old embryos (E12-E14) of Sprague-Dawley rats. The ventral portion of the cervical segments of the spinal cord was removed using tungsten needles and was kept in ice-chilled, sterile-filtered 0.01 M phosphate-buffered saline (PBS). The spinal cord tissues were then treated with 0.5% trypsin prior to being incubated at 37˚C for 15 min with frequent agitation. The spinal MNs were purified by centrifugation in a two-step metrizamide gradient, whose purity typically was shown to achieve 75-90% enrichment in MNs previously (24). Briefly, the partially dissociated cells were added to Leibowitz L15 medium (Invitrogen, Melbourne, Australia) and defined serum-free medium (Gibco, Grand Island, NY, USA), and further dissociated by running the mixture through a 1-ml pipette, followed by centrifugation (3,400 x g for 15 min; Beckman GS-6R centrifuge; Beckman Instruments, Fullerton, CA, USA) over a layer of 6.8% metrizamide (Sigma, St. Louis, MO, USA). Due to their large size, spinal MNs remained in the top half of the metrizamide, forming a visible white band, which was collected and added to 5 ml of L15 medium. A 4% bovine serum albumin (BSA) cushion was then gently added beneath the cells and centrifuged at 3,200 x g for 10 min (Beckman centrifuge). The supernatant was discarded, and the pellet was resuspended in complete medium. The complete medium (neurobasal medium; Gibco-BRL) was supplemented with 2 mM glutamate (Sigma Pharmaceuticals, Melbourne, Australia), 1% penicillin/streptomycin (CSL, Melbourne, Australia), 2% B-27 (Gibco-BRL), 7.2% glucose (BDH Chemicals, Australia), 2% horse serum (CSL), 4% BSA (CSL), 10 ng/ml GDNF (Cytolab) and 25 μM β-mercaptoethanol, and then filtered through a 50-μm nylon filter. Cultures were incubated in a CO_2 water-jacketed incubator with 37˚C and 5% CO_2 for 24 h. To enhance adhesion of the cells to the wall of the culture wells, the culture plates were precoated with polyornithine-laminin (Sigma).

Immunocytochemistry for MN and astrocyte markers. Several types of antibodies specific for the markers of MNs and glial cells were chosen to identify the components of the cultured cells. All antibodies used in the present study were purchased from Sigma. At three days *in vitro* 3 (DIV) and 6 DIV, complete culture medium were removed gently, and the cultured cells were washed once with 1-2 ml PBS before the cells were fixed with 4% paraformaldehyde for 15 min at 4˚C. The cells were then washed three times with a washing buffer (0.2% Triton X-100 in PBS) to remove any excess paraformaldehyde. The cultured cells were incubated at room temperature for 1-2 h with each of the primary antibodies, including neuron-specific enolase (NSE, 1:500), anti-75-kDa low affinity neurotrophin receptor (anti-p75NTR, 1:100), neurofilament H non-phosphorylated (SMI-32, 1:5000) and choline acetyltransferase (ChAT, 1:500). The presence of the glial cells and astrocytes in the cell cultures was determined by immunofluorescence of the astrocyte marker glial fibrillary acidic protein (GFAP, 1:1000) (25). After the cells were washed three times with washing buffer, the cultured cells were incubated for 2 h at room temperature with secondary antibodies. Following three additional rinses with washing buffer, the cells were incubated for 2 h in an avidin-biotin complex solution (Vectastain kit; Vector Laboratories, Burlingame, CA, USA) and were stained with diaminobenzidine (DAB; Vector Laboratories) for 2-5 min. Finally, the cells were washed three times, and a coverslip was placed over them for microscopic observation (Leica DM 2500B, Germany). For immunofluorescence, the cells were incubated with tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody at room temperature in the dark for 2 h. After three additional washes with washing buffer, the sections were mounted onto the slides, coverslipped in 0.5 M buffer bicarbonate (pH 9.5) containing 50% glycerin, and then examined via an inverted fluorescence microscopy (Leica DM 2500B). The negative controls were treated in the same way, except that each primary antibody or secondary antibody was omitted.

Spinal MN culture treatment with GDNF and a specific inhibitor of PLC-γ, 1-[6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl]-1H-pyrrole-2,5-dione (U73122). GDNF was added to the complete medium followed by plating cells into 96-well plates at the most optimal plating density. According to the different concentrations of GDNF, the cultured cells were divided into five groups as follows: 0.1, 1, 10 and 100 ng/ml. Another group of culture medium without GDNF was used as a control. Twenty-four hours later (1 DIV), the cells of each group were counted under the phase contrast optics of an inverted microscope (Leica DMI4000B), and the survival rate of the cultured MNs was compared among the cultures treated with various concentrations of GDNF.

The inhibitor of PLC-γ, U73122 (Calbiochem), was used to investigate the involvement of PLC-γ signaling in the development of embryonic MNs facilitated by GDNF. U73122 was dissolved in dimethyl sulfide (DMSO, 1 mg/ml, Sigma) and diluted in normal saline to the working solution concentration (2.5 μmol/l) prior to being applied to the cultured cells. The cultures were then randomly assigned to the U73122 + DMSO + GDNF, GDNF + DMSO and GDNF groups. U73122 was then added into the culture medium 30 min prior to GDNF application.

Quantification of MN survival. MN counting was carried out by two investigators who were blinded to the culture treatment groups. All attached cells showing visible nuclei and a phase bright halo and clearly defined limiting soma were considered to have survived (26,27). The counting of surviving cells was carried out at x400 magnification under an inverted microscope (Leica DM 2500B) according to our previous study (28). Six random microscopic fields per well were counted. The number of surviving MNs was counted in three randomly selected fields per well, and the mean value was obtained. In the present study, the surviving MNs at 4 h after initial seeding (time required for complete neuronal attachment) in each well were considered as the internal controls (27). Cells surviving

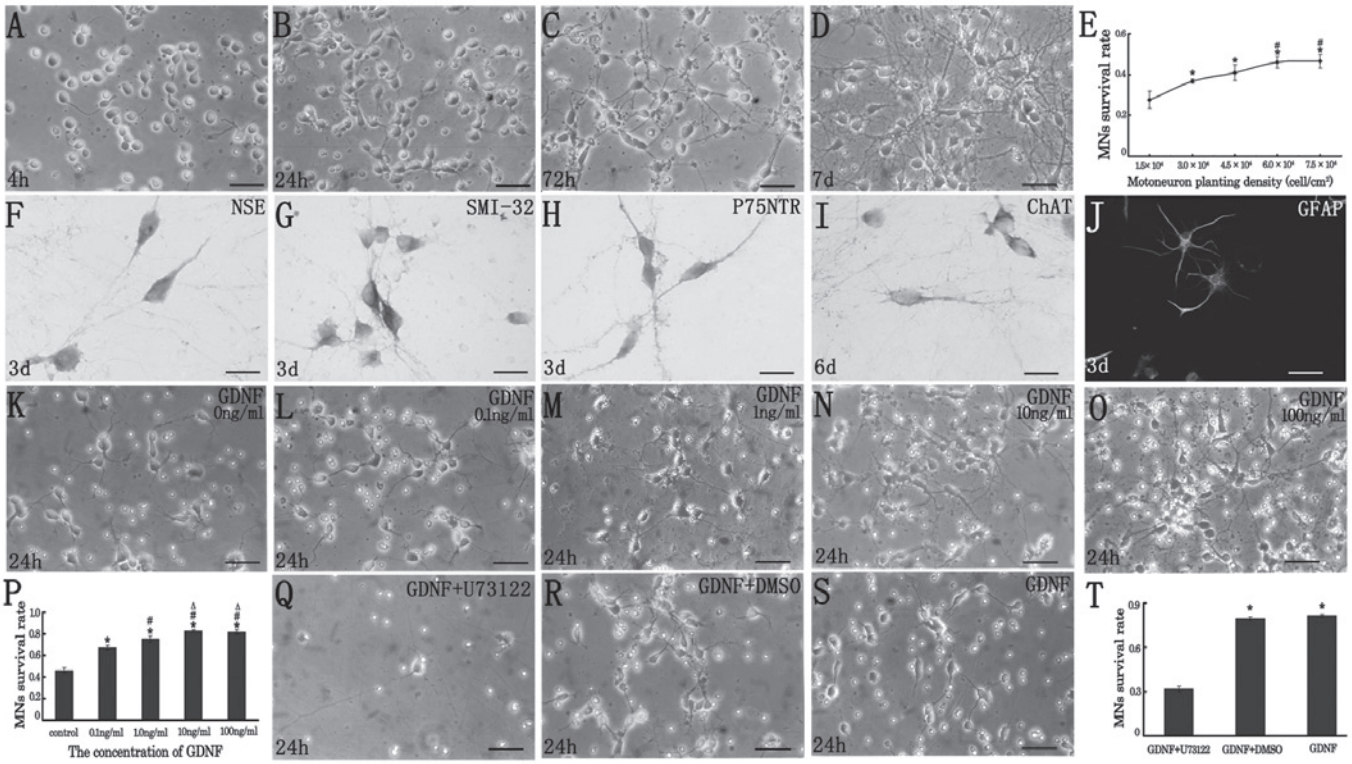

Figure 1. (A-D) Spinal motoneuron morphology depicted using phase contrast microscopy at (A) 4 h, (B) 24 h, (C) 72 h and (D) 7 days after plating. The live cells with motorneuronal morphology were predominantly from the 24 h culture, and the round and phase bright soma were associated with several primary neurites extending to twice the length of the cell soma occurring in the culture 72 h after plating. The presence of these MN-like cells was sustained for a period of up to 7 days. (E) When cultured at a gradually elevated plating density, the survival rate of the embryonic MNs at 24 h after plating increased correspondingly. *Indicates values that were significantly different compared with that at a density of $1.5x10^4$ cell/cm^2 ($^*p<0.05$); #indicated values that were significantly different compared with that at a density of $3.0x10^4$ cell/cm^2 ($^\#p<0.05$); bar, 50 μm. (F-I) Identification of the MN markers in the cultured cells by immunocytochemistry or immunofluorescence. The immuno-positive particles were distributed mainly in the cytoplasm and the neurite of the MNs. At day 3 after plating, the cultured embryonic spinal MNs were identified by (F) the neuronal marker NSE; (G) MN markers SMI-32 and (H) P75NTR. At day 6 after cell seeding, the cultured embryonic spinal MNs were identified by (I) the MN marker ChAT, and the presence of astrocytes was shown by (J) GFAP immunofluorescence. (F-I) Bar, 25 μm; (J) bar, 50 μm. (K-O) The dose-dependent effect of GDNF on cultured embryonic spinal MNs. Twenty four hours later, compared to the morphology of the cultured MNs in the complete culture medium (K), the spinal motoneruons cultured with GDNF at a concentration of (L) 0.1, (M) 1.0, (N) 10 or (O) 100 ng/ml typically possessed a phase bright, oval-shaped or multi-angular shaped soma with extensive, relatively bipolar neurites. Bar, 50 μm. (P) Twenty four hours later, the survival rate of the cultured spinal MNs was compared and the result showed that GDNF promoted spinal MN survival *in vitro*. *Indicated values that were significantly different compared with control ($^*p<0.05$); #indicated values that were significantly different compared with the 0.1 ng/ml GDNF-treated group ($^\#p<0.05$); △indicated values that were significantly different compared with the 1 ng/mL GDNF-treated group ($^\triangle p<0.05$). (Q-T) Effects of the PLC-γ inhibitor on spinal MN survival. U-73122 at a concentration of 2.5 μM/l, was added into the complete culture medium 30 min prior to GDNF (10 ng/ml) application. Twenty four hours later, the survival rate of the cultured spinal MNs was decreased in the (Q) GDNF + U73122-treated culture, while the survival rate was similar in the (R) GDNF + DMSO treated culture as compared to that in the (S) GDNF-treated culture. Bar, 50 μm. (T) Twenty four hours later, the survival rate of the cultured spinal MNs was significantly decreased by the PLC-γ inhibitor U73122. *Indicates values that were significantly different compared with the GDNF + U73122-treated culture ($^*p<0.05$).

at 24 h were compared with those in each well initially at 4 h after plating, which were taken arbitrarily as 100%. At 24 h after GDNF or GDNF + U73122 treatment, the number of surviving MNs with a neurite process length longer than the length which is two times the cell diameter, was counted. Data are expressed as a percentage of the internal control. Results were presented as the means ± standard error of the mean of triplicate samples from at least six separate experiments (n=6), as described in our previous studies (4,26,29).

Statistical analysis. The statistical calculations and data handling were performed using SPSS version 16.0. All variables were expressed as the means ± standard deviation (SD). A one-way ANOVA was applied to detect differences among groups followed by Tukey-Kramer multiple comparison tests. Differences were considered significant at p values <0.05.

Results

Identification of the MN markers in the cultured cells. Under a phase contrast microscope, the soma of these cells were phase bright and attached to the plate of the well after 4 h (Fig. 1A). The MN-like cells are predominantly solitary in their occurrence from culture 24 h after initial cell seeding (Fig. 1B). The round and phase bright soma were associated with several primary neurites, some of which have secondary and tertiary branches, extending to twice the length of the cell soma in the culture 72 h (Fig. 1C) after cell seeding. The presence of these MN-like cells was sustained for a period of up to seven days in the present study (Fig. 1D).

In order to ensure that the live cells were MNs *in vitro*, immunohistochemistry reactions for the neuronal marker NSE, MNs markers including p75NTR, SMI-32 and ChAT

were carried out. The result revealed that the MN-like cells expressed NSE (Fig. 1F), SMI-32 (Fig. 1G) and p75NTR (Fig. 1H) in culture 72 h after initial plating, and expressed ChAT (Fig. 1I) in culture 6 d after initial plating. The immuno-positive particles were distributed mainly in the cytoplasm and the neurite of the MNs (Fig. F-I). Thereafter, on the sixth day of culture, ChAT immuno-positive mature spinal MNs were characterized by darker brown cytoplasm and longer axon-like processes. From day three after initial plating, we also observed the presence of astrocytes, which expressed GFAP (Fig. 1J).

GDNF promotes spinal MN survival. Since previous studies observed that the survival rate of the cells *in vitro* was influenced by the plating density of the cells, it was, therefore, necessary to choose an optimal plating density prior to the application of GDNF and U73122. At first in our study, the cells were diluted and plated at five different densities: $1.5x10^4$, $3.0x10^4$, $4.5x10^4$, $6.0x10^4$ and $7.5x10^4$ cells/cm^2 in a 96-well plate. Twenty-four hours later, the surviving cells were counted. The survival rates were 27.67±4.25%, 36.87±1.02%, 40.97±3.86%, 46.11±2.73% and 46.61±3.38% at the densities of $1.5x10^4$, $3.0x10^4$, $4.5x10^4$, $6.0x10^4$ and $7.5x10^4$ cells/cm^2, respectively. Statistical analysis showed the lowest survival rate was at the density of $1.5x10^4$ cell/cm^2 (Fig. 1E), which was significantly lower than that at any of the other densities (all $P<0.05$). The highest survival rate occurred at the densities of $6.0x10^4$ and $7.5x10^4$ cell/cm^2. The survival rate was higher with the increase of the plating density. We took the density of $6.0x10^4$ cell/cm^2 as the most optimal plating density in the following study, as the cells tended to cluster and were hard to investigate at the density of $7.5x10^4$ cell/cm^2.

The GDNF, at a concentration of 0.1, 1.0, 10 or 100 ng/ml respectively, was added to the complete medium and applied to the plating cells at a density of $6.0x10^4$. The live cells cultured with GDNF typically possessed MN-like morphology, which showed a phase bright, oval-shaped or multi-angular shaped soma with extensive, relatively bipolar neurites (Fig. 1K-O). Twenty four hours later, the survival rate in the complete culture medium was 46.12±2.73% (Fig. 1K), which was lower than that in any of the GDNF-treated groups (all $p<0.05$). The survival rate of the culture cells was 67.35±1.77% (Fig. 1L), 75.15±2.80% (Fig. 1M), 83.19±1.07% (Fig. 1N) and 81.88±2.12% (Fig. 1O) in the culture with GDNF at the concentration of 0.1, 1.0, 10 or 100 ng/ml, respectively (Fig. 1P). The result showed that GDNF promotes spinal MN survival *in vitro*. Among the GDNF-treated groups, the survival rate was significantly higher at the concentration of 10 and 100 ng/ml (all $p<0.05$). Therefore, we chose 10 ng/ml as the optimal concentration of GDNF in the following study.

U73122 blocks the pro-survival effect of GDNF on spinal MNs. According to previous studies (30) and our preliminary experiment *in vitro*, the inhibitor of PLC-γ, U-73122, at a concentration of 2.5 μM/l, was added into the complete culture medium 30 min prior to GDNF (10 ng/ml) application. Twenty four hours later, the survival rate of the culture cells was only 31.87±2.17% in GDNF + U73122-treated cultures (Fig. 1Q), which was significantly lower than 79.39±1.22% in GDNF + DMSO- (Fig. 1R) and 81.38±1.13% in GDNF-treated cultures (Fig. 1S) (all $p<0.05$), while there was no significant difference of the survival rate between GDNF + DMSO- and GDNF-treated cultures ($p>0.05$). This result showed that the inhibitor of PLC-γ blocks the pro-survival effect of GDNF on the spinal MNs *in vitro*.

Discussion

The present data demonstrated a potent survival-promoting effect of GDNF on developing spinal MNs in culture. Furthermore, U73122, a pharmacological antagonist of PLC-γ, reversed the neuroprotection of GDNF.

Cell counting, adopted as a major method to evaluate MN survival, has been proven to be a credible and convincing tool and has been widely used in previous studies (27,31-33). In the present study, different MN survival rates were recorded by means of cell counting and it was found that PLC-γ is required for the embryonic MN survival in response to GDNF.

Neurotrophic factors play important roles in survival, development and maintenance of the nervous system. GDNF, one of the transforming growth factor (TGF)-β superfamily, was reported to not only reverse 6-hydroxydopamine (6-OHDA)-induced degeneration of dopaminergic neurons *in vivo* (34) but also rescue injured postnatal MNs (35). The results from present study further proved that GDNF exhibits a dose-dependent effect on the survival of embryonic rat spinal MNs in culture. This result was consistent with the previous study, which showed the ability of GDNF to promote chicken MN survival in culture (36).

The important finding of the present study is that the survival-promoting effect of GDNF on MN was reversed by the inhibitor of the PLC-γ signal. Since previous studies have demonstrated that PLC-γ acts as the downstream signal of the GDNF-TrkB pathway, which is involved in the transcription, translation and trafficking of proteins during neuronal and synaptic plasticity of the nervous system (10,11), our present results indicated that the GDNF-TrkB-PLC-γ signaling pathway plays a pro-survival role in the developing spinal MNs. In line with our results, many of the previous studies have also identified that the PLC-γ signal plays a role in numerous types of signaling transduction associated with neuroprotection. It has been shown that PLC-γ plays a pivotal role in survival promotion for many types of neurons in the central nervous system (CNS), facilitated by neurotrophins, such as the hippocampal neurons (37-39), cerebellar granule cells (17,40,41), cerebral cortical neurons and PC12 cells (42). In addition, the role of PLC-γ has been focused on the anti-apoptotic signaling pathway for neurons in the CNS. In the research for preventing retinal ganglion neurons from apoptosis induced by withdrawal of trophic additives, the activation of PLC-γ is required for the anti-apoptotic signaling pathway induced by apolipoprotein E-containing lipoproteins (LpE) (43). It has been shown that PLC-γ is one of the essential signals in the anti-apoptotic role of the lithium ion to prevent cortical neurons from neuronal apoptosis induced by neurotoxicity (44).

Furthermore, the literature suggests that PLC-γ is capable of regulating the development process in the CNS. For instance, the PLC-γ pathway is required for fibroblast growth factor-2 to stimulate retinal ganglion cell (RGC) axon outgrowth *in vitro* and *in vivo* (45). Besides, Xenopus spinal neuron growth cone turning in response to a netrin-1 gradient is dependent on

PLC-γ and PI3K co-activation (46). In the previous studies exploring the underlying neuroprotective effect of GDNF on Parkinson's disease, PLC-γ has also been recognized as one of the second messengers of intracellular signaling pathways activated by the GDNF-GFR α1-Ret system (19). Taken together, the present study suggested that PLC-γ is involved in the survival-promoting effect of GDNF on developing MNs.

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