THE RESPONSE OF MESENCEPHALIC DOPAMINE NEURONS IN CULTURE TO GROWTH FACTORS: EFFECTS ON DEVELOPMENT AND NEUROTOXICITY

by

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Abstract

THE RESPONSE OF MESENCEPHALIC DOPAMINE NEURONS IN CULTURE TO GROWTH FACTORS: EFFECTS ON DEVELOPMENT AND NEUROTOXICITY

by

Jyh-Gong G. Hou

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Parkinson's disease is characterized by dopaminergic neuronal degeneration in the substantia nigra. Several peptide neurotropnic factors have been shown to be able to maintain the survival and promote the recovery of injured dopaminergic neurons. I investigated the effects of two trophic factors, glial cell line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF). bFGF is an astroglial cell mitogen while GDNF is not, although it is secreted by glial cells. By using the experimental model of rat embryonic mesencephalic cultures, I tested whether or not these growth factors could modify the damage caused by the specific dopaminergic neurotoxins 1-methyl-4-phenylpyridinium (MPP^+) and 6-hydroxydopamine (6-OHDA) and help neurons recover following toxin-induced damage.

My results show that GDNF supports the growth of normally developing dopaminergic neurons without affecting glial proliferation. GDNF is unable to prevent the toxicity of MPP^+ or 6-OHDA, but it stimulates the survival and recovery of dopaminergic neurons after damage by MPP^+. bFGF also supports the development of

dopaminergic neurons. This effect is mediated through astroglial cells but is not due to secretion of GDNF by the stimulated glia. Like GDNF, bFGF does not prevent MPP^+ damage to the cultured neurons but effectively protects from 6-OHDA toxicity. This protection is again mediated by glial cells. bFGF-treated mesencephalic cultures have higher levels of reduced glutathione (GSH). Following 6-OHDA treatment GSH levels are further increased. The bFGF effects on GSH are also mediated by glia. Protection from 6-OHDA by bFGF is diminished when GSH levels in mesencephalic cultures are decreased by the GSH synthesis inhibitor L-buthionine sulfoximine (L-BSO).

Astrocytic glial cells synthesize and secrete factors such as GDNF to enhance the development and survival of dopaminergic neurons *in vitro*. The stimulation of glial cells by bFGF allows the upregulation of the antioxidant mechanisms to help cell survival after oxidative stress damage. The role of these neurotrophic factors may be significant for the clinical treatment of Parkinson's disease in the future.

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I. INTRODUCTION

Parkinson's disease is a neurodegenerative disorder. It is characterized by progressive degeneration of the substantia nigra dopaminergic neurons. The clinical manifestations begin to appear when more than 85% of dopaminergic neurons have degenerated. The patient's motor functions continue to deteriorate as the disease progresses. How to protect the nigrostriatal dopaminergic neurons from premature degeneration and slow down the rate of their death has become a major task for investigators who are attempting to prevent or delay the progression of Parkinson's disease.

The etiology of idiopathic Parkinson's disease is still not clear. The causes may be hereditary, viral, environmental neurotoxins, nutrition, ischemia, etc. The underlying mechanisms of neuronal degeneration that have been proposed include abnormal mitochondrial respiration, excessive oxidative stress and apoptosis (for reviews, see Agid, 1991; Oertel and Kupsch, 1993; Olanow, 1993; Uitti and Calne, 1993; Burkhardt and Weber, 1994; Mizuno et al., 1995; Schapira, 1995)

After the finding that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) caused clinical symptoms and pathological changes very similar to those of Parkinson's disease (Davis et al., 1979; Langston et al., 1983), the study of this disease was vastly helped by the establishment of MPTP-monkey model (Burns et al., 1983). The *in vitro* model of MPTP-treated mesencephalic cultures was established shortly afterward (Mytilineou and Cohen, 1984). Treating mesencephalic cultures from rat embryos with MPTP or its metabolite, 1-methyl-4-phenylpyridinium (MPP^+) could cause destruction of

dopaminergic (DA) neurons. 6-Hydroxydopamine (6-OHDA) is also another specific DA neurotoxin commonly used in animals or culture systems to induce DA neuron degeneration.

Many peptide growth factors have been shown to be able to stimulate the development, maintain the survival and promote the recovery of CNS or PNS neurons. Growth factors affecting the development of DA neurons include basic fibroblast growth factor (bFGF; Ferrari et al., 1989; Knusel et al., 1990), epidermal growth factor (EGF; Knusel et al., 1990; Casper et al., 1991; Ferrari et al., 1991;), brain-derived neurotrophic factor (BDNF; Hyman et al., 1991a; Hyman et al., 1991b), neurotrophin-4/5 (NT-4/5; Hynes et al., 1994), and glial cell line-derived neurotrophic factor (GDNF; Lin et al., 1993; Lin et al., 1994; Hou et al., 1996). The mechanisms by which these growth factors exert their effect supporting DAergic neurons are still unknown. However, astrocytic glial cells may in part mediate the DAergic neurotrophic effects, since EGF and bFGF were found to stimulate the proliferation of astrocytes (Casper et al., 1991; Engele and Bohn, 1991). Their effects disappeared when the glial growth was inhibited. Furthermore, GDNF was purified from B49, a rat glial cell line (Schubert et al., 1974). Glial cells thus may be able to secrete factors that are neurotrophic to DAergic neurons. Glia may also support the neuron growth and survival by other mechanisms. The antioxidant capability of glial cells is one of the most likely candidates. It has been shown that glutathione (GSH), a free radical scavenger, is largely maintained and regulated by glial cells (Raps et al., 1989; Sagara et al., 1993).

In this study, I characterized the effects of GDNF and bFGF on the development of DA neurons in rat embryo mesencephalic cultures, and examined whether treatment

with these factors could modify the damage caused by MPP^+ and 6-OHDA or help the DAergic neurons recover and survive after the damage. The role of astrocytic glial cells and the mechanisms of protection, particularly the involvement of the antioxidant glutathione, was also investigated.

A. Parkinson's Disease:

Parkinson's disease was first described by James Parkinson (1817) in "An essay on the shaking palsy." Clinically, it is an idiopathic, slowly progressive, degenerative CNS disorder with four characteristic features: (1) slowness and poverty of movement; (2) muscular rigidity; (3) resting tremor; and (4) postural instability. Approximately one in every one thousand adults above 50 years old are affected (see review, Berkow et al., 1987). Pathologically, the major findings are degeneration of the pigmented dopaminergic neurons in the substantia nigra pars compacta, resulting in striatal dopamine (DA) deficiency, and the presence of eosinophilic Lewy body inclusions in the cytoplasm of surviving substantia nigral neurons (Agid, 1991). When the reduction in the striatal DA content is greater than 80%, the clinical symptoms manifest.

Currently the two strategies used for treatment of Parkinson's disease are symptomatic and protective therapy (see review, Calne, 1993). Levodopa is the most common medicine for symptomatic therapy. It penetrates the blood brain barrier and is decarboxylated to dopamine in the brain. However, after prolonged treatment with levodopa, patients may have severe dyskinesia, often alternating with episodes of sudden unpredictable loss of mobility, and be refractory to drug treatment in the end. Neuroprotective therapy is designed to treat the underlying pathogenesis of Parkinson's

disease so that neurodegeneration may be prevented or delayed. Selegiline (deprenyl), an inhibitor of monoamine oxidase B, may decrease the generation of free radicals by reducing the oxidative metabolism of dopamine (Parkinson Study Group, 1989). However, it is still not clear whether its benefit arise from a symptomatic or a protective action. Brain transplantation of human fetal neurons from aborted fetuses is also undergoing investigation (Redmond et al., 1983; Olanow et al, 1994). So far, it has been only moderately successful. Furthermore, it is highly controversial because of the therapy's link with abortion. Exploring the mechanisms to protect neurons from degeneration and stimulate regrowth can provide important information for effective treatment of Parkinson's disease.

B. Dopaminergic Neurons:

Dopamine (DA), a monoamine neurotransmitter, belongs to the family of catecholamines. The main mass of DA neurons is located in the mesencephalon in the retrorubral (A8), substantia nigra (A9), and ventral tegmental (A10) groups (see review, Carpenter, 1991). These neurons convert tyrosine by the rate-limiting enzyme tyrosine hydroxylase (TH) to L-Dopa, which is subsequently catalyzed by L-aromatic amino acid decarboxylase to dopamine. In norepinephrine neurons, dopamine is further converted to norepinephrine. In epinephrine neurons, norepinephrine is further converted to epinephrine. Dopamine, norepinephrine, and epinephrine all belong to catecholamine family (Joh et al., 1984). Dopamine is stored within highly specialized granular vesicles in nerve endings. Upon an action potential, DA is released from the nerve terminal. This release is calcium-dependent and modulated by presynaptic autoreceptors. The principal

deposition of DA after release is by reuptake into the nerve endings. Approximately 80-90% of DA is reuptaken by nerve endings (Axelrod, 1972). Only 10-20% is metabolized by enzymes, MAO and COMT, into DOPAC and HVA, respectively (Persson, 1970).

The axons originating from DA neurons form three major different tracts with different physiologic functions. The nigrostriatal tract, which projects from A8 and A9 substantia nigral cells, is mainly responsible for initiation and execution of movement. Loss of its function is the cause of Parkinson's disease, which shows akinesia and rigidity. The mesolimbic-mesocortical tract, which projects from ventrotegmental cells (A10), is involved in thinking and emotion. It is the one implicated in DA theories of schizophrenia (see review, Snyder, 1982). The tuberoinfundibular tract, which projects from A11, A12, A13, and A14 in diencephalon, is considered to facilitate the release of oxytocin and vasopressin (see review, Björklund and Lindvall, 1984).

C. *In Vitro* Model of Dopaminergic Neuronal Degeneration:

The establishment of the appropriate model for Parkinson's disease relies on the causes of DA neuron degeneration. Unfortunately, the etiology of Parkinson's disease is still not clear. Genetic and environmental factors as triggers of the nigrostriatal neurodegeneration have been proposed for a long time. Since about 10-15% of Parkinson's disease cases have relatives similarly affected, inheritance may be an important factor. However, monozygotic twins are no more likely to share the illness when one has Parkinson's disease than are dizygotic twins (Ward et al., 1983). There are some reports of kindreds with familial Parkinson's disease with autosomal dominant

inheritance that have been autopsy-confirmed (Golbe et al., 1990; Golbe et al., 1993; and see review, Duvoisin, 1993). However, most of the Parkinson's cases remain idiopathic.

The environmental factor hypothesis is not strongly supported, either. People earlier thought that the clinical onset of the disease is due to long-term environmental insult. However, observations on twins or spouses did not support this idea, since the occurrence of conjugal Parkinson's disease is remarkably low among twins and spouses in marriages lasting for many decades (Agid, 1991).

In 1983, a human neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was identified (Langston et al., 1983). It was found to cause a clinical symptoms similar to Parkinson's desease itself, selectively destroying the substantia nigra pars compacta and inducing neuropathological and neurochemical changes very similar to Parkinson's disease (Ballard et al., 1985). Although the hypothesis of prolonged MPTP exposure is still not a convincing explanation to the etiology of the disease, MPTP has become an important tool for research in Parkinson's disease. Another important toxin is 6-hydroxydopamine (6-OHDA), which is a specific neurotoxin for catecholaminergic neurons (see review, Kostrzewa and Jacobowitz, 1974) and has long been used for animal models.

(I) MPTP and MPP^+

Since its identification in 1983, MPTP has been widely used to produce experimental models of Parkinson's disease in various animals (Langston et al., 1983). Species differences in sensitivity to MPTP was found. *In vivo,* primates are very sensitive to the toxic effects of MPTP, while mice and particularly rats are rather resistant (Chiueh

et al., 1984; Heikkila et al., 1984). However, Mytilineou and Cohen found that in culture system, rat embryos' mesencephalic dopaminergic neurons were destroyed by treatment with MPTP (Mytilineou and Cohen, 1984). This finding established the basis for studying MPTP toxicity and its effects on neurodegeneration in vitro.

MPTP is a protoxin. It must be first converted by MAO-B to the intermediate, 1-methyl-4-phenyl-2,3-dihydropyridinium ($MPDP^+$) and subsequently to 1-methyl-4-phenylpyridinium (MPP^+) to have the neurotoxic action (Chiba et al., 1985). This conversion is believed to be outside the dopamine neurons, probably by MAO-B in astrocytes (Westlund et al., 1985). Inhibitors of MAO-B such as deprenyl and pargyline are capable of blocking the neurotoxicity of MPTP *in vivo* (Langston et al., 1984; Cohen et al., 1994). MPP^+ is selectively accumulated within dopaminergic neurons via the high affinity DA uptake carrier system (Javitch et al., 1985). This was proven by the fact that DA uptake inhibitors such as mazindol and GBR-12909 were able to protect against MPTP-induced neurotoxicity (Chiba et al., 1985; Javitch et al., 1985; Sanchez-Ramos et al., 1986; Schinelli et al., 1988; Snape et al., 1988).

The mechanism of MPTP neurotoxicity is still not completely clear, although there are two possible explanations (Sayre, 1989; Gerlach et al., 1991). The first hypothesis is that MPP^+ accumulates in the mitochondria and inhibits the NADH-linked mitochondrial respiratory chain at the level of Complex I, causing cell destruction (Nicklas et al., 1985; Ramsay and Singer, 1986; Krueger et al., 1990). A similar deficiency in complex I activity has been found in the substantia nigra of patients with Parkinson's disease (Schapira et al., 1990). The second hypothesis is that MPP^+ can induce oxidative stress (Johannessen et al., 1986). According to these studies, free radicals like superoxide

and hydroxyl radicals can be generated intracellularly as a result of MPP^+ redox cycling and lead to cellular damage. A recent study showed that antioxidants, such as glutathione, ascorbic acid and catalase could protect mitochondria from the inhibition of complex I activity caused by MPP^+ (Cleeter et al., 1992). It is possible that free radicals are generated following intracellular biochemical changes due to inhibition of mitochondria respiration and cause further cell damage.

(II) 6-Hydroxydopamine

First described by Tranzer and Thoenen (1967 & 1968), 6-hydroxydopamine (6-OHDA) is a specific neurotoxin to catecholaminergic neurons. Its structure is an analogue of catecholamines. It is selectively taken up and accumulated within catecholaminergic neurons by the high-affinity transport system (Breese and Traylor, 1970). It produces dopaminergic neuronal degeneration in experimental animals *in vivo* by direct injections into the substantia nigra (Ungerstedt, 1968; Breese and Traylor, 1970; Uretsky and Iversen, 1970). Bilateral injections of 6-OHDA produce animals with a profound motor deficit similar to that in Parkinson's disease (Jonsson, 1976). *In vitro,* mesencephalic neuron cultures are also damaged by addition of 6-OHDA (Mytilineou and Danias, 1989).

6-OHDA neurotoxicity results from its autoxidation to form quinones, hydrogen peroxide and cytotoxic free radical species like superoxide and hydroxyl radicals (Heikkila and Cohen, 1972; Sachs et al., 1975; Graham et al., 1978). This neurotoxicity is potentiated by ascorbic acid which helps the recycling of quinones back to 6-OHDA (Heikkila and Cohen, 1972). In the cytoplasm and the axonal membrane, quinone covalently binds to neuronal proteins (Johsson, 1976) and the free radicals cause

oxidation of sulfhydryl groups of enzymes and membrane lipid peroxidation, thus damaging neuronal structures. Recently, there has been evidence showing that the injection of 6-OHDA into the striatum of rats causes reduction in the level of GSH, superoxide dismutase (SOD) and GSH peroxidase, and decreased the membrane fluidity, while elevating intracellular calcium concentration (Kumar et al., 1995), further suggesting the involvement of 6-OHDA in free radical generation.

D. Dopaminergic Neurotrophic Factors:

The first neurotrophic factor discovered is nerve growth factor (NGF; Bueker, 1948) It is called "neurotrophic" because it regulates survival and neurite growth of sympathetic and sensory neurons. Molecules that regulate neuronal growth, differentiation, and survival are referred to as neurotrophic factors.

Growth factors affecting the development of DA neurons include basic fibroblast growth factor (bFGF; Ferrari et al., 1989; Knusel et al., 1990), epidermal growth factor (EGF; Knusel et al., 1990; Casper et al., 1991; Ferrari et al., 1991;), brain-derived neurotrophic factor (BDNF; Hyman et al., 1991a; Hyman et al., 1991b) neurotrophin-4/5 (NT-4/5; Hynes et al., 1994), and glial cell line-derived neurotrophic factor (GDNF; Lin et al., 1993; Lin et al., 1994; Hou et al., 1996).

(I) GDNF:

Lin et al. purified and cloned a potent neurotrophic factor from the rat B49 glial cell line that enhanced survival of midbrain dopaminergic neurons (Lin et al., 1993; Lin et al., 1994). This glial cell line-derived neurotrophic factor (GDNF) is a N-glycosylated,

disulfide-bonded homodimer distantly related to the TGF-β superfamily (< 20% homology). The recombinant human GDNF (rhGDNF) promoted the survival and morphological differentiation of dopaminergic neurons in cultures and increased their dopamine uptake.

The initial findings by Lin et al. (1993), showed that there are several specific characteristics of GDNF:

(1) It only increased high-affinity dopamine uptake of dopaminergic neurons; it did not increase transmitter uptake by GABAergic and serotonergic neurons.

(2) It did not increase total neuron or astrocyte numbers.

(3) It was rather potent in exhibiting its EC_{50} (half-maximal effective concentration) at 36 pg/ml, compared to those of EGF and FGF at the 1 to 100 ng/ml range.

The distribution and expression of GDNF mRNA was detected by reverse transcriptase followed by PCR. Scharr et al. (1993) found that GDNF was expressed by substantia nigra (SN) and basal forebrain type 1 astrocytes in rat CNS. Neuronal cultures of embryonic SN also expressed GDNF mRNA. Using PCR, GDNF mRNA was also found in striatum, hippocampus, cortex and spinal cord in both adult rat and human (Springer et al., 1994). However, *in situ* hybridization showed no GDNF mRNA in adult rat (Stromberg et al., 1993). This suggests that GDNF is expressed in higher levels during embryonic stages to help the development of neurons, and gradually decreases its expression after maturation.

The *in vivo* effects of GDNF were extensively studied. Hoffer et al. (1994) injected GDNF intranigrally four weeks *after* ipsilateral 6-OHDA lesion in rats and found that it produced a marked decrease in apomorphine-induced rotations. Using HPLC,

Bowenkamp et al. (1995) observed that in the 6-OHDA lesioned site of SN, GDNF produced DA levels that were not statistically different from the control. In addition, GDNF injection *before* 6-OHDA protected the intranigral or intrastriatal lesions (Kearns et al., 1995). Similar to 6-OHDA, Tomac et al. (1995) found GDNF protected from MPTP toxicity if injected over the SN or striatum prior to MPTP injection. If injected after MPTP, it significantly restored DA levels and fiber densities. These experiments show that GDNF both protects and helps the regeneration of neurons in SN and striatum of rats, against 6-OHDA or MPTP neurotoxcity.

GDNF is also a potent survival factor for motoneurons present in cranial motor nuclei and spinal cord (Henderson et al., 1994; Zurn et al., 1994). It stimulates fiber formation and survival in cultured neurons from peripheral autonomic ganglia (Ebendal et al., 1995) and is an age-specific survival factor for sensory and autonomic neurons (Buj-Bello et al., 1995).

(II) bFGF:

Fibroblast growth factor (FGF) is a family of angiogenic factors consisting of aFGF, bFGF, kFGF, int-2, FGF-5, FGF-6 and KGF (see review, Westermann et al., 1990). They are found in the brain as well as in many mesenchymal tissues outside the nervous system. bFGF and its receptors are both expressed in the embryonic (Caday et al., 1990; Kalcheim and Neufeld, 1990) and adult brain (Emoto et al., 1989), indicating that this factor not only plays a role in the development of the CNS but also in the maintenance of neural architecture and function. *In vitro,* bFGF supports the survival and growth of neurons in cultures from different brain regions (Westermann et al., 1990). It

was found to be synthesized by astrocytes *in vitro* (Ferrara et al., 1988; Hatten et al., 1988). However, it is also mitogenic (Morrison and De Vellis, 1981; Perraud et al., 1988) and induces morphological and biochemical changes to astrocytes (Perraud et al., 1988). This implies that bFGF may have an autocrine function to astrocytes.

bFGF also plays an important role in neuroprotection. *In vivo* experiments showed enhanced recovery of the nigrostriatal dopaminergic system in MPTP-treated mice following intrastriatal injection of bFGF (Matsuda et al., 1992; Date et al., 1993). Many *in vitro* studies also showed that bFGF protected from toxicity of MPP^+ (Park and Mytilineou, 1992; Otto and Unsicker, 1993), iron (Zhang et al., 1993) and glutamate (Skaper et al., 1993). Although earlier studies suggested that the support of survival and growth of DA neurons *in vitro* by bFGF was independent of glial cell proliferation (Walicke and Baird, 1988), it was subsequently shown that bFGF increases glial division and that its neurotrophic activity is eliminated by inhibition of cell proliferation (Knusel et al., 1990; Engele and Bohn, 1991; Engele et al., 1991; O'Malley et al., 1994). Mesencephalic glia and medium conditioned by mesencephalic astrocytes promote the survival and growth of DA neurons, suggesting the secretion of soluble neurotrophic factor(s) by astrocytes (Engele et al., 1991; O'Malley et al., 1991; Gaul and Lubber 1992; Dong et al., 1993; O'Malley et al., 1994; Takeshima et al., 1994a; Takeshima et al., 1994b). Thus, the neurotrophic effect of bFGF is likely to be mediated, at least in part, by other soluble factor(s) secreted by bFGF-stimulated glia. Although the glial-secreted neurotrophic molecule(s) have not yet been completely characterized, GDNF should be regarded as one of the candidates since it was originally isolated from the medium conditioned by B49 glial cell line as previously mentioned (Lin et al., 1993; Lin et al.,

1994). In my thesis study I examined whether secretion of GDNF by glial cells plays a role in the neurotrophic effect of bFGF on DA neurons in dissociated embryonic mesencephalic cell cultures. Combinations of the two growth factors were used to determine whether their effects on DA neurons were additive. In addition, polyclonal antibodies neutralizing the neurotrophic action of GDNF were used in conjunction with bFGF to determine whether they would modify the bFGF effect on DA neurons.

E. Antioxidant Mechanisms on DA Neuron Protection

Brain cells consume a large proportion of the body's oxygen, as they get almost all the energy from oxidative metabolism during mitochondrial respiration. As a result, they are very vulnerable to oxidative stress from hydrogen peroxide (H_2O_2) and free radicals---superoxide anion ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$)---which are generated as byproducts of metabolic processes that utilize molecular oxygen (O_2) (see review, Fahn and Cohen, 1992; Olanow, 1993).

There are defense mechanisms in the human body to remove oxidants such as peroxidase and free radicals. A major defense mechanism involves glutathione (GSH; Meister and Anderson, 1983; Cohen, 1983). Its reduced form, GSH, reacts with H_2O_2 and lipid peroxides via the enzyme GSH peroxidase to form H_2O and oxidized form of glutathione (GSSG). GSSG is reduced by GSSG reductase to GSH. This cycle needs NADPH to be maintained. Glutathione is thus called a 'scavenger' for free radicals. It is involved in protection against oxidative stress and conjugation of various toxic substances. Measuring the levels of GSH is one of the indexes in determining the function of antioxidant defenses.

The mechanisms of dopamine cell death in substantia nigra in Parkinson's disease are not known but is has been postulated that free radical species and oxidative stress are involved (see review, Fahn and Cohen, 1992). There is evidence demonstrating that postmortem tissues from patients with Parkinson's disease have reduced levels of GSH (Perry et al, 1982; Kish et al., 1985; Slivka et al., 1987; Di Monte et al., 1992; Jenner et al., 1992), altered iron metabolism (Dexter et al., 1989; Riederer et al., 1989), increase of lipid peroxides (Ambani et al., 1975; Dexter et al., 1989), and decreased complex I activity of the mitochondrial respiratory chain (Schapira et al., 1990a; Schapira et al., 1990b). Experiments also showed that transgenic mice over-expressing SOD were resistant to MPTP toxicity (Przedborski et al., 1992). One could thus expect that up-regulating the antioxidant defense systems may be a key to prevent neuronal loss in Parkinson's disease.

Neurotrophic factors can protect neurons against oxidative insults (see review, Mattson et al., 1993). It was shown that NGF up-regulated catalase and protected human neuroblastoma cells from damage by direct addition of H_2O_2 (Jackson et al., 1990). Recently, it was reported that NGF also elevated GSH level in PC12 cells (Pan and Perez, 1993; Jackson et al., 1994). Another growth factor, BDNF, elevated GSSG reductase, prevented a rise in GSSG, and protected mesencephalic cultures against 6-OHDA and MPP^+ neurotoxicity (Spina et al., 1992a; Spina et al., 1992b). bFGF, NGF, and BDNF each increased the activity of SOD and GSSG reductase (Mattson et al., 1995).

Glial cells seem to play a paradoxical role on neurotoxicity. First, glia transform MPTP to MPP^+ which is taken up by DA neurons to evoke a neurotoxic action (Chiba, 1984). Second, microglia actively secrete superoxide and H_2O_2 upon stimulation (Colton

et al., 1990). However, there is increasing evidence showing that glia plays a role in antioxidant mechanisms. It was found that in CNS the glial cells have the greatest abundance of GSH and might protect neurons against oxidative insults (Slivka et al., 1987). Makar et al. (1990) reported that vitamin E and glutathione levels are higher in astrocytes. Sagara et al. (1993a, 1993b) found that glutathione level was maintained by glial cells by actively taking up cystine, a precursor of glutathione. Cytochemical evidence showed high levels of GSH in radial glial cells in the rabbit retina (Pow and Crook, 1995) as well as glial cells and neuropil in rat brain (Slivka et al., 1987). Viña et al. (1992) found that astrocytes in primary cultures could maintain higher GSH content than neurons even under oxidative stress created by tert-butyl hydroperoxide. The presence of an abundance of GSH in glial cells suggests that glia play a critical role in regulating the content of potentially damaging oxidative species in brain.

II. EXPERIMENTAL PROCEDURES

A. Preparation and Maintenance of Dissociated Mesencephalic Neuronal Cultures

Neuronal cultures were established from rat embryonic mesencephalic tissue on the 14.5 ± 0.5th day of gestation. Female pregnant Sprague-Dawley rats, purchased from Taconic Farms (Germantown, NY), were sacrificed in a container with CO_2. Cesaerian section (a one-inch horizontal incision on the rat lower abdomen) was performed. The uteri containing the embryos were removed and placed in a petri dish containing Minimal Essential Medium (MEM; Gibco). Embryos were then taken out from the uteri under sterile environment and placed in dishes containing Earle's balanced salt solution (BSS; Gibco) with 1% horse serum and penicillin-streptomycin (Sigma). Embryos were kept in this solution for 15 minutes before moved to another identical preparation of fresh BSS solution with serum for the total of three, 15-minute rinses. After these rinsing periods, embryos were considered sterilized and cleaned from contaminating blood. Embryos were then moved to another dish containing defined medium without serum and ready for dissection.

Mesencephalic tissues were dissected out and separated from skin and meningial tissues under a dissecting microscope. They were collected in a dish containing Ca^{+2}- and M^{+2}- free phosphate buffer saline (PBS; Sigma) at 4°C. After all the mesencephalic brain tissues were collected, they were minced into small fragments and mechanically

dissociated in chemically defined medium by mild trituration with a small-bore Pasteur pipette. The number of viable cells was determined by exclusion of trypan blue dye (1:1 dilution with 4% solution in PBS) and counted with a hemocytometer. 0.5 ml of cell suspension was then plated into 35mm Falcon plastic dishes with 1 ml of chemically defined medium at the density of 1 X 10^6 cells/cm^2. Final volume in each dish was 1.5 ml. The Falcon 35mm plastic dishes were pre-coated with L-polyornithine (0.1 mg/ml; Sigma), washed and rinsed with Earle's balanced salt solution (BSS; Gibco) 24 to 48 hours before plating neuronal cells.

The serum-free chemically defined medium, modified from Bottenstein and Sato (1979) and DiPorzio et al. (1980), consisted of Dulbeco's modified Eagle's medium/F12 (1:1) with Glutamax I (Gibco), glucose (33mM), $NaHCO_3$ (44.6mM), apo-transferrin (100 μg/ml; Sigma), insulin (25 μg/ml; Sigma), putrescine (60 nM; Sigma), sodium selenite (30 nM), and progesterone (20 nM; Sigma).

Cultures were maintained at 37°C in an atmosphere of 5% CO_2 / 95% air and 100% relative humidity. In experiments designated to grow the cells in serum-containing medium, the medium was changed to MEM with 200μM glutamine plus 10% fetal bovine serum (HyClone) and 10% horse serum (Gemini, CA) three hours after plating.

B. Experimental Treatments of Cultures:

(I) Growth Factors:

All cultures were refed three hours after plating with fresh chemically defined medium or with medium containing various concentrations (0.01, 0.1, 1, and 10 ng/ml) of

GDNF (recombinant human GDNF; gift from Dr. L-F. H. Lin) and/or bFGF (0.1, 1 and 10 ng/ml, recombinant human bFGF; gift from Dr. L-F. H. Lin). All growth factors were stored in 0.1% BSA stock. The same amount of 0.1% BSA without growth factors is added in the medium in controls. Growth factors were present throughout the experiments including during treatment of cultures with toxins.

(II) Astrocytic Glial Cell Division (Proliferation) Inhibitor:

The mitotic inhibitor 5-fluoro-2'-deoxyuridine (FUDR) and uridine (Sigma) at 8 μM and 20 μM, respectively, were added to the feeding medium 24 hours after plating cultures. FUDR was added again at each refeeding and was present throughout the experiment.

(III) L-buthionine-[S,R]-sulfoximine (L-BSO) Treatment

To inhibit the synthesis of GSH in the cultured cells, sterile L-BSO (Chemical Dynamics Co.; South Plainfield, NJ) solution was directly added into the cultures at the final concentration of 10 μM on DIV 7. L-BSO remained in the culture medium for 24 hours inculding during the treatment with 6-OHDA on day 8. Fresh medium containing the appropriate amount of L-BSO was again added into the cultures after 6-OHDA treatment until the time of GSH assay or [^{3}H]-DA uptake measurement (on DIV 9).

(IV) Anti-GDNF Antibodies Treatment

Sterile polyclonal GDNF antisera raised in rabbit (gift of Dr. L-F. H. Lin,) at a 1:1000 dilution was added concurrently with growth factors. This antiserum has been

tested and shown to cross react with the GDNF from rat B49 and the human recombinant GDNF. The same amount of pure rabbit serum was added in controls. Fresh GDNF antiserum was added to the cultures every other day to increase the probability that antibodies were present throughout the experiment.

(V) Neurotoxins:

A. 1-Methyl-4-Phenylpyridine (MPP^+)

Cultures were exposed to freshly prepared 100 μM 1-methyl-4-phenylpyridine (MPP^+) (Research Biochemicals , Inc.) in the feeding medium on the designated day for 60 minutes, or 0.1 to 10 μM MPP^+ for 24 hours. Control groups received the same amount of sterile double distilled water into the medium. After exposure, cultures were washed with BSS. Fresh defined medium containing 10μM MK-801 (Research Biochemical Inc.), to prevent damage to neurons following medium change (Driscoll et al., 1993), was then added and the cultures were maintained for another 24 hours. The addition of MK-801 was solely to prevent glutamate toxicity and it did not interfere with MPP^+ toxicity (Michel and Agid, 1992; Finiels-Marlier et al., 1993). Damage to dopaminergic neurons was determined by tyrosine hydroxylase (TH) immuno-cytochemistry and by measuring the uptake of [^{3}H]-DA (New England Nuclear, Boston; 29.5 Ci/mmol).

B. 6-Hydroxydopamine (6-OHDA)

6-OHDA was prepared in 0.01N HCl containing ascorbic acid (200μM final concentration in the cultures) and kept on ice prior to use. Cultures were exposed to 30 μM 6-OHDA for 45 minutes in the 37°C incubator. Control groups received the same

amount of 0.01N HCl containing ascorbic acid without 6-OHDA. After washing with BSS, cultures were refed with fresh chemically defined medium containing 10μM MK-801 and analyzed 24 hours later for neurotoxic damage to the dopaminergic neurons by TH immunostaining and by uptake of [^{3}H]-DA.

C. Analytical Procedures:

(I) High Affinity Dopamine Uptake:

After one rinse and a 10-minute incubation with fresh Krebs-Ringer buffer with ascorbic acid (0.2mg/ml), cultures were incubated with the same buffer containing [^{3}H]-DA (0.5 μCi /ml; 29.5 Ci/mmol; New England Nuclear) for 10 minutes at 37°C. Cultures were then washed twice and incubated for 5 minutes at 37°C with fresh buffer to remove remaining extracellular [^{3}H]-DA in the buffer. The [^{3}H]-DA accumulated was released by incubating with 1ml 95% ethanol at 37°C for 30 minutes. The [^{3}H]-DA containing ethanol was then put into scintillation vials filled with 10 ml Ecoscint (National Diagnostics) and counted in a scintillation spectrometer. Nonspecific uptake values were determined by adding 10 μM mazindol (Sandoz Pharmaceuticals) into culture groups to block the neuronal dopamine uptake.

(II) Immunocytochemistry:

To visualize tyrosine hydroxylase, cultures were fixed in 4% paraformaldehyde for 10 minutes at room temperature and then 20 minutes at 4°C. After three washes and

permeabilization by a 30-minute 0.2% Triton X-100 treatment, cultures were exposed to tyrosine hydroxylase antibodies (1:1000; monoclonal from Boehringer-Mannheim; polyclonal from Eugene Tech). Cultures were allowed to react with antibodies at 4°C overnight (in PBS containing 2% horse or goat serum). After the reaction period, they were stained with the peroxidase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories) and 3', 3'-diaminobenzidine (DAB) as a chromagen.

For demonstration of glia, cultures were stained by the same method described above except that the primary antibodies were against glial fibrillary acidic protein (GFAP antibodies; 1:1000; Accurate Chemical).

(III) Cell Counts and Morphometry:

The number of dopaminergic neurons in cultures was determined by counting the cells positively immunostained with TH antibodies. One hundred fields (0.5 X 0.5 mm) in two transverse strips across the diameter of the dish, representing 2.5% of the total area, were counted using a Nikon inverted microscope at 200X magnification. The number of astrocytes was determined the same way by counting GFAP-immunoreactive cells in 100 random fields. The length of the longest neurite of a TH immunopositive (TH+) neuron was estimated at 400X magnification by counting the number of its intersections with a reticule grid (0.25 X 0.25 mm, subdivided into 100 squares). A total of 60 cells (3 dishes, 20 cells in each) were selected at random for measurements in each treatment group.

(IV) Glutathione (GSH) Assay

The cell culture plates were kept on ice and the medium was removed. Following two rinses with 1 ml of sterile phosphate-buffered saline (PBS), the cells were scraped off into 1.2 ml of buffered saline with a cell scraper (Falcon) and transferred to plastic microcentrifuge tubes. Samples were centrifuged at 4,000 g for 15 minutes, and the supernatant was discarded. Addition of 250 µl of 0.4 M perchloric acid was followed by sonication for 10 seconds with setting at 4 (Vibra-Cell model V1A; Sonics and Materials, Danbury, CT) and centrifugation at 18,000 g for 15 minutes. The pellet was reserved for protein assay. The supernatant (40 µl) was assayed for glutathione with a modification (Slivka et al., 1987) of the enzymatic recycling assay of Tietz (1969), ussing GSSG reductase, NADPH, and 5,5'-dithiobis-(2-nitrobenzoic acid). Results are reported in terms of the protein content of the samples (µg GSH/ mg protein).

(V) Protein Determination

Protein in the precipitated pellet (0.4 M perchloric acid) was quantified by the method of Lowry et al. (1951) with bovine serum albumin used as the standard. The protein pellet was first solubilized in 0.5 ml of 0.2 M NaOH with sonication, and 100µl was taken for assay (final assay volume, 2.0 ml).

(VI) Statistical Analysis:

Data (means ± SEM) are expressed as % of untreated controls. Significance between two groups was tested by independent *t*-test. For multiple comparisons, analysis of variance (ANOVA) followed by post-hoc test were performed. The computer program Instat Version 2.0 (GraphPad Software, San Diego, CA) was used for statistical analysis.

III. RESULTS

A. Dopaminergic Neurotrophic Effect of GDNF

Cultures maintained in chemically defined medium were treated with 0.01, 0.1, 1, and 10 ng/ml GDNF at the time of plating (DIV 0). High affinity [^{3}H]-DA uptake and immunostaining for TH were used to determine the *in vitro* development of DA neurons on DIV 6. No growth stimulating effect was observed at 0.01 ng/ml GDNF, while 0.1, 1 and 10 ng/ml GDNF significantly increased DA uptake (Fig. 1A). At 10 ng/ml, DA uptake was lower, compared to 1 ng/ml concentration. Similar to DA uptake, the number of TH-positive (TH+) cells also was significantly higher at 0.1 and 1 ng/ml but not at 10 ng/ml GDNF (Fig. 1B). On DIV 10, the DA uptake showed a remarkable increase at 0.1 ng/ml, reaching the plateau above 1 ng/ml (Fig. 1A). The number of TH+ cells was significant lower than that of DIV 6 due to serum deprivation, but GDNF 1 and 10 ng/ml effectively reduced the loss of TH+ cells over this period in vitro. These data indicate that GDNF requires an optimal concentration for maximal neurotrophic effect, at around 1 ng/ml under our experimental conditions.

The length of neuritic processes of DA neurons was also increased following GDNF treatment. This was evident by microscopic observation of cultures stained with antibodies to TH (Fig. 2 A & B). The morphological observations were confirmed by measurement of the longest neurite of 60 randomly selected TH+ cells on DIV 6. Table 1 shows the average length of the longest neurite was 185 ± 13 μm in controls and 350 ±

29 μm in GDNF treated cultures ($p<0.001$; N=60). In addition to the increase of neuritic length, the number of neurites per TH+ neuron was increased in GDNF treated cultures (total number of neurite branches per TH+ cell was 3.41 ± 0.19 in controls and 5.70 ± 0.26 in GDNF treated cultures; $p<0.001$; N=60). The increase in the number and length of TH+ fibers in the GDNF-treated cultures indicates that the observed increases in [^{3}H]DA uptake reflect process outgrowth rather than just increased numbers of DA transporters.

GDNF treatment did not cause an increase in the number of GFAP+ astrocytes present in the cultures (Fig 2C & D and Table 1). The number of GFAP+ astrocytes per cm^2 at DIV 7 was 1501 ± 134 in controls compared to 1438 ± 178 in GDNF-treated cultures and there was no significant difference between the two groups. This is in agreement with previous findings (Lin et al., 1993), demonstrating that the growth stimulating effects of GDNF were not due to an increase in the number of astrocytes. The neurotrophic effect of GDNF on DA neurons was not modified by maintaining the cultures in serum containing medium, which stimulates the growth of glial cells. In serum containing medium, the value of [^{3}H]DA uptake in 1 ng/ml GDNF-treated cultures on DIV 11 was 182.1 ± 8.0 % of control, compared to 183.6 ± 7.4 % of control in serum free medium.

To determine whether stimulation of growth depends on the age of neurons at the time of GDNF treatment, we applied GDNF (1 ng/ml) during DIV 0-5, DIV 5-10 or DIV 0-10 and measured [^{3}H]-DA uptake on DIV 10. Under all GDNF treatment paradigms [^{3}H]-DA uptake was higher than that in control cultures and it reached the similar levels on DIV 10 (Fig. 3). This indicates that a single addition of GDNF is sufficient to exert

neurotrophic activity on DA neurons and that GDNF is effective when applied at different times of *in vitro* development.

B. Effect of GDNF on MPP^+ Neurotoxicity

To determine whether GDNF has protective effect against MPP^+, cultures were treated with GDNF concentrations of 0.1, 1 and 10 ng/ml in serum free medium on DIV 0. They were exposed to 100 μM MPP^+ for 1 hour on DIV 5. MPP^+ was washed from the cultures and [^{3}H]-DA uptake was measured 24 hours later. As expected, in cultures not treated with MPP^+, GDNF increased [^{3}H]-DA uptake at 0.1 and 1 ng/ml concentrations (Fig. 4). Exposure to MPP^+ resulted in a reduction of [^{3}H]-DA uptake to 37% of control values in cultures without GDNF treatment. MPP^+-treated cultures that had been exposed to 1 ng/ml GDNF had higher absolute values of [^{3}H]-DA uptake than MPP^+-treated controls. However, when MPP^+ uptake values were compared to the appropriate non-MPP^+-treated controls, there was no significant improvement in the reduction of uptake at any of the GDNF concentrations. Comparison of the % values shown in Fig. 4 indicated no significant difference between the groups. GDNF did not prevent TH+ neuronal loss due to MPP^+ toxicity. MPP^+ caused 26.9 ± 6.2 % reduction in TH+ cell numbers in 1 ng/ml GDNF-treated cultures compared to 27.2 ± 6.0 % reduction in controls (N=8 for each group).

The effect of GDNF on the toxicity produced by different concentrations of MPP^+ was also tested. Cultures were treated with 1 ng/ml GDNF on DIV 0. Different concentrations of MPP^+ were applied to the cultures on DIV 5 for 24 hours, and then washed away on DIV 6 and [^{3}H]-DA uptake was measured 24 hours later (DIV 7). Figure

5 shows that in both GDNF-treated and control groups the values of [^{3}H]-DA uptake decreased as MPP^+ concentration increased. The concentrations of MPP^+ required to cause 50% reduction in DA uptake (IC_{50}) were 0.77 μM for control and 0.79 μM for GDNF-treated group, indicating that even though DA uptake values were higher in the corresponding GDNF-treated cultures, GDNF did not protect against loss of DA uptake at any concentration of MPP^+ used.

The effect of GDNF on the surviving TH+ neurons after termination of MPP^+ treatment was investigated. Cultures maintained in serum-containing medium were used in these experiments because serum supplement increases the survival of DA neurons in normally developing cultures. Cultures were exposed to 100 μM MPP^+ for 1 hour on DIV 6 and [^{3}H]-DA uptake and TH+ neuron survival were determined 24 hours and 5 days later (DIV 7 and DIV 11, as depicted in Fig. 6). In cultures that had not been exposed to GDNF prior to MPP^+ treatment, there was a continuous decrease in [^{3}H]-DA uptake following MPP^r removal, resulting in significantly reduced values on DIV 11 (from 22.6 ± 1.9 to 14.0 ± 1.1% of control, $p<0.05$; Fig. 7A1). If , however, GDNF (1 ng/ml) was added to these cultures after removing MPP^+, the further reduction in [^{3}H]-DA uptake seen on DIV 11 was prevented (Fig. 7A1) There was also a significant decrease in TH+ cell survival 5 days after MPP^+ treatment (from 74.4 ± 11.3 to 42.9 ± 8.7% of control; Fig. 7A2), but the addition of GDNF after MPP^+ could not prevent this reduction. In contrast to the untreated cultures, cultures maintained in GDNF showed recovery in uptake values from 20.0 ± 2.5% on DIV 7 tc 57.3 ± 4.0% of control on DIV 11 ($p<0.001$ Fig. 7B1). If GDNF was removed from the medium after the end of MPP^+ treatment, recovery in [^{3}H]DA uptake still occurred in these cultures, although to a lesser degree

than when GDNF was present (Fig. 7B1). In cultures pre-treated with GDNF there was no further loss of TH+ neurons after removal of MPP^+ (Fig. 7B2) whether or not GDNF was added after the end of MPP^+ treatment. Thus although GDNF cannot prevent MPP^+ induced neurotoxicity, pre-treatment with GDNF or addition of the growth factor after MPP^+ can stimulate recovery after damage.

C. Dopaminergic Effect of bFGF and Its Interaction with GDNF

The dopaminergic effect of bFGF in culture was previously tested (Park and Mytilineou, 1992). In my study, bFGF caused dose dependent increase in [^{3}H]DA uptake which was significant at 1 and 10 ng/ml bFGF ($p<0.001$, Table 3A). TH+ cell number was also increased by 208.5 ± 22.2% on DIV 7 ($p < 0.001$, $N = 9$ from three experiments). The striking difference between bFGF and GDNF was that bFGF strongly stimulated the proliferation of glial cells in culture by more than four-fold (Fig. 8C & D and Table 2). This suggests that the dopaminergic neurotrophic effect of bFGF may be mediated by glial cells.

To further demonstrate that the presence of glia is necessary for the neurotrophic of bFGF on DA neurons, I added FUDR (8 μM) 24 hours after plating cells and analyzed on DIV 7. Glial cell growth was inhibited in both control and bFGF-treated cultures with the presence of FUDR (Table 2). DA uptake and TH+ immunostaining showed that the DA neurotrophic effect of bFGF was totally abolished by FUDR (Fig. 9).

To examine whether secretion of GDNF by glial cells plays a role in the neurotrophic effect of bFGF on DA neurons in dissociated embryonic mesencephalic cell

cultures, combinations of the two growth factors were used to determine whether their effects on DA neurons were additive. I used [^{3}H]DA uptake to examine the growth promoting effects of bFGF and GDNF when administered together. As shown in Table 3A, addition of bFGF caused a dose dependent increase in [^{3}H]DA uptake, which was significant at 1 and 10 ng/ml bFGF ($p<0.001$). GDNF (0.1 ng/ml) when added alone resulted in a 36% increase in [^{3}H]DA uptake ($p<0.05$). In the presence of bFGF, GDNF caused further increases in [^{3}H]DA uptake, which were significant ($p<0.05$) with 0.1 and 1 but not 10 ng/ml bFGF. I then examined whether higher concentrations of GDNF could potentiate the effect of 10 ng/ml bFGF on DA uptake (Table 3B). No significant increase in uptake over the bFGF effect was observed at any GDNF concentration tested.

I further examined the possibility that GDNF could be involved in the neurotrophic effect of bFGF by using antibodies able to neutralize the neurotrophic activity of GDNF (Fig. 10). In cultures that were not treated with any growth factors, addition of antibodies to GDNF had no effect on [^{3}H]DA uptake (Fig. 10A) or TH+ neuron survival (Fig. 10B). GDNF (1 ng/ml) treatment increased the uptake values and the number of TH+ neurons (to 207.7 $\pm$ 9.9% and 162.8 $\pm$ 8.9% of control, respectively). Addition of GDNF antibodies completely blocked the GDNF-induced increases in uptake and TH+ neuron numbers. Treatment with bFGF (10 ng/ml) also resulted in increased DA uptake and TH+ neuron survival of a similar magnitude as GDNF (217.0 $\pm$ 11.7% and 146.0 $\pm$ 14.2% of control, respectively). However, addition of GDNF antibodies did not suppress the bFGF-induced increases (DA uptake 194.2 $\pm$ 8.1% of control and TH+ neurons 139.3 $\pm$ 11.7% of control). The inability of GDNF-neutralizing antibodies to modify the bFGF trophic activity on DA neurons provides

strong evidence that secretion of GDNF by glial cells is not responsible for the bFGF trophic effects.

D. Effect of bFGF on MPP^+ Neurotoxicity

To determine whether bFGF has protective effect against MPP^+, cultures were treated with 10 ng/ml of bFGF in serum free medium on DIV 0. Similar to the GDNF-treated cultures described earlier, they were exposed to 100 μM MPP^+ for 1 hour on DIV 5. MPP^+ was washed from the cultures and [^{3}H]-DA uptake was measured 24 hours later. Figure 11 shows that bFGF did not have significant protection against MPP^+ toxicity. In control cultures, MPP^+ caused 63% decrease in [^{3}H]-DA uptake while it caused 67% decrease in bFGF-treated cultures.

E. Effect of GDNF and bFGF on 6-OHDA Neurotoxicity

To test another dopaminergic specific neurotoxin, 6-OHDA, cultures were treated with either GDNF (1 ng/ml) or bFGF (10 ng/ml) three hours after initial plating (DIV 0) and throughout the duration of the experiments. On DIV 5, cultures were treated with 30 μM 6-OHDA for 45 minutes at 37°C. Cultures were then washed twice with sterile BSS and fresh medium with appropriate concentrations of growth factors was added. On DIV 6, 24 hours after 6-OHDA treatment, survival of dopaminergic neurons was monitored by measuring the high affinity [^{3}H]DA uptake levels and by TH immunoreactivity. As shown in Figure 12, GDNF increased the uptake value to 154% of control and TH+ number to 144% of control. bFGF had a stronger neurotrophic effect, it increased both the

uptake value and TH+ number by about 2-fold. 6-OHDA treatment caused significant reduction of dopamine uptake by 70% in control (no factors added) and 75% in GDNF-treated groups. TH+ number also dropped by 62% in control and 58% in GDNF-treated groups. However, bFGF showed strong ability to protect cultures from 6-OHDA toxicity. The dopamine uptake value decreased only by 37% and no significant decrease in TH+ cell number was seen in the bFGF-treated group.

F. Involvement of Astrocytic Glial Cells and GSH on the bFGF Protection Against 6-OHDA

The significant protective effect of bFGF against 6-OHDA toxicity could be mediated by glial cells, which are stimulated by the addition of bFGF. FUDR was added to prevent the growth of glia to test the protective effect of bFGF on 6-OHDA toxicity. [^{3}H]-DA uptake measurement and TH immunostaining showed that the DAergic neurotrophic effect and the protective effect of bFGF against 6-OHDA toxicity were completely abolished by FUDR treatment (Fig. 13). This indicates that glial cells were responsible for the DAergic neurotrophic effect of bFGF and were providing the protection from 6-OHDA toxicity.

Glial cells contain high concentrations of reduced glutathione (GSH) (Raps et al., 1989) and provide cysteine for neurons to synthesize GSH (Sagara et al., 1993). To test if there is a relationship between the protective effect of bFGF and the glial-facilitated generation of GSH, I first measured the GSH levels in GDNF- and bFGF-treated mesencephalic cultures. GDNF did not increase GSH levels, while bFGF caused 1.5-fold increase over control on DIV 8 and about 2-fold on DIV 9 (Fig. 14). To test if this

increase in GSH level was due to the presence of glial cells, FUDR was added into cultures on DIV 1 to prevent the mitosis of glial cells and the GSH level was measured on DIV 9. Addition of FUDR totally prevented the increase of GSH level by bFGF (Fig. 16). This indicates that the glial cells were involved in the increased GSH levels in cultures.

It is possible that the oxidative stress of 6-OHDA treatment will induce the cellular protective mechanisms to generate GSH in order to remove excessive free radical species. It has been shown that catecholamines such as L-DOPA or dopamine increased the GSH level in mesencephalic cell cultures (Mytilineou et al., 1993; Han et al., 1996). To test this hypothesis, I measured the GSH levels at the following time points: (1) immediately before the treatment with 6-OHDA; (2) immediately after the 45-minute-6-OHDA treatment; (3) 2 hours after 6-OHDA; and (4) 4 hours after 6-OHDA treatment. Figure 15 shows that in bFGF-treated cultures, the GSH level was significantly increased 4 hours after 6-OHDA treatment. By contrast, in control cultures, GSH remained at about the same level and was not increased after the 6-OHDA insult. This demonstrated that cultures treated with bFGF up-regulate the GSH levels after 6-OHDA treatment and this may be one of the key factors for the protection from 6-OHDA.

To test if this up-regulation of GSH was mediated by glial cells, again FUDR was added to prevent glial proliferation. As shown in Figure 17, the up-regulation of GSH in the bFGF-treated group was completely blocked by FUDR. This showed that the glial cells stimulated by bFGF played a role in increasing GSH level.

As mentioned earlier glial cells have multiple functions that can support the growth and survival of neurons like nutritional support, ion buffering, glutamate uptake, neurotrophic factor secretion, antioxidant mechanism, etc. I examined whether depleting

GSH in cultures but still having glial cells present would affect the DAergic neurotrophic and neuroprotective effect of bFGF. On DIV 7, L-buthionine-[S,R]-sulfoximine (L-BSO), an inhibitor of glutamyl cysteine synthetase, was added to deplete GSH levels. Figure 18 shows that after 24-hour of treatment with L-BSO (10μM), the GSH level dropped about 50% in control and about 36% in bFGF-treated cultures. On day 9, 48 hours after adding L-BSO, the GSH level was further reduced to only 18% in control and 34% in bFGF-treated cultures. This shows that L-BSO at 10μM is effective in reducing the GSH levels in cultures even with bFGF treatment. The [^{3}H]-DA uptake and the TH+ cell number were not affected by the decrease of GSH (Fig. 19), indicating that in my experimental situation, depletion of L-BSO at this level did not affect normal neuronal survival. However, when 6-OHDA was added to the cultures on DIV 8, 24 hours after L-BSO was added, not only the GSH levels decreased in both groups, but the up-regulation of GSH level within 4 hours in the bFGF group was also inhibited (Figure 20), indicating that L-BSO effectively diminishes the ability of glial cells to produce GSH.

The inhibition of GSH synthesis by L-BSO affected the protective effect of bFGF against 6-OHDA. As described, L-BSO was added to the cultures on DIV 7. 6-OHDA was added for 45 minutes on DIV 8. Figure 21 shows the results of [^{3}H]-DA uptake. L-BSO caused a significant attenuation in the protection from 6-OHDA in the bFGF-treated group (from 82% down to 53%, $p < 0.05$). This suggested that the protection from damage by glia may depend upon GSH.

IV. DISCUSSION

A. Dopaminergic Neurotrophic and Neuroprotective Effects of GDNF in Cultures

(I) Neurotrophic Effect of GDNF

Glial cell line-derived neurotrophic factor (GDNF) was the neurotrophic factor first ever isolated and purified from the medium conditioned by a glial cell line, B49 (Lin et al., 1993; Lin et al., 1994). It is a polypeptide growth factor that belongs to the transforming growth factor-β superfamily. It does not stimulate the growth of astrocytes and it has very specific and potent (at picomolar concentrations) neurotrophic effect on DA neurons.

My present studies confirm the neurotrophic activity of GDNF for dopaminergic neurons. Treatment with GDNF reduced the rate of dopaminergic neuronal cell death, which occured when mesencephalic cells were maintained *in vitro* in the absence of serum and glial support (Casper et al., 1991; Hyman et al., 1991a; Takeshima, 1994) and increased [^{3}H]-DA uptake of surviving dopaminergic neurons. The increase in [^{3}H]-DA uptake was primarily due to expanded process outgrowth, as demonstrated by measuring the number of processes and neurite length after staining with TH antibodies. The neurotrophic effect of GDNF was fully expressed even if the growth factor was added only once, suggesting that continuous exposure to GDNF was not necessary to maintain the long term changes induced by the growth factor. Furthermore, dopaminergic neurons remained sensitive to the neurotrophic effects of GDNF for at least several days *in vitro*.

Our data indicate that there was an optimal concentration of GDNF for maximal support of dopaminergic neurons. After 6 days *in vitro*, GDNF concentrations of 0.1 and 1 ng/ml resulted in higher [^{3}H]-DA uptake levels and survival of dopaminergic neurons than 10 ng/ml GDNF. However, at 10 days *in vitro*, the effect of GDNF reached a plateau at 1 ng/ml and remained at the same level with 10 ng/ml concentration. These findings were similar to those reported by Lin el at. (1993), who measured the neurotrophic effect of GDNF at DIV 12, showing that it reached a plateau at 0.1 ng/ml and remained at the same level with concentrations up to 100 ng/ml.

In agreement with the previous report by Lin et al. (1993), our experiments show that the neurotrophic effect of GDNF did not result from stimulation of astrocyte proliferation in the cultures, since there was no change in the number of GFAP-positive cells after GDNF treatment. In addition, the presence of glial cells did not modify the effectiveness of GDNF to dopaminergic neurons, indicating that GDNF acted independently of glial-mediated trophic mechanisms in mesencephalic cultures. In this respect GDNF differs from bFGF and EGF, whose neurotrophic effects on dopaminergic neurons result from stimulation of glial proliferation (Engele and Bohn 1991: Casper et al., 1991; Park and Mytilineou, 1992). BDNF, a member of the neurotrophin family, has also been shown to support dopaminergic neuron survival and growth without affecting glial cell proliferation (Hyman et al, 1991a). However, unlike GDNF, BDNF shows neurotrophic activity for both dopaminergic and GABAergic neurons in mesencephalic cultures (Hyman et al, 1991b). GDNF is by far the most specific DAergic neurotrophic factor *in vitro* and it seems to directly exert its neurotrophic effect on DA neurons, independent from the mediation of glial mediated trophic effects. Nevertheless, people

recently have shown that GDNF is also trophic to sensory and autonomic neurons (Buj-Bello et al., 1995), motoneurons (Henderson et al., 1994; Zurn et al., 1994; Li et al., 1995) and Purkinje cells cells (Mount et al., 1995).

(II) Neuroprotection from MPP^+ by GDNF

The goal of this study was to determine whether GDNF could protect dopaminergic neurons from damage caused by neurotoxins such as MPP^+. Our experiments indicate that treatment with GDNF (0.1 ng/ml to 10 ng/ml) could not prevent damage caused by MPP^+, whether or not the medium contained serum (that is, whether or not glial cells were present in the cultures). This was true whether MPP^+ treatment consisted of a one-hour exposure to a concentration of 100 μM, or 24-hour exposure to concentrations from 0.1 to 10 μM.

Although GDNF could not prevent MPP^+-induced neurotoxicity, once the toxin was removed from the cultures, the benefits of the neurotrophic actions of GDNF on the damaged dopaminergic neurons became apparent. The most pronounced toxic effect of MPP^+ was the reduction of DA uptake (approximately 80% reduction in the present studies), which was the consequence of dopaminergic fiber degeneration (Park and Mytilineou, 1992). The loss of dopaminergic cell somata was less pronounced (20-30% reduction). After removal of MPP^+, there was a continuous loss of dopaminergic neurons. while the surviving ones failed to show recovery in DA uptake for several days (Park and Mytilineou, 1992; Hou et al., 1996). In contrast, in cultures continuously exposed to GDNF the number of dopaminergic neurons was not further reduced after removal of MPP^+ and the surviving neurons recovered their uptake capacity to about 57.3 % of

control levels in 5 days. A similar protective effect was found even if GDNF was added to the cultures only once at the time of plating and not after the end of the toxin treatment, but the recovery of uptake under these conditions was less potent. Maintenance of DA uptake in neurons surviving MPP^+ treatment could also be achieved if GDNF was added only after the end of MPP^+ treatment. Thus GDNF offered significant protection to MPP^+-damaged neurons by preventing continuous cell death and stimulating recovery in DA uptake.

Previous reports have indicated that the neurotrophins BDNF and NT-4/5 protected mesencephalic DA neurons from MPP^+ toxicity *in vitro* (Hyman et al., 1991a & b; Spina et al, 1992b; Hynes et al., 1994). Protection was apparent at the end of the treatment or 24 hours after MPP^+ removal, suggesting that the toxicity of MPP^+ was attenuated. However, in a separate study, Beck et al. (1992) showed that BDNF did not prevent actual MPP^+-induced damage, since there were no differences between control and BDNF-treated cultures immediately after exposure to MPP^+, but it prevented further cellular deterioration and death following MPP^+ treatment. These observations were similar to mine with respect to GDNF, and in this context GDNF can be considered a protective agent against long term destructive effects of MPP^+.

In a recent in vivo study, GDNF given intracranially 24 hours before or 7 days after MPTP administration to mice caused a significant protection of the nigrostriatal dopaminergic system, indicated by increased DA neuron survival and higher DA levels (Tomac et al., 1995). These data are not necessarily in conflict with our own, as the protection from toxicity in the above study was evaluated 7 days after MPTP treatment. Prevention of neurotoxic damage and stimulation of survival and/or regrowth by GDNF

are difficult to delineate when the damage is not evaluated immediately after the end of treatment with the toxin. In another in vivo study, GDNF was shown to protect from DA neuron loss that occurred following axotomy by transection of the medial forebrain bundle (Beck et al., 1995).

In my experiments, the inability of GDNF to prevent cell damage caused by MPP^+ treatment, in spite of its potent dopaminergic neurotrophic activity, should not be surprising. The increase in process outgrowth and capacity for DA uptake, which resulted from exposure to GDNF, could counteract any influence towards improved survival. The toxicity of MPP^+ was directly related to its ability to accumulate at high concentrations in the dopaminergic neurons by using the high affinity DA uptake (Javitch et al., 1985). Consequently, after GDNF treatment higher concentrations of MPP^+ would be expected to accumulate in each dopaminergic neuron, which would create greater challenge for survival. However, as my data show, when the neurotoxin was no longer present, GDNF could ameliorate the consequences of exposure to MPP^+.

(III) Neuroprotection from 6-OHDA by GDNF

6-OHDA is also a specific DAergic neurotoxin since it is structurally related to dopamine and will be selectively taken up by DA neurons (Breese and Traylor, 1970). 6-OHDA is very unstable and is rapidly autooxidating to quinone and hydrogen peroxide. This reaction is potentiated by ascorbic acid which facilitates the recycling of the quinone back to 6-OHDA (Heikkila and Cohen, 1971). This auto-oxidative conversion of 6-OHDA may happen both intra- and extracellularly. In my experimental conditions, when 6-OHDA (30μM) was added for 45 minutes, the cells did not show any damage under the

microscope until 6 hours after treatment with 6-OHDA. However, there was major damage to the neurons 24 hours later when the analyses began. This time suggests that the delayed cell death caused by 6-OHDA probably results from cellular damage by oxidative stress.

It is not surprising that GDNF did not protect neurons from 6-OHDA. There is no known evidence of anti-oxidant mechanisms induced by GDNF treatment. Some in vivo studies show that GDNF promoted the survival of nigral dopaminergic neurons following intracranial 6-OHDA lesion (Hoffer et al., 1994; Bowenkamp et al., 1995; Sauer et al., 1995). These results were similar to my in vitro data that GDNF promoted DA neuron survival and regrowth after damage. Kearns and Gash (1995) reported that injections of GDNF prior to 6-OHDA infusion in rats were able to protect nigral dopamine neurons against toxicity. This result may seem to conflict with my own, however, the condition in the live brain tissue is very different from the conditions in pure mesencephalic neurons with no glial cells in culture. In my experimental condition, GDNF by enhancing the neurite outgrowth of DA neurons in mesencephalic cultures could cause more uptake of 6-OHDA into the cells. This may explain why the damage of 6-OHDA to GDNF-treated cultures was about the same or even greater than that of non-GDNF treated controls (Fig. 12).

B. Dopaminergic Neurotrophic and Neuroprotective Effects of bFGF in Cultures

(I) Neurotrophic Effect of bFGF:

The DAergic neurotrophic effect of bFGF has been characterized (Ferrari et al., 1989; Knusel et al., 1990). In my study, bFGF increased the [^{3}H]-DA uptake and TH+ reactivity, similar to the effect of GDNF in the cultures. However, bFGF also increased GFAP+ cell number by 3-4 fold over the controls. The increase in DA uptake and TH+ cell number was totally blocked when GFAP+ cell growth was inhibited by treatment with the mitotic inhibitor FUDR. Inhibition of the bFGF neurotrophic effect by inhibiting glial growth was reported previously (Knusel et al., 1990; Engele and Bohn 1991; Engele et al., 1991; and O'Malley et al., 1994).

Mesencephalic glia and medium conditioned by mesencephalic astrocytes promote the survival and growth of DA neurons, suggesting the secretion of soluble neurotrophic factor(s) by astrocytes (Engele et al., 1991; O'Malley et al., 1991; Gaul and Lubber, 1992; Dong et al., 1993; O'Malley et al., 1994; Takeshima et al., 1994a; Takeshima et al., 1994b). Although this neurotrophic factor has not yet been identified, it was shown that it is not bFGF (O'Malley et al., 1994). Cortical astrocytes also secrete molecules that promote the differentiation of mesencephalic DA neurons. Analysis of the conditioned medium has ruled out the possibility that bFGF, BDNF, NT-3, Il-1, Il-6, S100β and α_2-macroglobulin are involved in the neurotrophic effect (Gaul and Lubber, 1992).

The fact that GDNF was purified from a glial cell line conditioned medium, as well as the expression of GDNF mRNA in substantia nigra astrocytes (Schaar et al., 1993; Schaar et al., 1994) and the high sensitivity of DA neurons to this growth factor made GDNF the most likely candidate for the yet unidentified factor secreted by glial

cells. However, in my study (Hou and Mytilineou, 1996), GDNF-neutralizing antibodies failed to modify the bFGF-induced trophic effect (Fig. 10). This indicates that secretion of GDNF by glial cells cannot account for the neurotrophic effects of bFGF on DA neurons, and that GDNF alone is not the neurotrophic factor responsible for the glial-mediated stimulation of DA neuron growth.

An additional observation in my study was that the presence of GDNF-neutralizing antiserum had no effect on the development of DA neurons in control cultures. This indicates that, in spite of its potent neurotrophic activity, the presence of GDNF in the extracellular space is not a requirement for the survival and growth of DA neurons.

(II) Neuroprotection from MPP^+ and 6-OHDA by bFGF; Involvement of Glial Cells and Glutathione:

My study shows that bFGF was not able to prevent the MPP^+ damage to DA neurons. MPP^+ treatment on DIV 5 caused a 63% reduction in DA uptake in the control group and a 67% reduction in the bFGF-treated group (Fig. 11). This is in agreement with the results shown by Park and Mytilineou (1992). When they treated the cultures with MPP^+ on DIV 4, bFGF was not able to prevent its toxicity. It was not until DIV 10, when the glial cells in the cultures had been confluent, then they observed some protection from bFGF. I treated cultures with MPP^+ on DIV 5. It is possible that at this stage, although glial cells had begun to proliferate, they were not enough to prevent MPP^+-caused destruction on DA neurons.

In comparison, bFGF was able to reduce the effect of 6-OHDA toxicity even when 6-OHDA treatment was as early as DIV 5. This indicates that if the protection from 6-OHDA was mediated by glia, the mechanisms must be different than those protecting from MPP^+ toxicity.

It is highly possible that the bFGF protective mechanisms against 6-OHDA are due to the antioxidant ability of glial cells. As mentioned earlier, there are many reports showing that astrocytes contain higher glutathione content than neuronal cells (Makar et al., 1990; Pileblad, E., 1991; Sagara et al., 1993; Pow and Crook, 1995). Although there is still no evidence showing that glial cells directly transport GSH to the neurons, it has been shown that the cultured astrocytes were able to release GSH into the medium (Yudkoff et al., 1990; Sagara et al., 1996). Sagara et al. (1993b) found that glial cells actively took up cystine, and converted it into cysteine inside the cells. Cysteine was the rate-limiting precursor of GSH and was secreted into the medium. Neurons were not able to take up cystine. However, cysteine was taken up rapidly by both neurons and glial cells and accounted for the synthesis of neuronal glutathione. This suggests that neurons maintain their GSH level by taking up cysteine provided by glial cells. My present study supports this hypothesis. With the addition of FUDR that blocked the proliferation of glial cells, the GSH level of the bFGF-treated cultured cells dropped to a level not significantly different from that of controls. The neurotrophic activity of bFGF disappeared and the toxicity of 6-OHDA could not be prevented.

The role GSH plays in detoxifying 6-OHDA remains not completely clear. GSH does not prevent the auto-oxidation of 6-OHDA to form quinone and hydrogen peroxide or the recycling of the reaction in the presence of ascorbic acid. However, GSH may form

glutathionyl conjugates (catechol-thioethers) with the oxidation products of 6-OHDA and shifts the oxidation reaction away from potentially toxic eumelanin precursors to the production of non-toxic pheomelanin (Prota, 1988; Nappi and Vass, 1994).

My data have shown that an increase in the synthesis of GSH can be induced within four hours after the oxidative stress of 6-OHDA. This induction is only seen in bFGF-treated cultures and not in controls or in bFGF + FUDR groups. This suggests that the induction requires the presence of glial cells. Although it is not known whether in bFGF-treated cultures, GSH levels increase in both neurons and glia or just in glial cells, the fact that this GSH increase plays a role in preventing 6-OHDA damage can not be easily disputed. Another group also showed a marked increase in GSH content when the cells in neural cultures were exposed to oxidative insults, such as sodium arsenite, cadmium chloride, and glucose/glucose oxidase (Sagara et al., 1996), and L-DOPA (Mytilienou et al., 1993; Han et al., 1996). Sagara et al. (1996) found that the increase in GSH content was attributed to the induction of the cystine transport activity. Once GSH concentration inside the cells reaches toward saturation, glia then increase the efflux of GSH to the extracellular space. This GSH in the medium, whether taken up by neurons or not, can shift away the recycling oxidative pathway of 6-OHDA to protect cells from oxidative stress.

To obtain further support for the involvement of GSH in protection from 6-OHDA toxicity, I used L-buthionine-[S,R]-sulfoximine (L-BSO) to inhibit the synthesis of GSH in the presence of glial cells. L-BSO is an inhibitor of γ-glutamylcysteine synthetase, the enzyme catalyzing the first step in GSH synthesis (Griffith and Meister, 1979). Slivka et al. (1988) reported a moderate decrease in mouse brain GSH following

repeated injections of high doses of L-BSO. Pileblad and Magnusson (1988 and 1989) were able to markedly reduce the rat brain glutathione following intracerebroventricular administration of L-BSO. The same group further showed that reduction of brain GSH by L-BSO potentiated the DA-depleting action of 6-OHDA in rat striatum (Pileblad et al., 1989). In my study, L-BSO was able to reduce the GSH level in culture. Although the reduction in bFGF-treated cultures was not as great as that in control, L-BSO still effectively reduced the GSH to about two thirds in 24 hours and one third in 48 hours (Fig. 18). In addition to reducing the overall levels of GSH in bFGF-treated cultures, L-BSO also prevented the upregulation of GSH after exposure to 6-OHDA (Fig. 20). Following treatment with L-BSO, the protection of bFGF against 6-OHDA toxicity was decreased. DA uptake values after 6-OHDA dropped from 82% in the bFGF-treated cultures to 53% in the bFGF and L-BSO combination (Fig. 21). These results suggest that protection from 6-OHDA toxicity in the bFGF-treated cultures is linked to the ability of the cells to upregulate the levels of GSH.

It should be noted that the presence of L-BSO alone did not reduce the DA uptake or TH+ cell numbers in cultures, although GSH levels were reduced. This suggests that under my experimental conditions, DA neurons survived with lower levels of GSH, if they were not subjected to excessive oxidative stress. It was also observed that although DA uptake following 6-OHDA treatment was reduced in cultures treated with bFGF + L-BSO, the number of TH+ neurons was not affected. Thus, the reduction of GSH levels did not completely eliminate the protective effect of bFGF. It is likely that although DA neurites were damaged, as refected by the lower DA uptake, the DA neurons were able to survive in the bFGF + L-BSO-treated cultures.

Treatment with FUDR, which inhibited glial growth, had a more profound effect than the reduction in GSH levels in eliminating the growth promoting and neuroprotective action of bFGF. This does not necessarily mean that the presence of GSH is not vital to the trophic and protective effects of bFGF. In my experiments, L-BSO at 10 μM did not completely deplete GSH levels. In control cultures after 48 hours of L-BSO treatment, there was still 18% of GSH remaining and this was sufficient to maintain the survival of the cells. However, neuronal processes first became susceptible to the oxidative stress caused by 6-OHDA when GSH was reduced, although the bodies of neurons still remained viable. This was reflected in my experiments showing that 10 μM L-BSO reduced DA uptake but not TH+ cell number.

Glial cells may have additional physiologic functions other than just increasing the GSH level to protect neuronal cells. Glial cells may be able to regulate other free radical scavenging systems, especially superoxide dismutase (SOD). Some reports have shown that the SOD levels correlated with the number of glial cells in vivo and in vitro. One report shows that both GFAP reaction and CuZnSOD were decreased in ALS patients (O'Reilly et al., 1995). Another study demonstrated that cysteamine induced astrocyte hypertrophy as well as upregulated MnSOD and heat shock proteins (Manganaro et al., 1995). However, the role of glial cells in cultures stimulated by bFGF on SOD levels is still not known. Furthermore, the above reports did not directly demonstrated that increased SOD level would protect cells from 6-OHDA. There was a report stating that addition of SOD into the mesencephalic cultures potentiated the 6-OHDA toxicity to dopamine neurons (Mytilineou and Danias, 1989), but this effect was

believed to result from the delay of 6-OHDA oxidation in the medium by SOD and increased 6-OHDA accumulation in DA neurons.

Glial cells might also be secreting neurotrophic factors that protect DA neurons from toxicity. In my study (Hou and Mytilineou, 1996) I have ruled out that the secretion of GDNF by bFGF-stimulated glial is responsible for the trophic effect of bFGF. However, there may be still other unidentified neurotrophic factors secreted by glial cells responsible for the trophic and protective effects of bFGF. Park and Mytilineou (1992) had forwarded this hypothesis in their study of MPP^+ protective effect of bFGF. Recently, Schaar et al. (1994) showed that a new protein other than GDNF present in the mesencephalic type I astrocyte conditioned medium helped the survival of DA neurons in cultures. In addition, NGF provided protection from 6-OHDA treatment in the SH-SY5Y neuroblastoma cell line (Tiffany-Castiglioni and Perez-Polo, 1981). BDNF also had been shown to protect DA neurons against 6-OHDA and MPP^+ toxicity by increasing the activity of GSSG reductase and preventing the rise in GSSG (Spina et al., 1992b). However, there is no evidence that NGF or BDNF was secreted by bFGF-stimulated glial cells. NGF increased the activity of catalase (Jackson et al., 1990 and 1994), and BDNF increased the activity of GSSG reductase, while none of these enzymes were increased in bFGF-treated cultures (Spina et al., 1992b). Furthermore, neither NGF nor BDNF stimulates the proliferation of glial cells like bFGF does. This indicates that NGF, BDNF and bFGF up-regulate antioxidant defenses by different mechanisms. In this sense, the search for the existence of such new neurotrophic factors remains an open field for researchers to accomplish.

V. CONCLUSION:

My research has been focused on the study of the mechanisms of dopaminergic neuronal degeneration in Parkinson's disease. Finding ways to prevent or delay the process of neurodegeneration is one of the top priorities for effective treatment of this disease. In my studies, using the mesencephalic dopaminergic culture system, certain neurotrophic factors were shown to be promising for the future clinical treatment of Parkinson's disease. Glial cell line-derived neurotrophic factor (GDNF), purified from a rat glial cell line, is a very potent trophic factor to dopaminergic neurons. My studies show that it supports the growth of normally developing dopaminergic neurons and stimulates their survival and recovery after damage by MPP^+, a specific dopaminergic neuronal toxin. Another neurotrophic factor, basic fibroblast growth factor (bFGF), is also a potent dopaminergic neurotrophic factor whose effects are mediated through stimulation of astroglial proliferation. bFGF not only supports the development of dopaminergic neurons but also prevents the toxicity of 6-hydroxydopamine. The glial-mediated trophic effects of bFGF are not due to secretion of GDNF by the stimulated glial cells. Upregulation of reduced glutathione (GSH) by bFGF-stimulated glia is involved in the neuroprotective effects. Oxidative stress is a possible cause of dopaminergic neurodegeneration in Parkinson's patients. GDNF and bFGF, as well as other yet unidentified trophic factors that are released by glial cells, or factors that upregulate antioxidant mechanisms, may serve an important role in the treatment of Parkinson's disease or other neurodegenerative disorders.

Table 1

GDNF Effects on Dopaminergic Neuritic Length, Neurite Number per Neuron, and Glial Cell Number in Culture

	Control	GDNF 1 ng/ml
Average Length of the Longest TH+ Neurite (μm ± SEM)	185 ± 13	350 ± 29***
Average Number of Neurite Branches per TH+ Cell (N ± SEM)	3.41 ± 0.19	5.70 ± 0.26***
GFAP+ Cells (N/cm^2 ± SEM)	1501 ± 134	1438 ± 178

Cultures were treated with the GDNF 1 ng/ml or bFGF 10 ng/ml beginning at the time of plating until DIV 6, when they were immunostained with tyrosine hydroxylase (TH) antibodies or glial fibrillary antibody protein (GFAP). Values represent the means ± S.E.M.
*** $p < 0.001$ compared to the corresponding control. (N=60 per group from three experiments).

Table 2

The Effect of GDNF and bFGF on Glial Cell Growth and the Effect After Treatment of 5-Fluoro-2'-Deoxyuridine (FUDR)

GFAP(+) Number ($N / cm^2 \pm SEM$)	Defined Medium	GDNF 1 ng/ml	bFGF 10 ng/ml
Control	1501.2 ± 134.4	1438.2 ± 177.8	6388.0 ± 380.1^a
FUDR	1156.4 ± 44.3^b	1025.6 ± 54.8^b	918.5 ± 86.7^c

Cultures were treated with the GDNF 1 ng/ml or bFGF 10 ng/ml beginning at the time of plating until DIV 8, when they were immunostained with glial fibrillary antibody protein (GFAP). Values represent the means of cell numbers ± S.E.M. (N=12 per group from three experiments).

$^a p < 0.001$ compared to control with no growth factor; $^b p < 0.01$, $^c p < 0.001$ compared to its corresponding control with no FUDR.

Table 3

Effect of Co-administration of bFGF and GDNF on the Development of DA Uptake in Mesencephalic Cultures

(A)

	GDNF concentration (ng/ml)	
bFGF (ng/ml)	**0**	**0.1**
0	100.0 ± 6.7	135.6 ± 10.2$^{\&}$
0.1	131.2 ± 7.4	163.3 ± 9.8$^{\&}$
1	172.5 ± 8.6***	253.9 ± 13.9$^{\&}$
10	321.0 ± 18.4***	381.2 ± 22.7

Cultures were treated with the indicated concentrations of the growth factors begining at the time of plating until DIV 10, when they were analyzed for [^{3}H]DA uptake. Uptake values are presented as % of untreated control. (N=8 per group from 2 experiments).
$^{***}p<0.001$ compared to no bFGF; $^{\&}p<0.05$ compared to the corresponding groups not treated with GDNF (GDNF 0).

Table 3

Effect of Co-administration of bFGF and GDNF on the Development of DA Uptake in Mesencephalic Cultures

(B)

	bFGF concentration (ng/ml)	
GDNF (ng/ml)	**0**	**10**
0	100.0 ± 3.4	$188.1 \pm 5.1^{\&\&\&}$
0.1	$143.0 \pm 5.9^{**}$	$201.8 \pm 7.9^{\&\&\&}$
1	$153.9 \pm 8.2^{***}$	$187.5 \pm 7.9^{\&\&}$
10	124.7 ± 7.6	$155.5 \pm 8.0^{\&}$

Cultures were treated with the indicated concentrations of the growth factors begining at the time of plating until DIV 8, when they were analyzed for [^{3}H]DA uptake. Uptake values are presented as % of untreated control (N=18 per group from 4 experiments).
$^{***}p<0.001$, $^{**}p<0.01$ compared to no GDNF; $^{\&\&\&}p<0.001$, $^{\&\&}p<0.01$, $^{\&}p<0.05$ compared to the corresponding groups not treated with bFGF (bFGF 0).

Figure 1

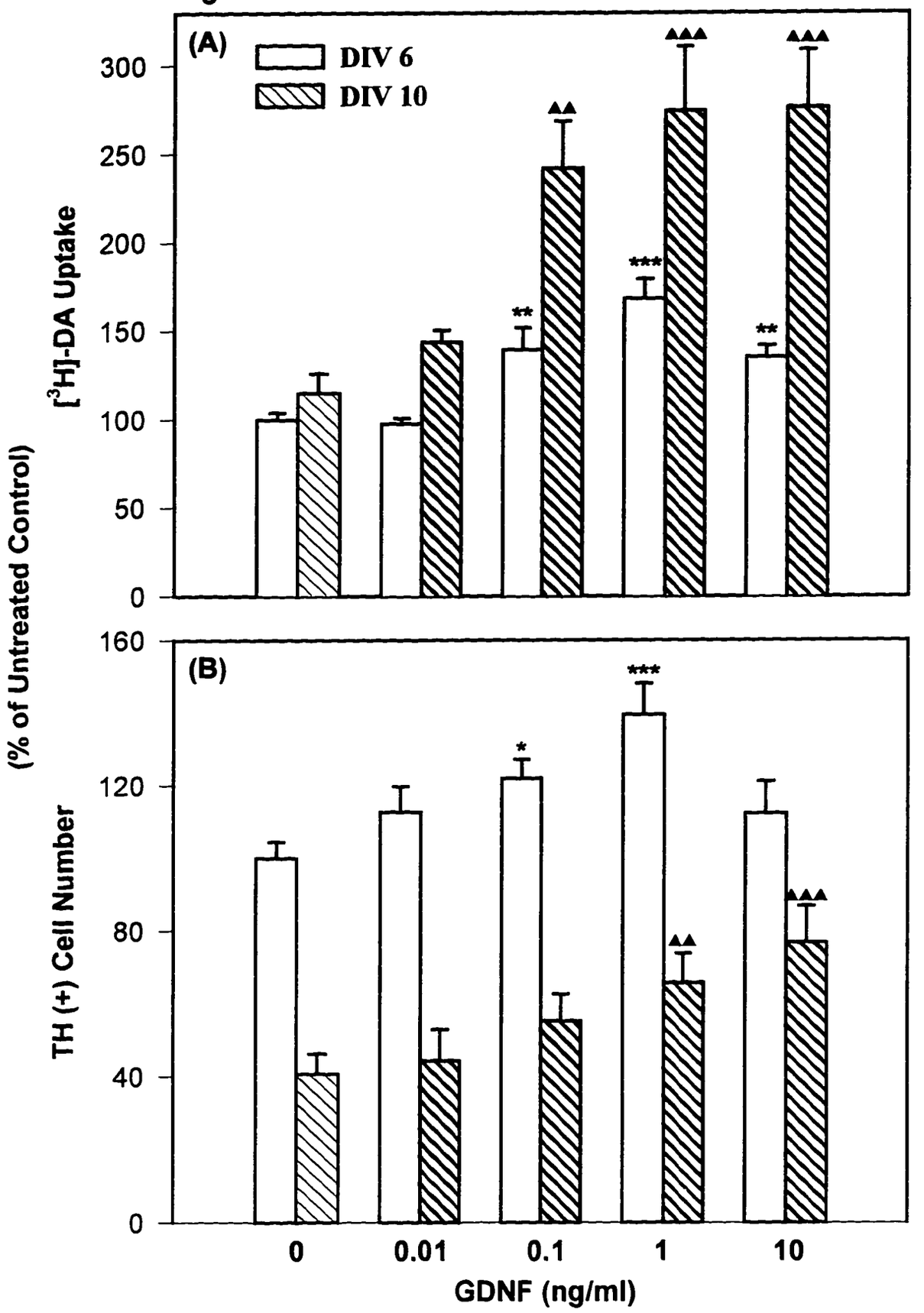
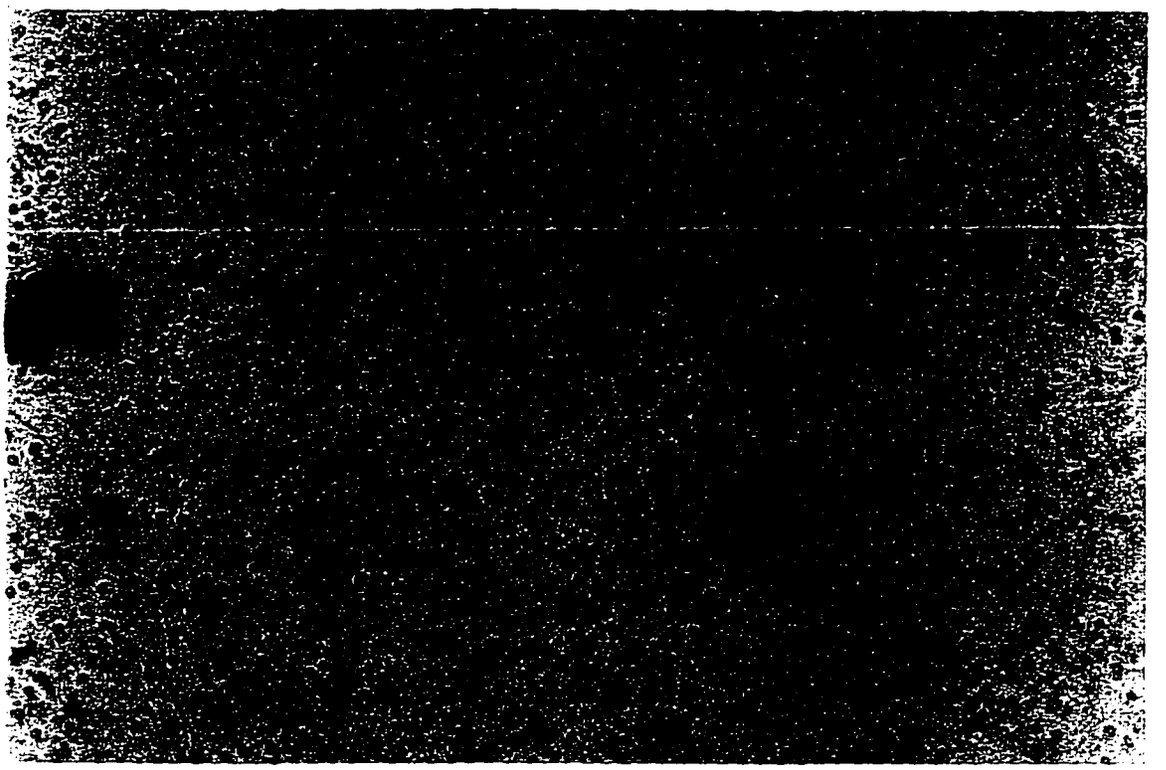


Figure 1

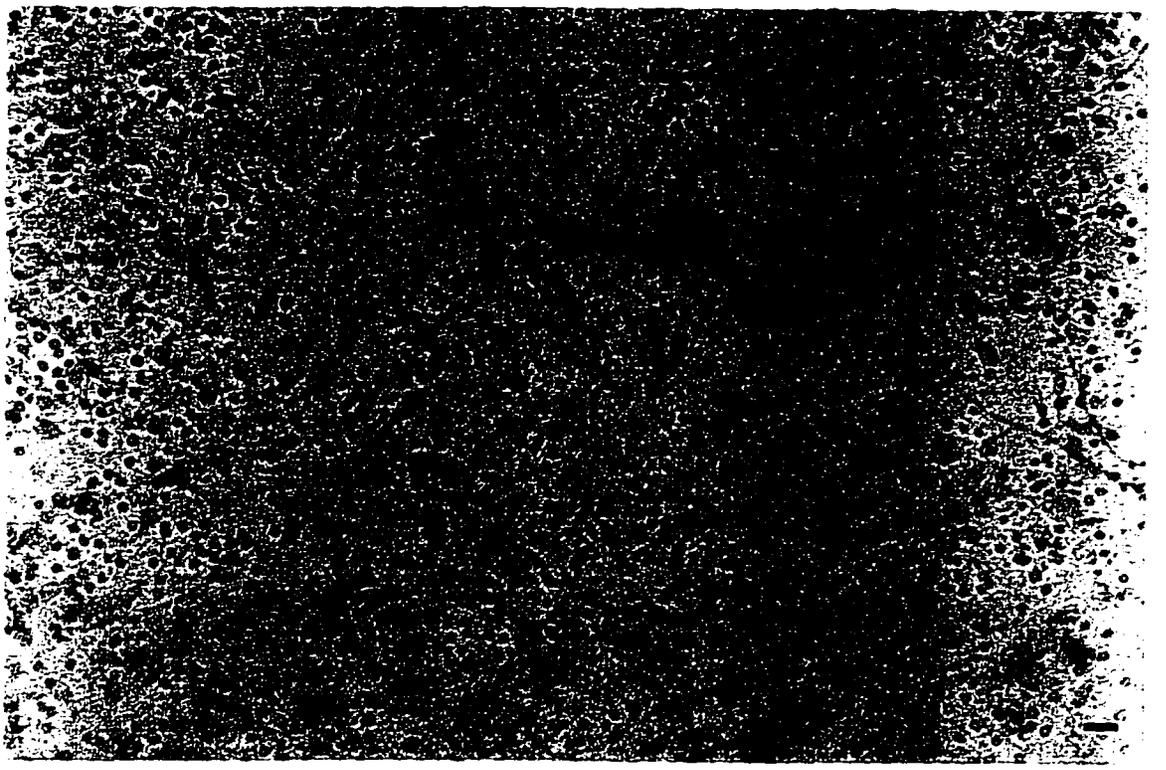
Effect of different concentrations of GDNF on (A) [^{3}H]DA uptake and (B) number of TH-positive neurons in mesencephalic cultures. Cultures from embryonic day 14 ± 0.5 rat embryos were treated with GDNF (0.01, 0.1, 1 or 10 ng/ml), DA uptake and TH immunocytochemistry were assayed on DIV 6 and 10. Data are mean ± SEM values presented as percentages of untreated controls (n = 21) from four experiments. Actual values of controls varied between experiments from 264.6 to 431.1 fmol/10 min per culture on uptake and from 301 to 506 cells/cm^2 on TH-positive cell number. Statistical analysis was by ANOVA followed by Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control on DIV 6; ▲$p < 0.05$, ▲▲$p < 0.01$, ▲▲▲$p < 0.001$ versus control on DIV 10.

Figure 2

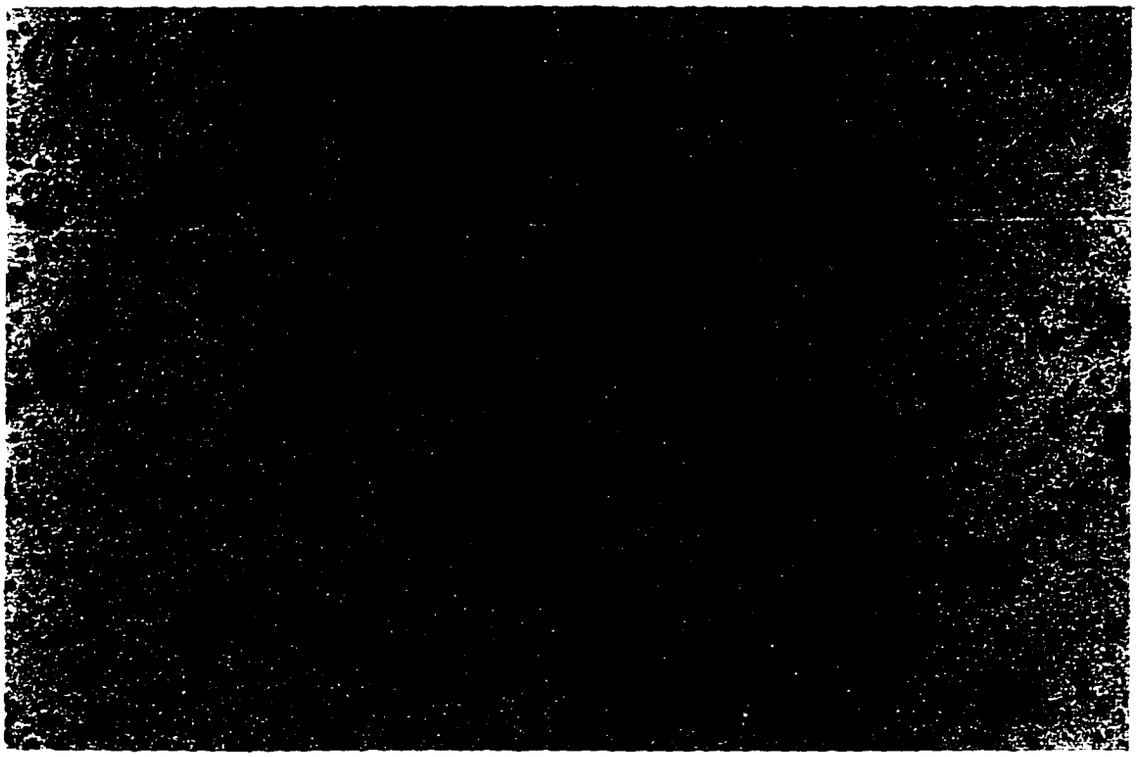
(A)



(B)



(C)



(D)

Figure 2

(A) and (B): TH immunocytochemistry of (A) control and (B) GDNF-treated mesencephalic cultures on DIV 6. Increased neuritic length and more prominent and darker staining of neuronal somata were evident after treatment with 1 ng/ml of GDNF compared with the control.

(C) and (D): GFAP immunoreactive astrocytes in (C) control and (D) GDNF-treated mesencephalic cultures on DIV 6. There was no difference in the number of GFAP-positive cells or intensity of the GFAP staining reaction in the cultures treated with 1 ng/ml of GDNF compared with controls. Bar = 25μm.

Figure 3

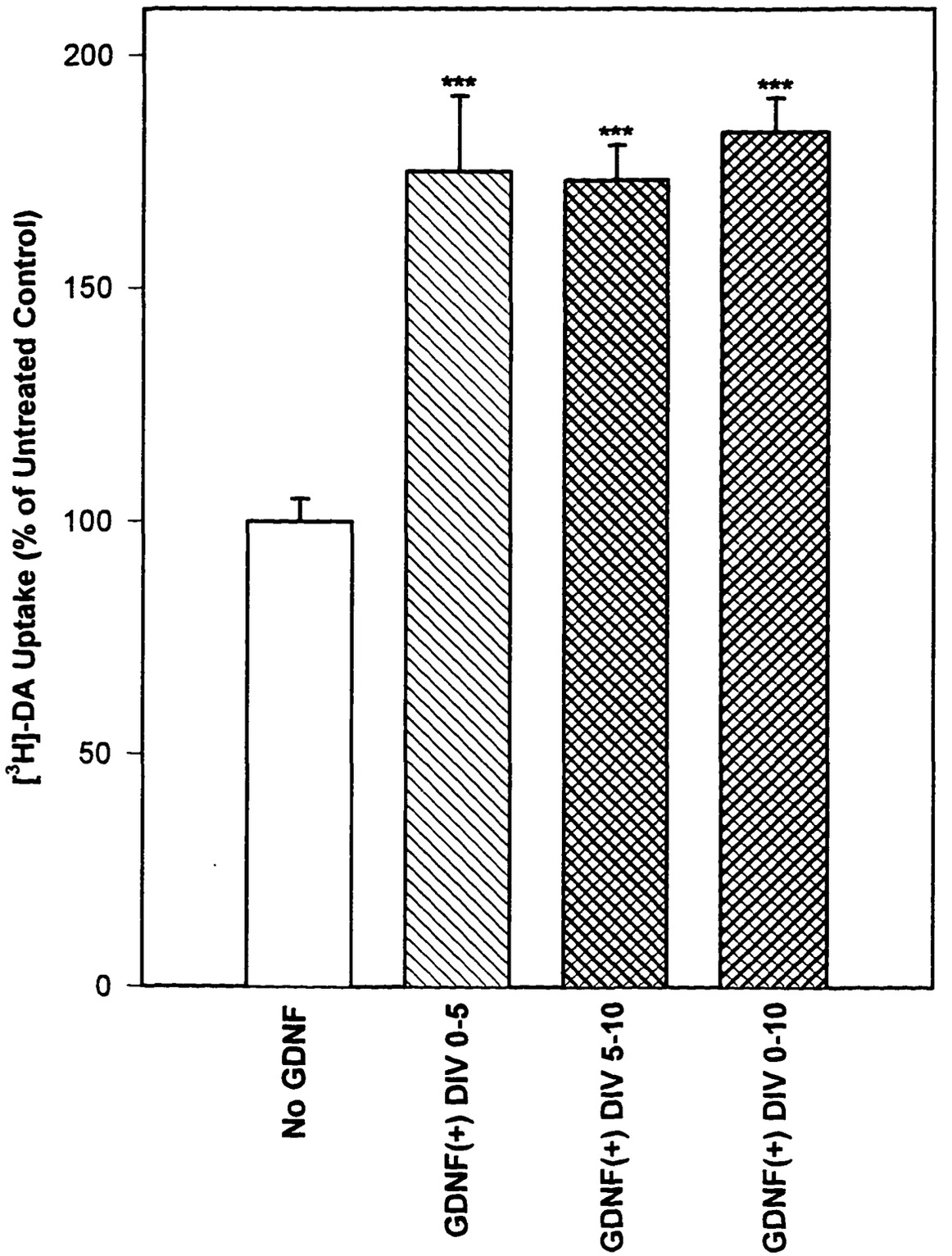


Figure 3

Effect of GDNF on [^{3}H]DA uptake applied at different times of development in mesencephalic cultures. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were treated with GDNF 1 ng/ml from DIV 0 to 5, 5 to 10, or 0 to 10. Data are mean ± SEM values on DIV 10 presented as percentages of untreated controls from 17 samples based on three experiments. Actual values in controls varied between experiments form 237.0 to 478.5 fmol/10 min per culture. Statistical analysis was by ANOVA followed by Tukey's post hoc test compared with the control on DIV 10: ***$p < 0.001$.

Figure 4

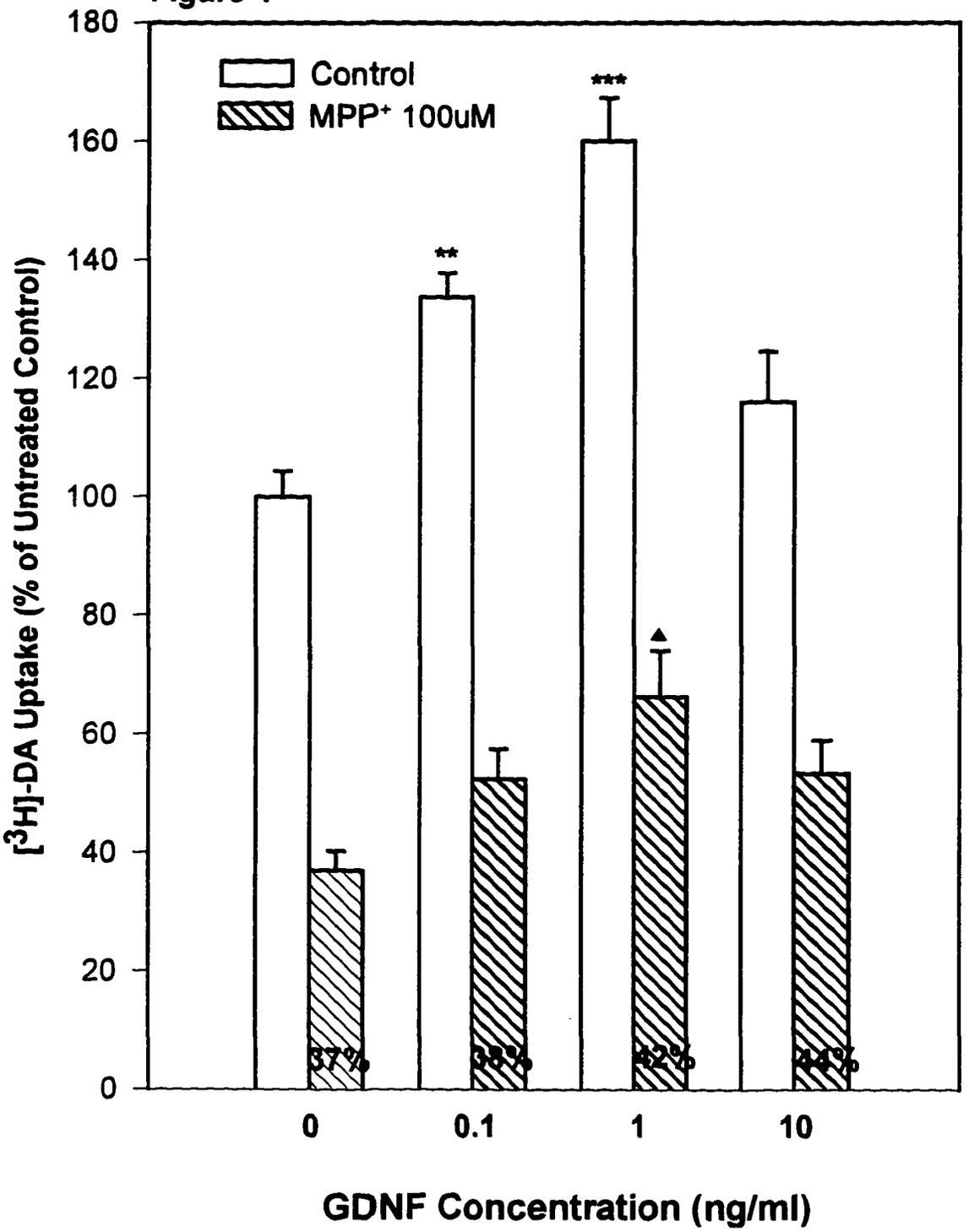


Figure 4

Effect of GDNF against MPP^+ toxicity on [^{3}H]DA uptake in mesencephalic cultures. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of GDNF (0.1, 1 or 10 ng/ml). MPP^+ (100 μM) was added to cultures on DIV 5 for 1 hour. [^{3}H]DA uptake was measured on DIV 6. Data are mean ± SEM values expressed as percentage of the untreated control (n = 16) based on 3 experiments. Actual values of untreated controls varied between experiments from 286.8 to 671.0 fmol/10 min per culture. The numbers on the bars indicate the percentile values of MPP^+-treated cultures when compared with the corresponding non-MPP^+-treated controls. Statistical analysis was by ANOVA followed by Tukey's post hoc test: $^{**}p < 0.01$, $^{***}P < 0.001$ versus non-GDNF control; ▲▲$p < 0.01$ in MPP^+-treated group versus non-GDNF-treated control.

Figure 5

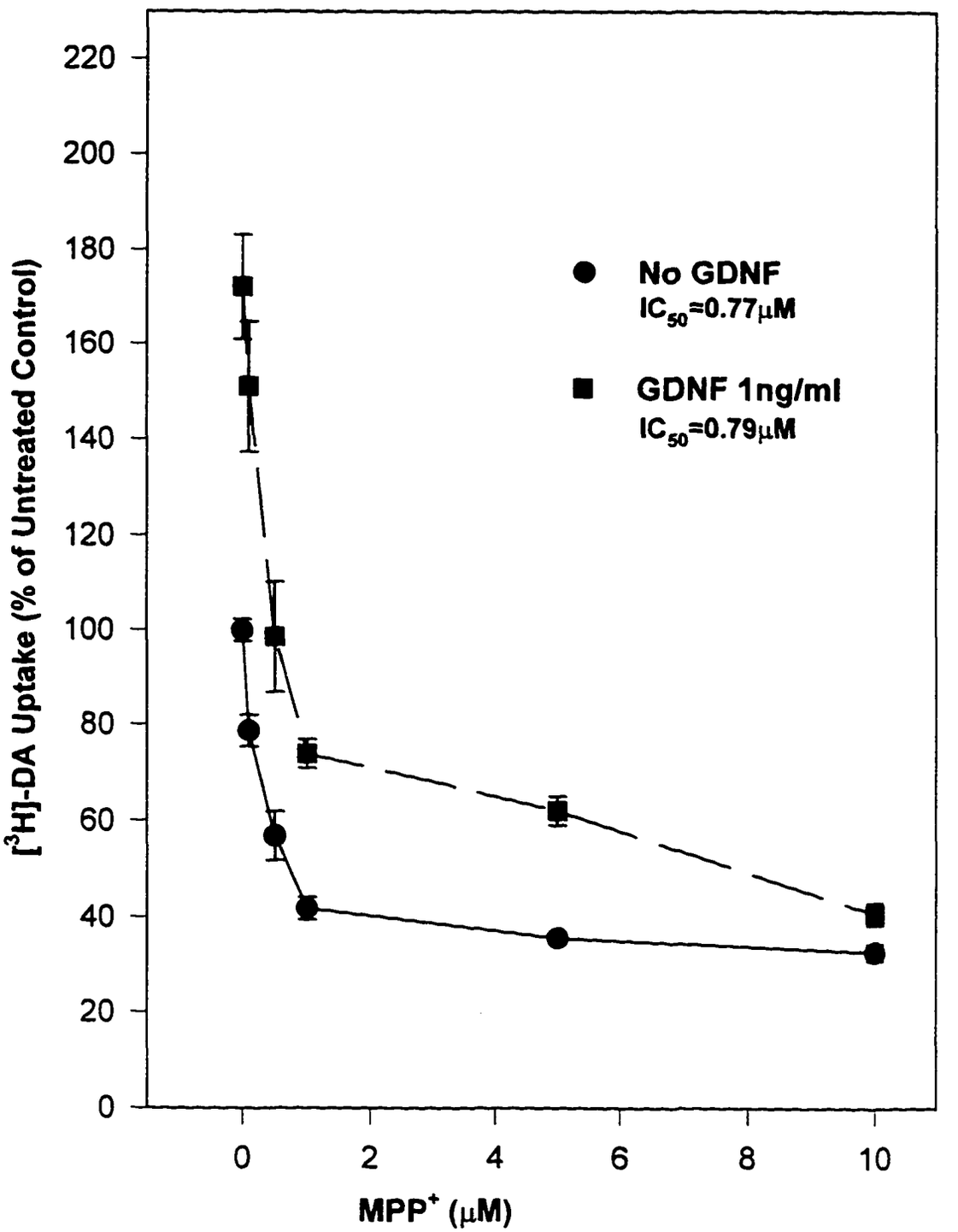


Figure 5

The effect of GDNF on [^{3}H]-DA uptake in cultures treated with different concentrations of MPP^+. Mesencephalic cultures from E14 ± 0.5 rat embryos were maintained in the presence of 1 ng/ml GDNF. MPP^+ (0.1, 0.5, 1, 5 and 10 μM) was added to cultures on DIV 5 for 24 hours and then washed off. DA uptake was measured on DIV 7. Data are mean ± SEM values for [^{3}H]DA uptake after MPP^+ treatment expressed as percentage of the corresponding control group from nine samples based on three experiments. Actual values of controls varied between experiments from 305.2 to 353.1 fmol/10min/culture. The concentrations of MPP^+ needed to cause a 50% decrease in uptake (IC_{50}) were calculated by linear regression using the uptake values from 0 to 1 μM MPP^+.

Figure 6: Design of Experiments Testing the Regrowth and Survival Effect of GDNF on Mesencephalic Cultures After MPP^+ Treatment

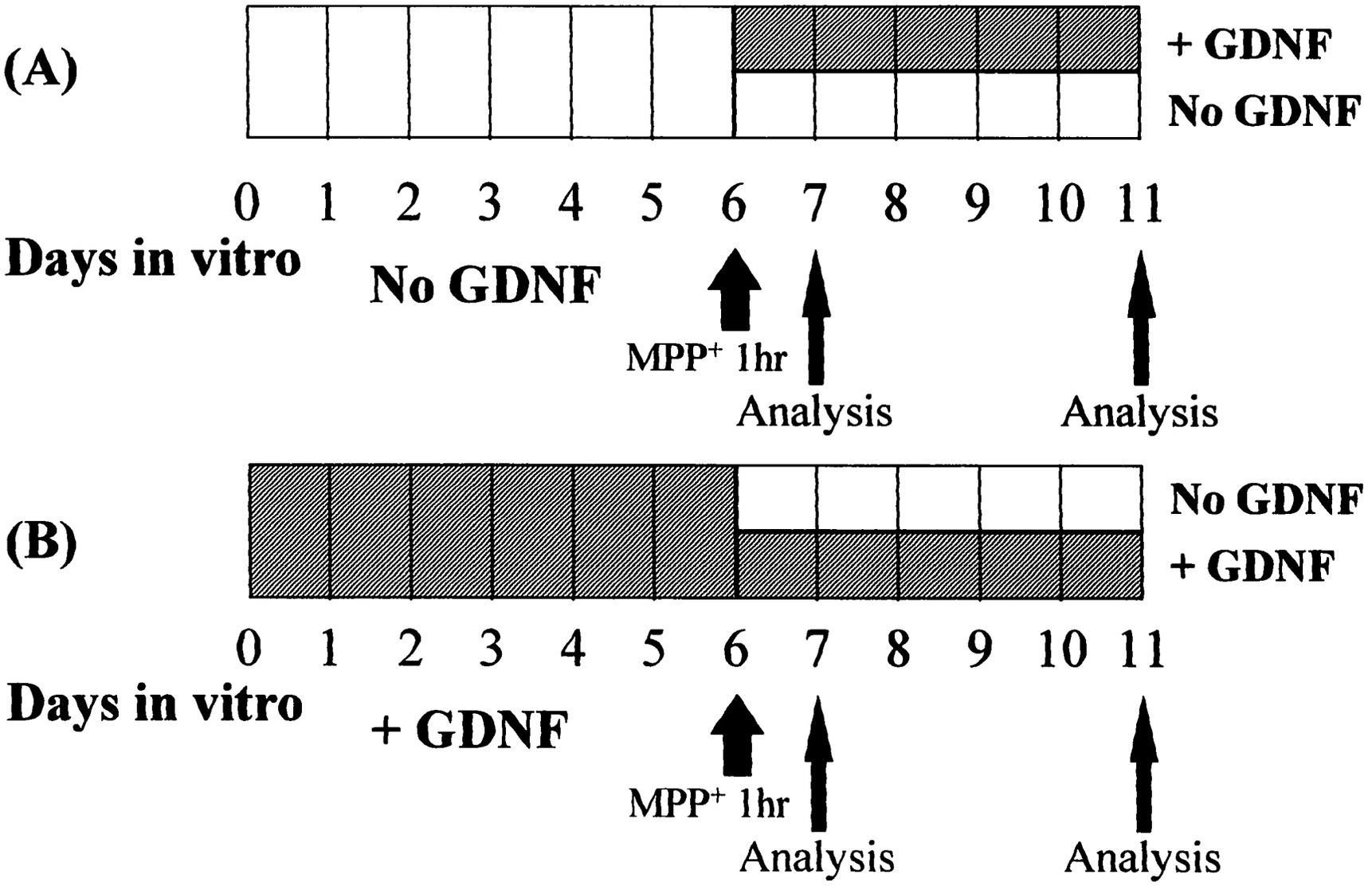


Figure 6

Design of experiments testing the regrowth and survival effect of GDNF on mesencephalic cultures after MPP^+ treatment. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence (green) or absence (red) of GDNF (1 ng/ml). Cultures were treated with MPP^+ (100 μM) on DIV 6 for 60 min. At the end of treatment, medium with MPP^+ was washed way and refed with fresh medium containing with GDNF (1 ng/ml; green) or in the absence of GDNF (red) in each group. DA uptake and TH+ cell counts were performed on DIV 7 and 11.

Figure 7

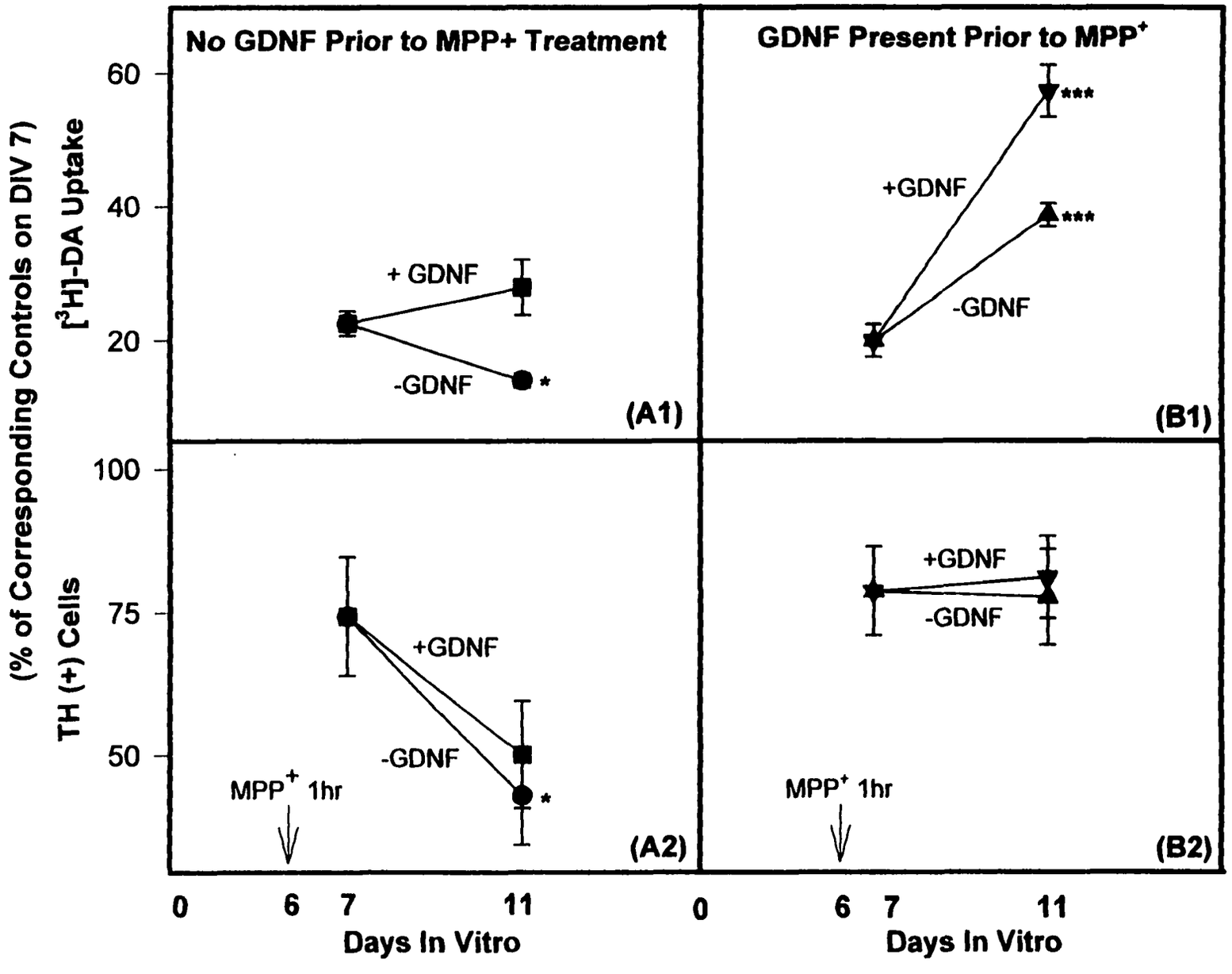
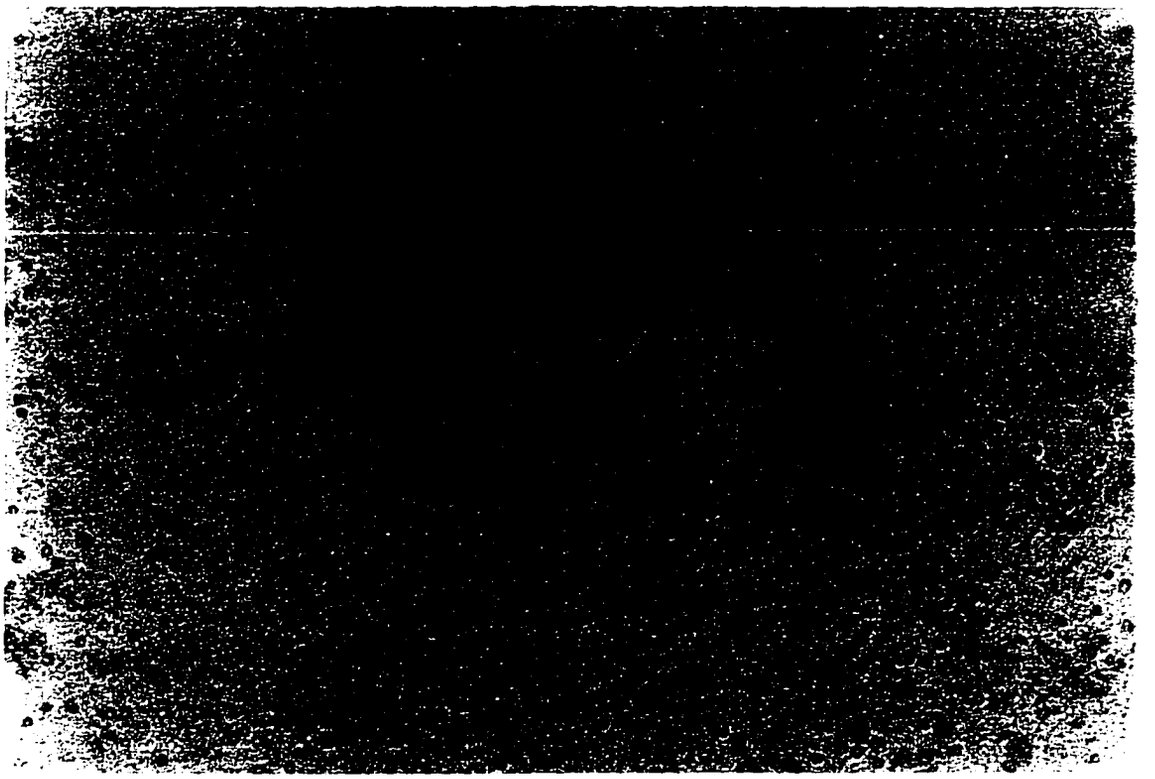


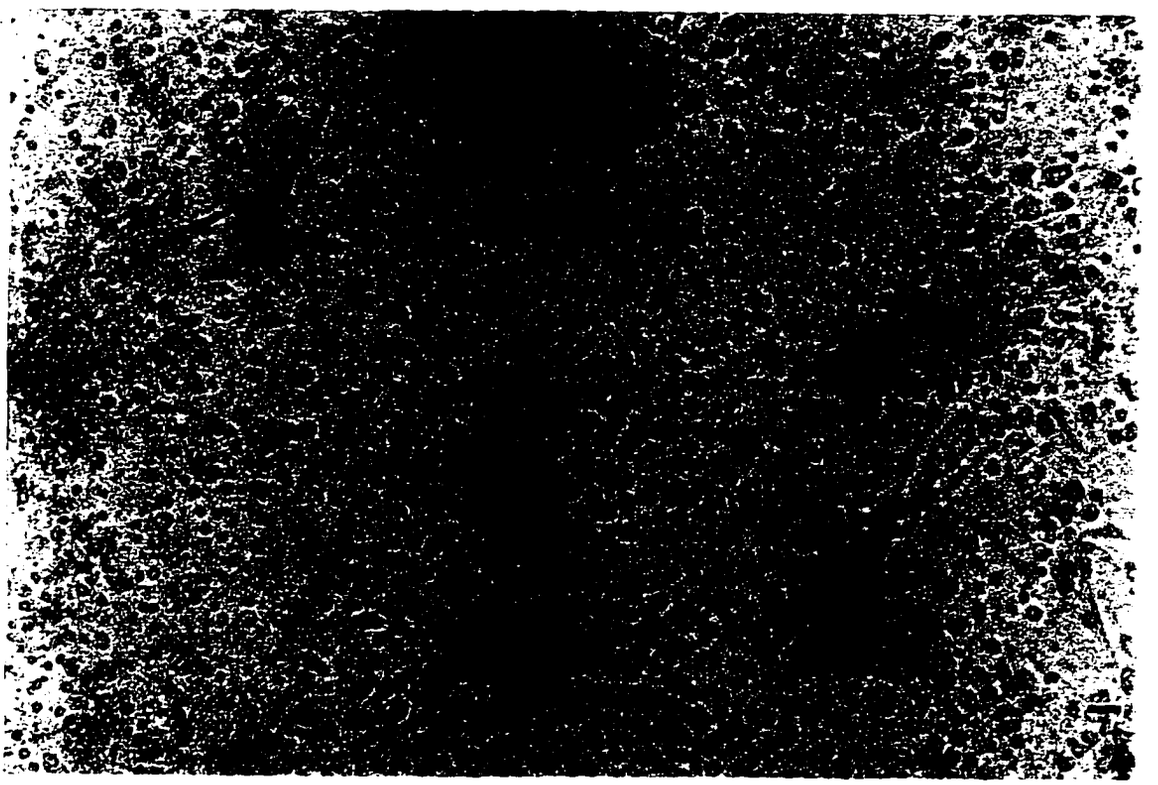
Figure 7

Effect of GDNF on the regrowth and survival of dopaminergic neurons after MPP^+ treatment. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence (B1 & B2) or absence (A1 & A) of GDNF (1 ng/ml). Cultures were treated with MPP^+ (100 μM) on DIV 6 for 60 min. DA uptake (A1 & B1) and TH+ cell counts (A2 & B2) were performed on DIV 7 and 11. Data are mean ± SEM values of MPP^+-treated groups expressed as percentage of corresponding controls (n = 22) from 5 experiments. Actual values in controls on DIV 7 varied between experiments from 248.7 to 310.1 fmol/10min per culture for [^{3}H]DA uptake and 162 to 222/cm^2 for TH+ cells. Statistical analysis was by ANOVA followed by Tukey's post hoc test: * $p < 0.05$, *** $p < 0.001$.

(A)



(B)



(C)

(D)

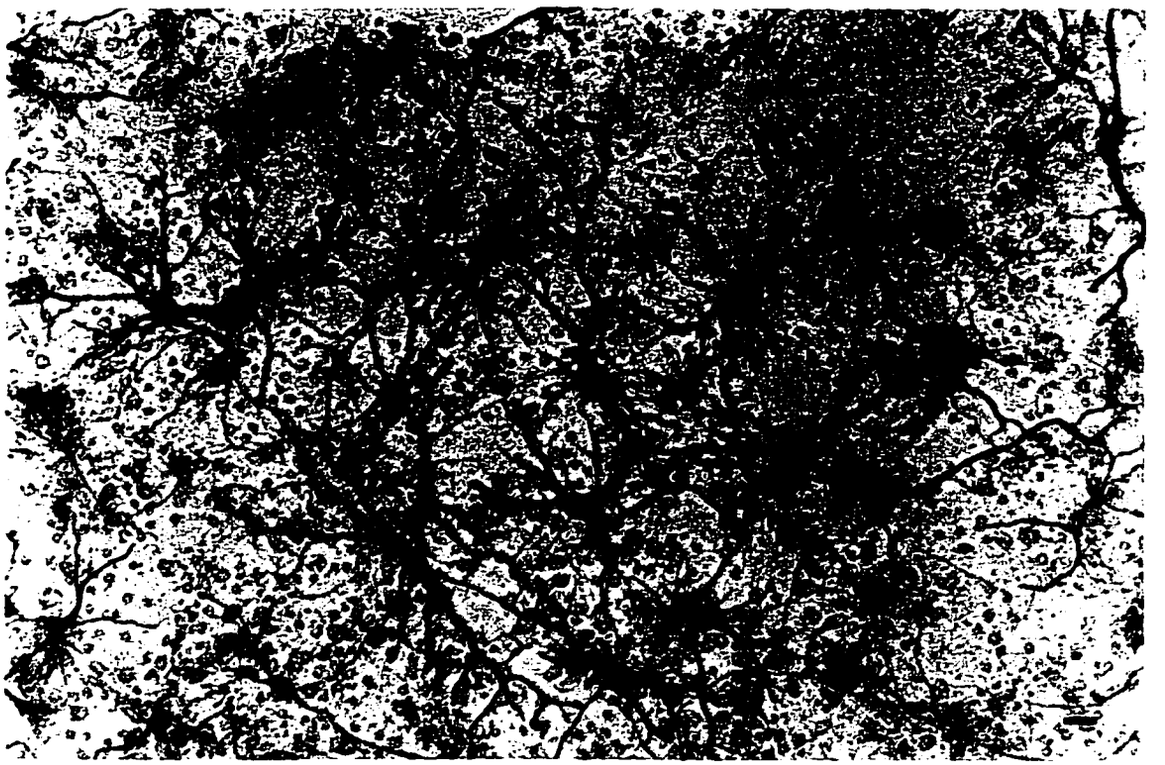


Figure 8

(A) and (B): TH immunocytochemistry of (A) control and (B) bFGF-treated mesencephalic cultures on DIV 6. Increased neuritic fiber number and more prominent and darker staining of neuronal somata were evident after treatment with 10 ng/ml of bFGF compared with the control.

(C) and (D): GFAP immunoreactive astrocytes in (C) control and (D) bFGF-treated mesencephalic cultures on DIV 6. There was a great difference in the number of GFAP-positive cells or intensity of the GFAP staining reaction in the cultures treated with 10 ng/ml of bFGF compared with controls. Bar = 25μm.

Figure 9

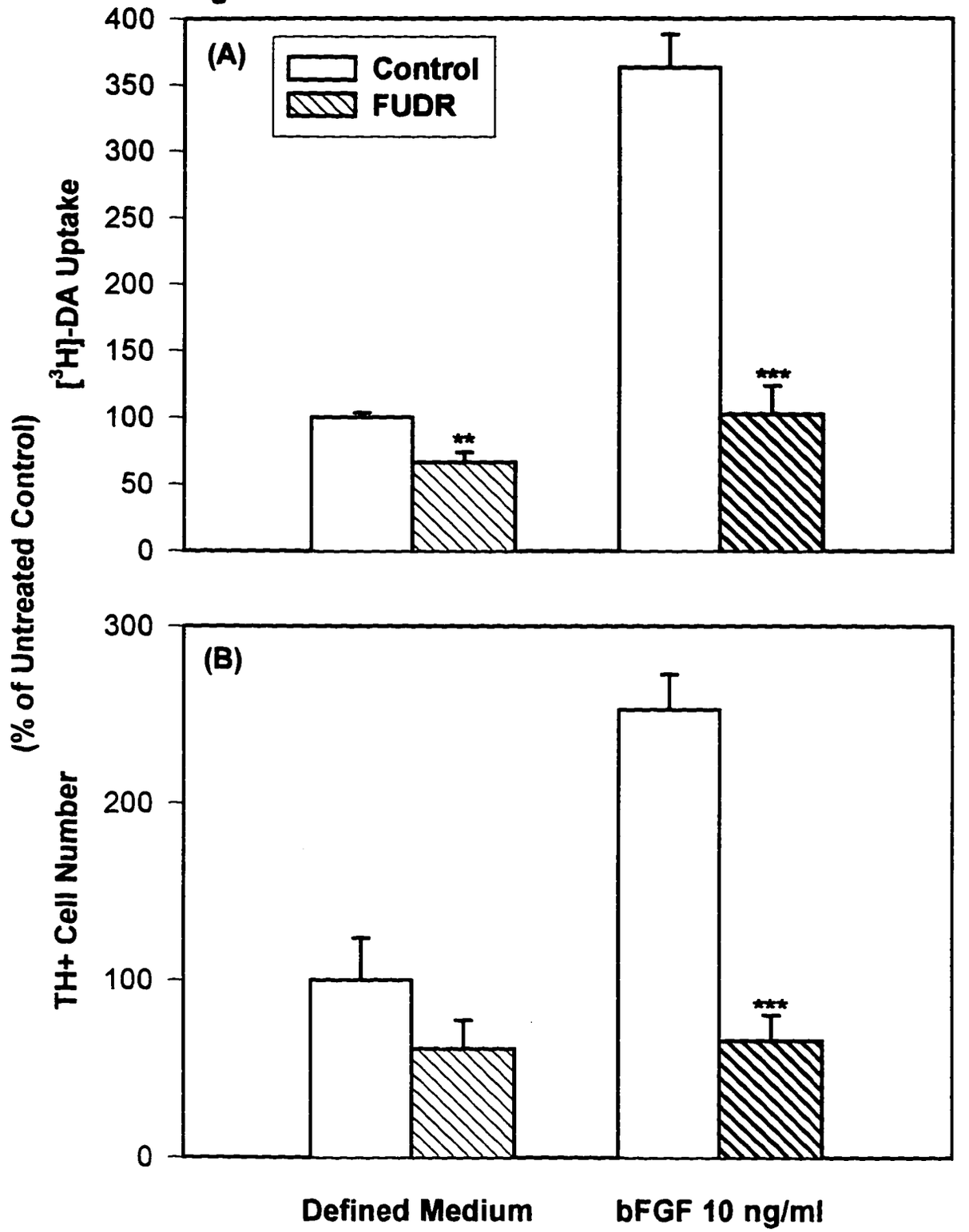


Figure 9

Effect of FUDR treatment on the neurotrophic effects of bFGF on (A) [^{3}H]DA uptake and (B) number of TH-positive neurons in mesencephalic cultures. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. FUDR 8 μM / uridine 20 μM was added to the cultures 24 hours after plating. [^{3}H]DA uptake and TH immunocytochemistry were assayed on DIV 8. Data are mean ± SEM values expressed as percentage of the untreated control (n = 15 in DA uptake and n = 9 in TH staining) based on three experiments. Actual values of untreated controls varied between experiments from 236.8 to 447.8 fmol/10 min per culture in uptake and 164 to 320/cm^2 in TH+ cell numbers. Statistical analysis was by independent t-test: **p < 0.01, ***P < 0.001 compared with corresponding non-FUDR control in each group.

Figure 10

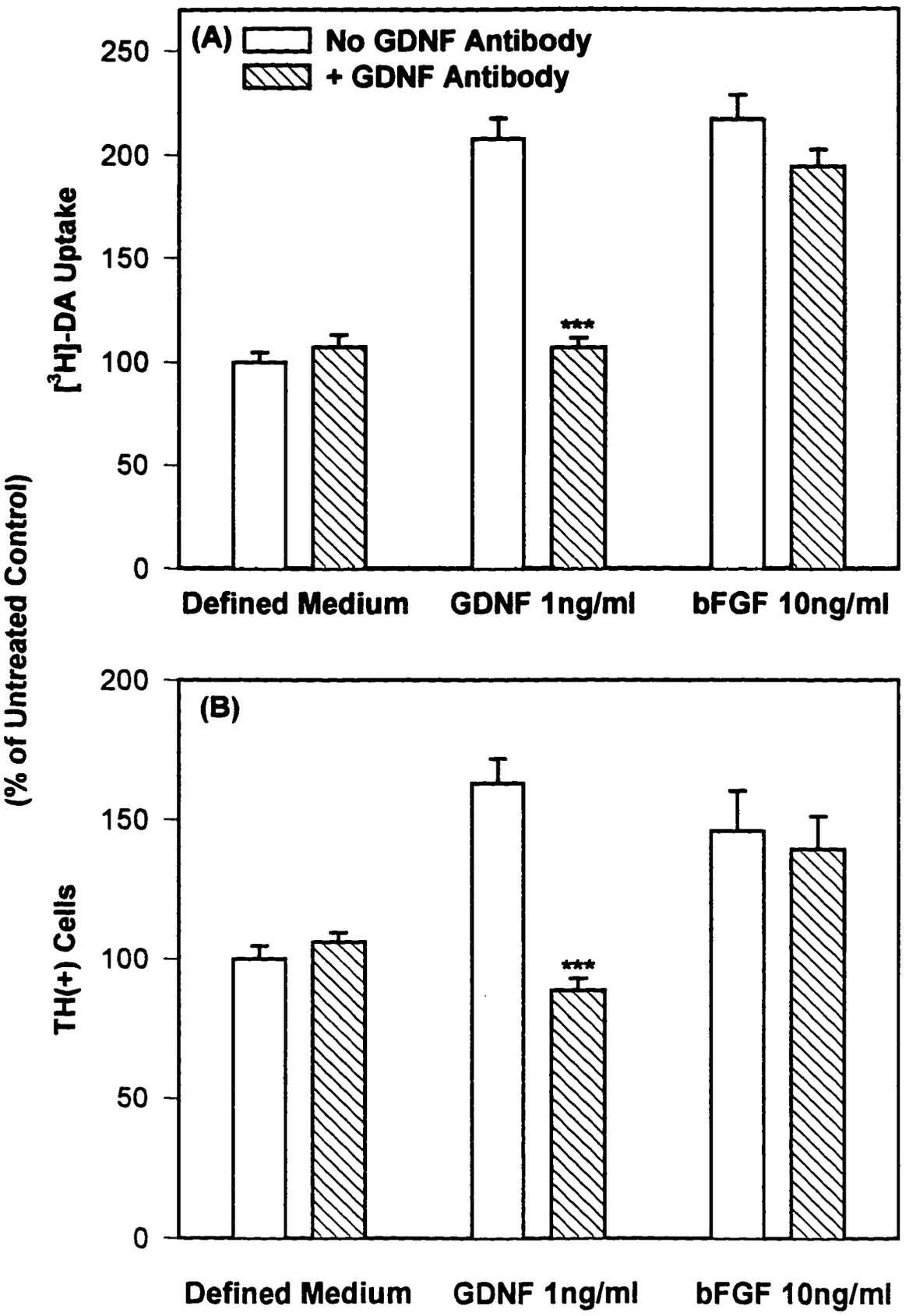


Figure 10

Effect of GDNF-neutralizing antisera in mesencephalic cultures treated with GDNF and bFGF on (A) [^{3}H]DA uptake and (B) number of TH-positive neurons. Cultures from embryonic day 14 ± 0.5 rat embryos were treated with GDNF 1 ng/ml and bFGF 10 ng/ml. Affinity-purified polyclonal antibodies against GDNF were added to the cultures concurrently with GDNF or bFGF at a dilution of 1:1000. Same amount of rabbit serum was added to controls. (A) [^{3}H]DA uptake and (B) number of TH-positive neurons on DIV 10. Data are means ± SEM presented as percentages of untreated controls (n = 18 for DA uptake; n = 8 for TH immunostaining). Actual values of controls varied between experiments from 334.1 to 877.6 fmol/10 min per culture on uptake and from 174 to 458 cells/cm^2 on TH-positive cell number. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***$p < 0.001$ versus non-antibodies control.

Figure 11

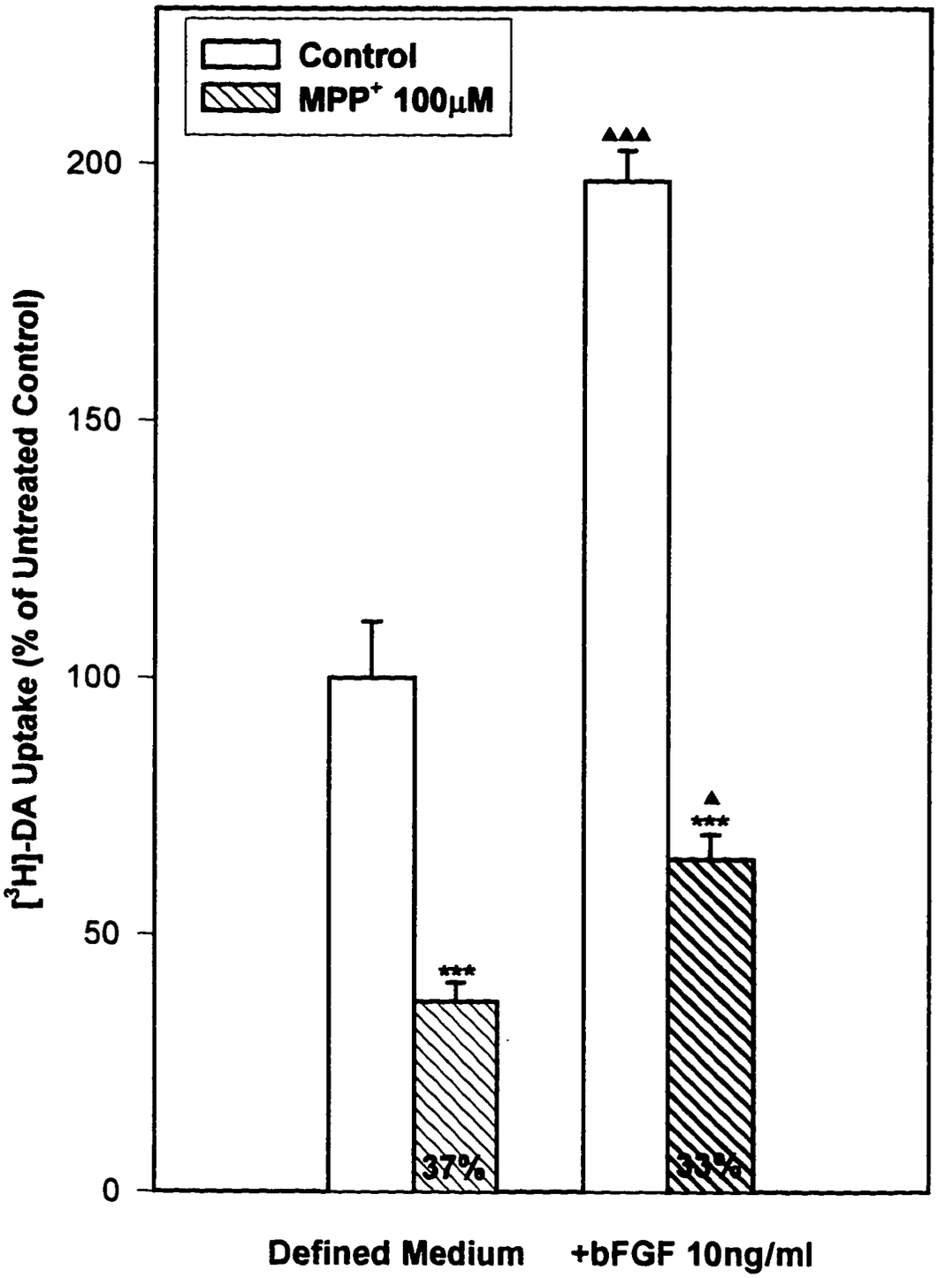


Figure 11

Effect of bFGF against MPP^+ toxicity on [^{3}H]DA uptake in mesencephalic cultures. Mesencephalic cultures from E14 ± 0.5 rat embryos were maintained in the presence of bFGF (10 ng/ml). Cultures were treated with 100 μM MPP^+ on DIV 5 for 60min and then washed and kept with fresh medium plus corresponding concentrations of bFGF or equal amount of 0.1% BSA for control. DA uptake was performed on DIV 6. Data are means ± SEM presented as percentage of untreated controls (n = 12) based on three experiments. Actual values of controls varied between experiments from 286.8 to 671.0 fmol/10 min per culture. The % values on the graph represent the remaining uptake after MPP^+ treatment compared to the corresponding non-MPP^+-treated control. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***$p < 0.001$ versus corresponding non-MPP^+ control; ▲$p < 0.05$, ▲▲▲$p < 0.001$ versus non-bFGF control.

Figure 12

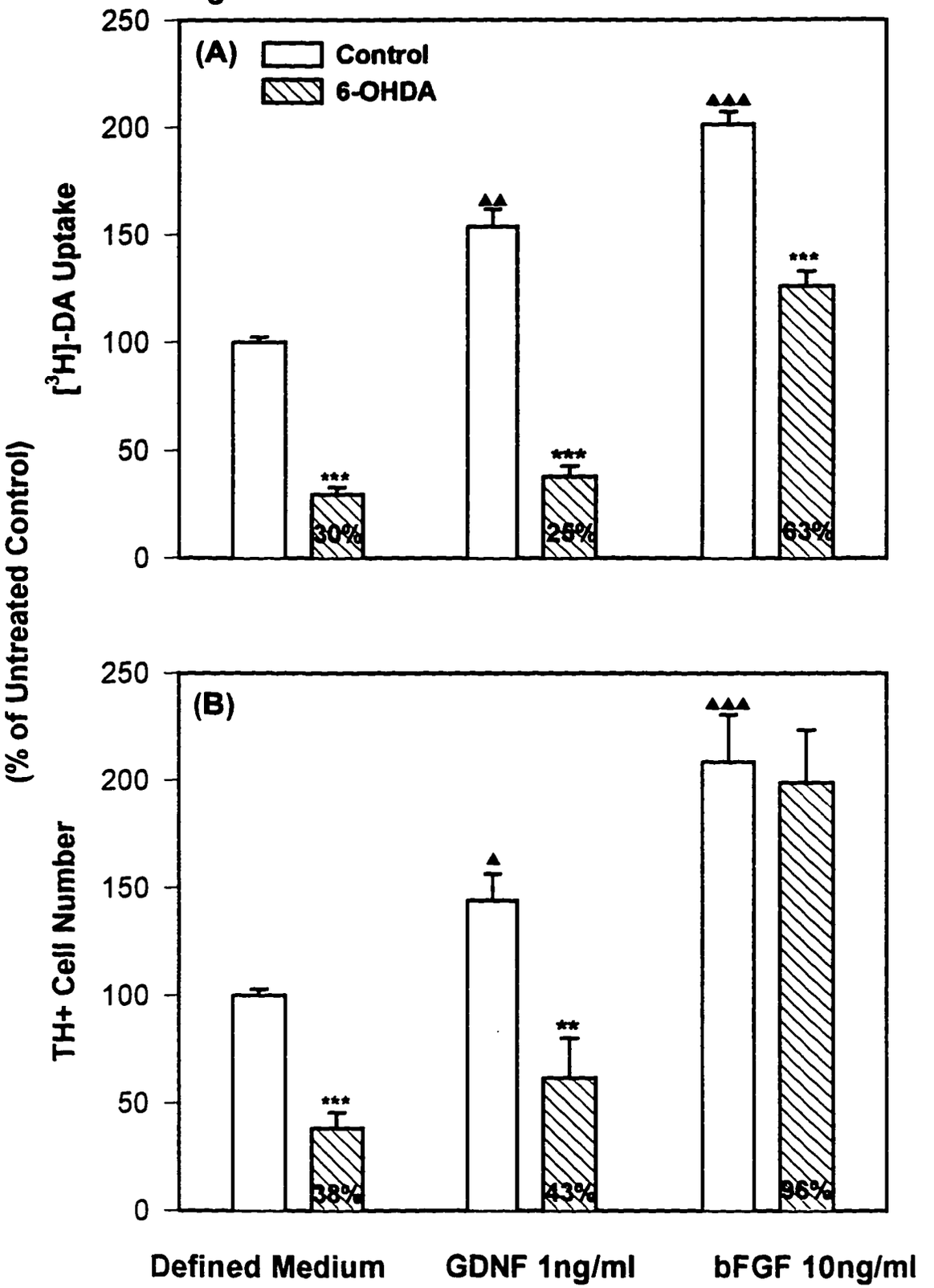


Figure 12

Effect of GDNF and bFGF against 6-OHDA toxicity on (A) [^{3}H]DA uptake and (B) number of TH-positive neurons in mesencephalic cultures. Mesencephalic cultures from E14 ± 0.5 rat embryos were maintained in the presence of GDNF (1 ng/ml) or bFGF (10 ng/ml). Cultures were treated with 30 μM 6-OHDA dissolved in 200μM ascorbic acid or equal amount ascorbic acid solution in control on DIV 5 for 45min, then washed and refed with fresh medium plus corresponding concentrations of GDNF or bFGF or equal amount of 0.1% BSA in control. DA uptake and TH immunostaining were performed on DIV 6. Data are means ± SEM presented as percentage of untreated controls (n = 25 in uptake and n = 12 in TH staining) based on five experiments. Actual values of controls varied between experiments from 357.3 to 657.9 fmol/10 min per culture in uptake and from 174 to 361 cells/cm^2. The % values on the graph represent the remaining uptake after 6-OHDA treatment compared to the corresponding non-6-OHDA-treated control. Statistical analysis was by ANOVA followed by Tukey's post hoc test: **$p < 0.01$, ***$p < 0.001$ versus corresponding non-6-OHDA control; ▲$p < 0.05$, ▲▲$p < 0.01$ ▲▲▲$p < 0.001$ versus non-growth control.

Figure 13

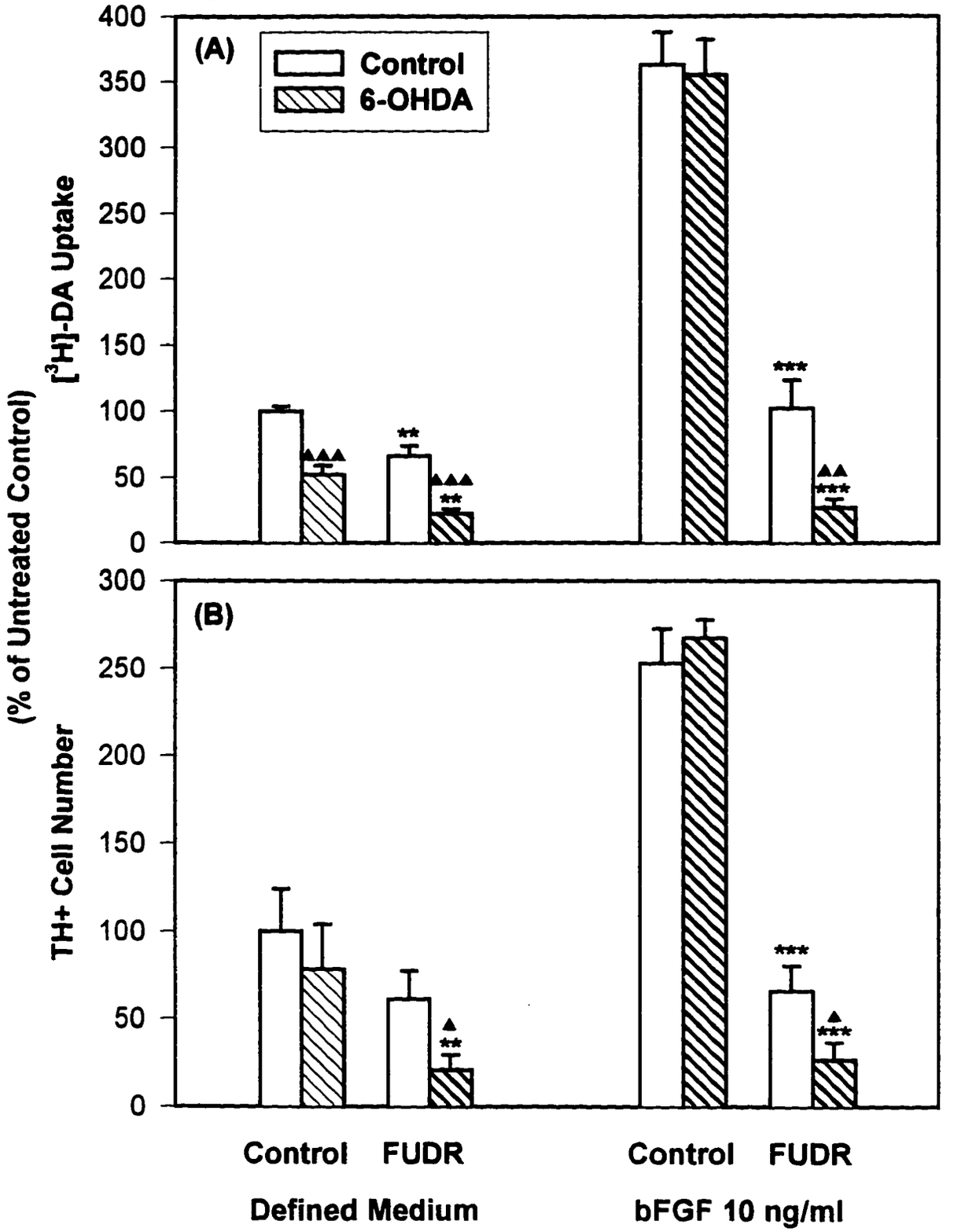


Figure 13

Effect of FUDR on the bFGF protection against 6-OHDA toxicity on (A) [^{3}H]DA uptake and (B) number of TH-positive neurons in mesencephalic cultures. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. FUDR 8 μM/ uridine 20 μM was added to the cultures 24 hours after plating. 6-OHDA 30 μM with 200 μM ascorbic acid was treated on DIV 7 for 45 minutes. Medium was then washed away and replaced with fresh medium containing appropriate concentrations of bFGF and FUDR. [^{3}H]DA uptake and TH immunocytochemistry were assayed on DIV 8. Data are mean ± SEM values expressed as percentages of the untreated control (n = 15 in DA uptake and n = 9 in TH staining) based on three experiments. Actual values of untreated controls varied between experiments from 236.8 to 447.8 fmol/10 min per culture in uptake and 164 to 320/cm^2 in TH+ cell numbers. Statistical analysis was by ANOVA multiple comparisons followed by Tukey's post hoc test: **p < 0.01, ***P < 0.001 compared with corresponding non-FUDR control in each group; ▲p < 0.05, ▲▲p < 0.01 ▲▲▲p < 0.001 versus corresponding non-6-OHDA-treated control in each group.

Figure 14

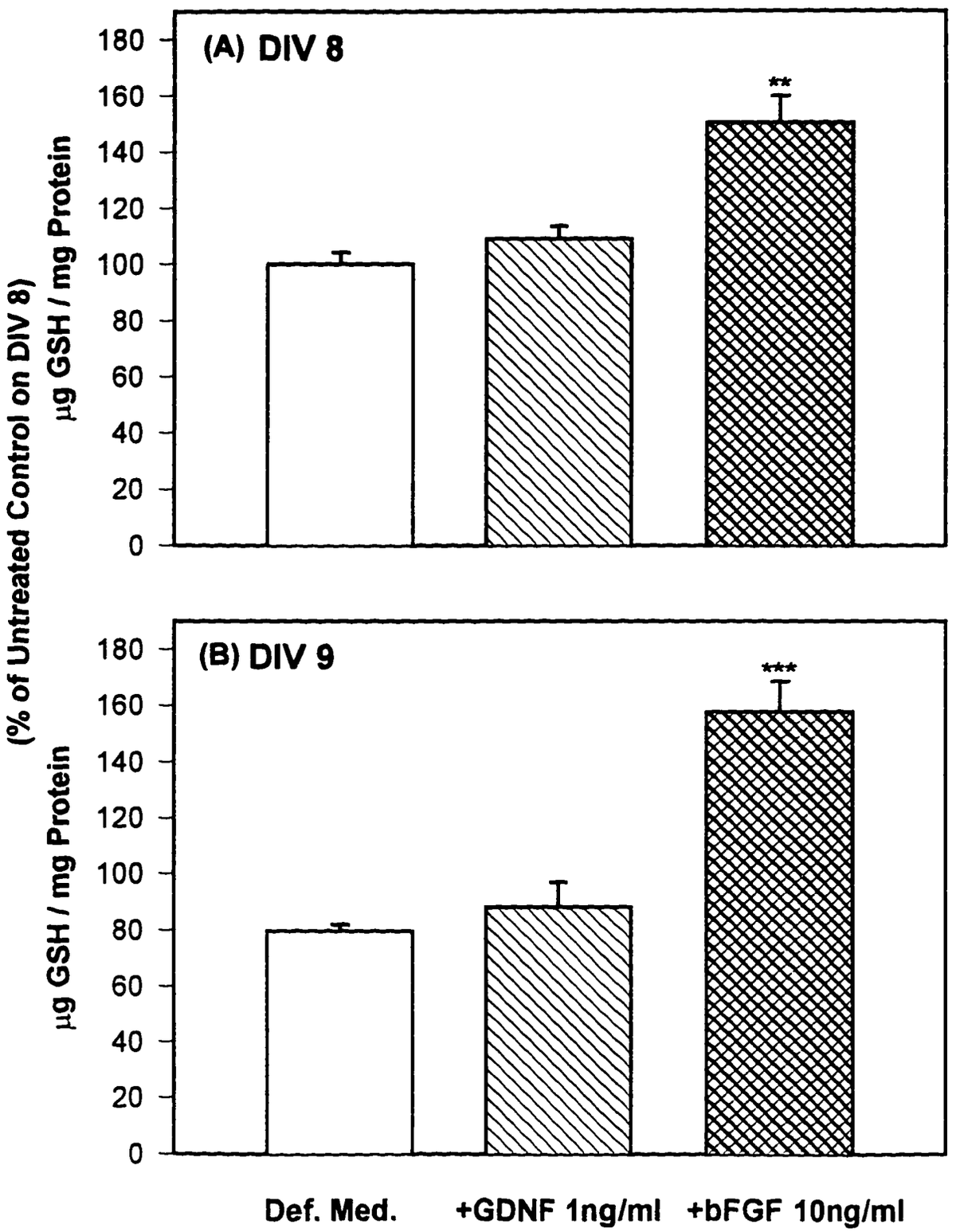


Figure 14

Effect of GDNF and bFGF on GSH levels in mesencephalic cultures at (A) day 8 and (B) day 9 in vitro. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of GDNF 1 ng/ml or bFGF 10 ng/ml. GSH level was assayed on DIV 8 and DIV 9. Data are mean ± SEM values expressed as percentage of the untreated controls on DIV 8 (n = 9) based on three experiments. Actual values of untreated controls varied between experiments from 0.93 to 1.55 μg GSH / mg protein. Statistical analysis was by ANOVA followed by Tukey's post hoc test: **p < 0.01, ***P < 0.001 versus non-growth factor treated control.

Figure 15

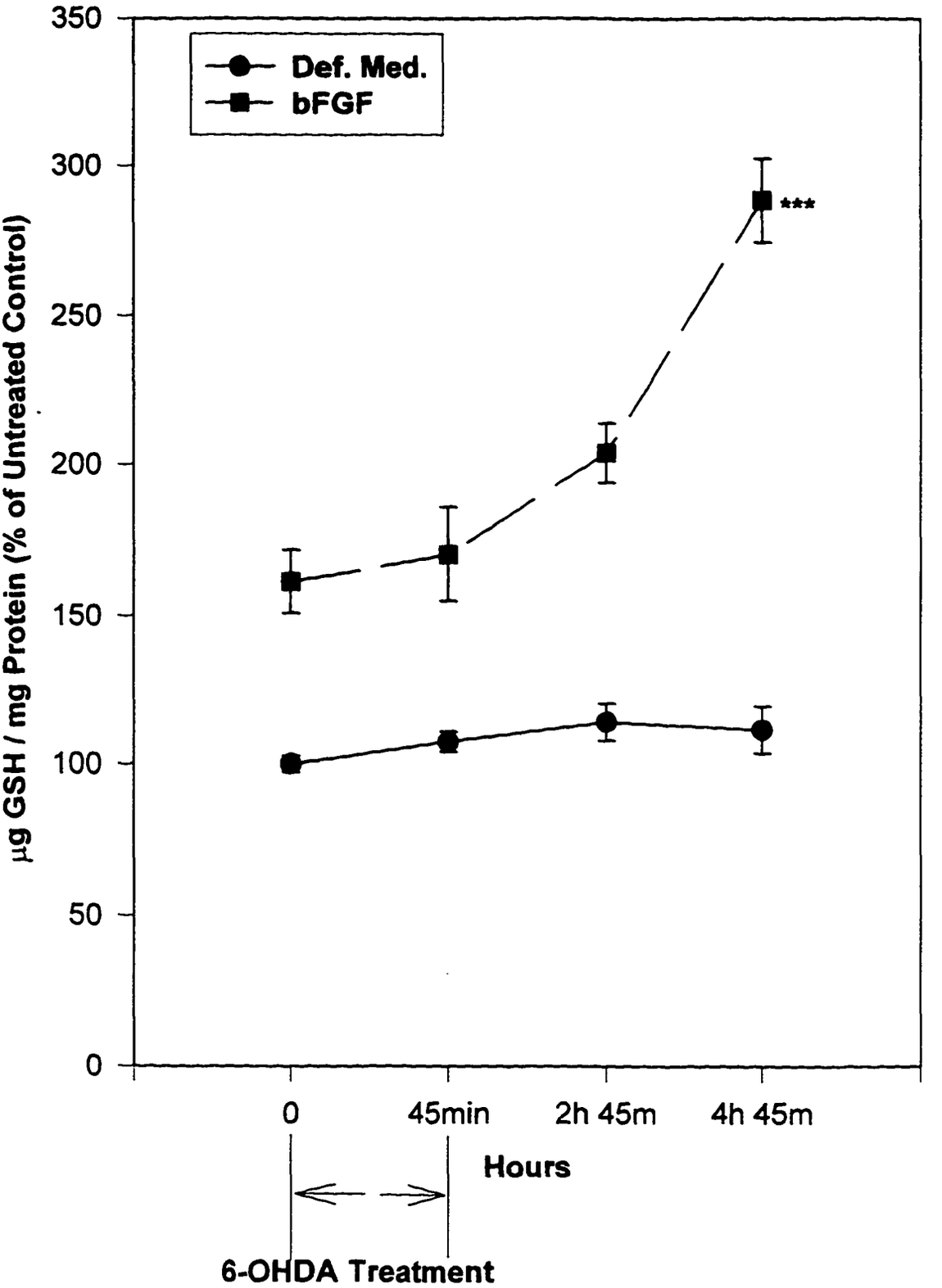


Figure 15

Effect of 6-OHDA treatment on GSH levels in mesencephalic cultures treated with bFGF. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. 6-OHDA 30 μM with 200 μM ascorbic acid was added on DIV 8 for 45 minutes. Medium was then removed and refed with fresh medium containing appropriate concentrations of bFGF. GSH levels were measured on before (0 hr); immediately after (45 min); 2 hours after (2h 45min); and 4 hours (4h 45min) after 6-OHDA treatment. Data are mean ± SEM values expressed as percentage of untreated controls (n = 15) based on five experiments. Actual values of untreated controls varied between experiments from 1.39 to 1.72 μg GSH / mg protein. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***$P < 0.001$ versus the GSH level before 6-OHDA treatment (0 hour).

Figure 16

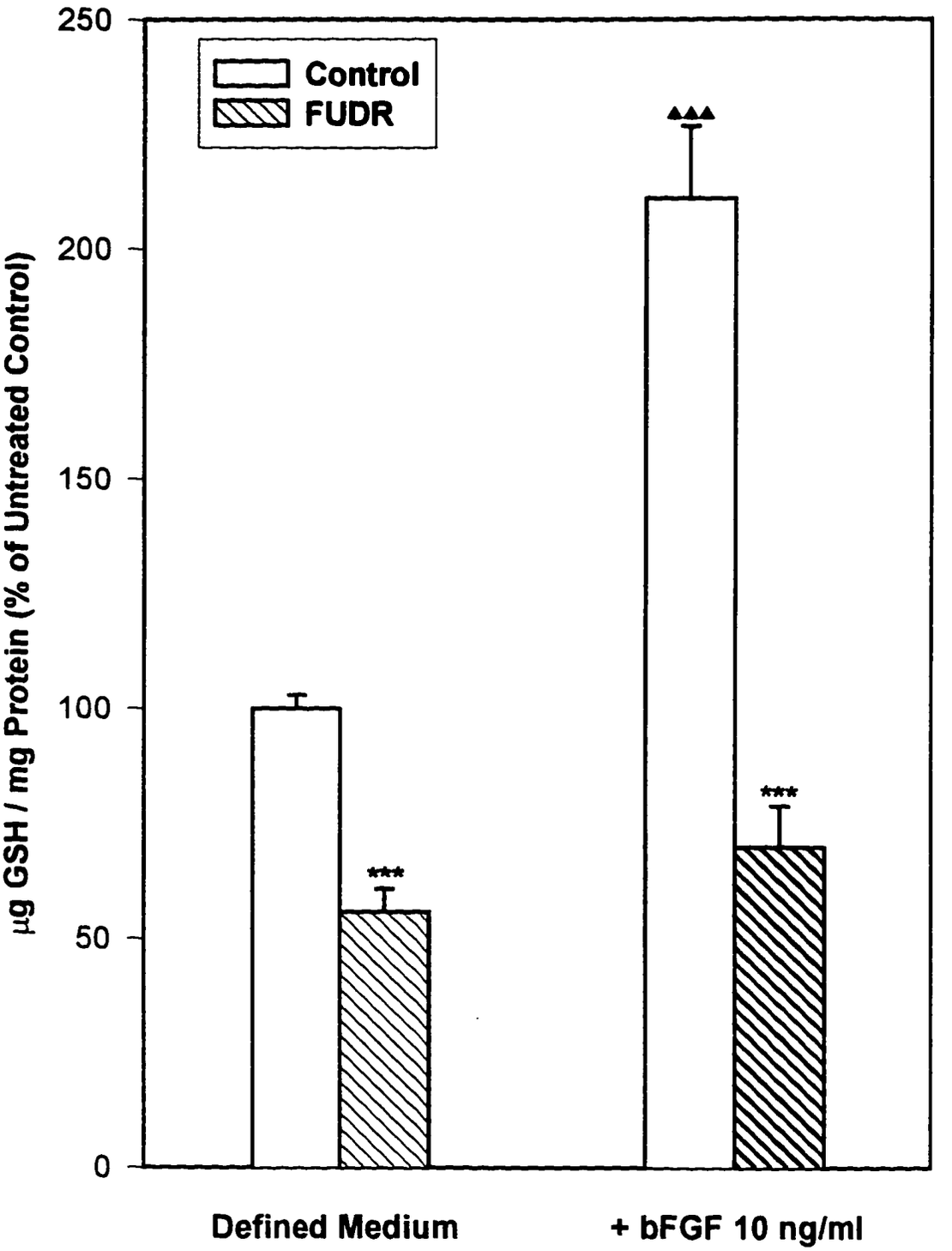


Figure 16

Effect of FUDR on GSH level in the mesencephalic cultures treated with bFGF. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. FUDR 8 μM / uridine 20 μM was added to the cultures 24 hours after plating. GSH levels were measured on DIV 8. Data are mean ± SEM (bars) values expressed as percentage of the untreated control (n = 9) based on three experiments. Actual values of untreated controls varied between experiments from 1.14 to 1.72 μg GSH / mg protein. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***P < 0.001 versus corresponding non-FUDR treated control; ▲▲▲p < 0.001 versus corresponding non-bFGF treated control.

Figure 17

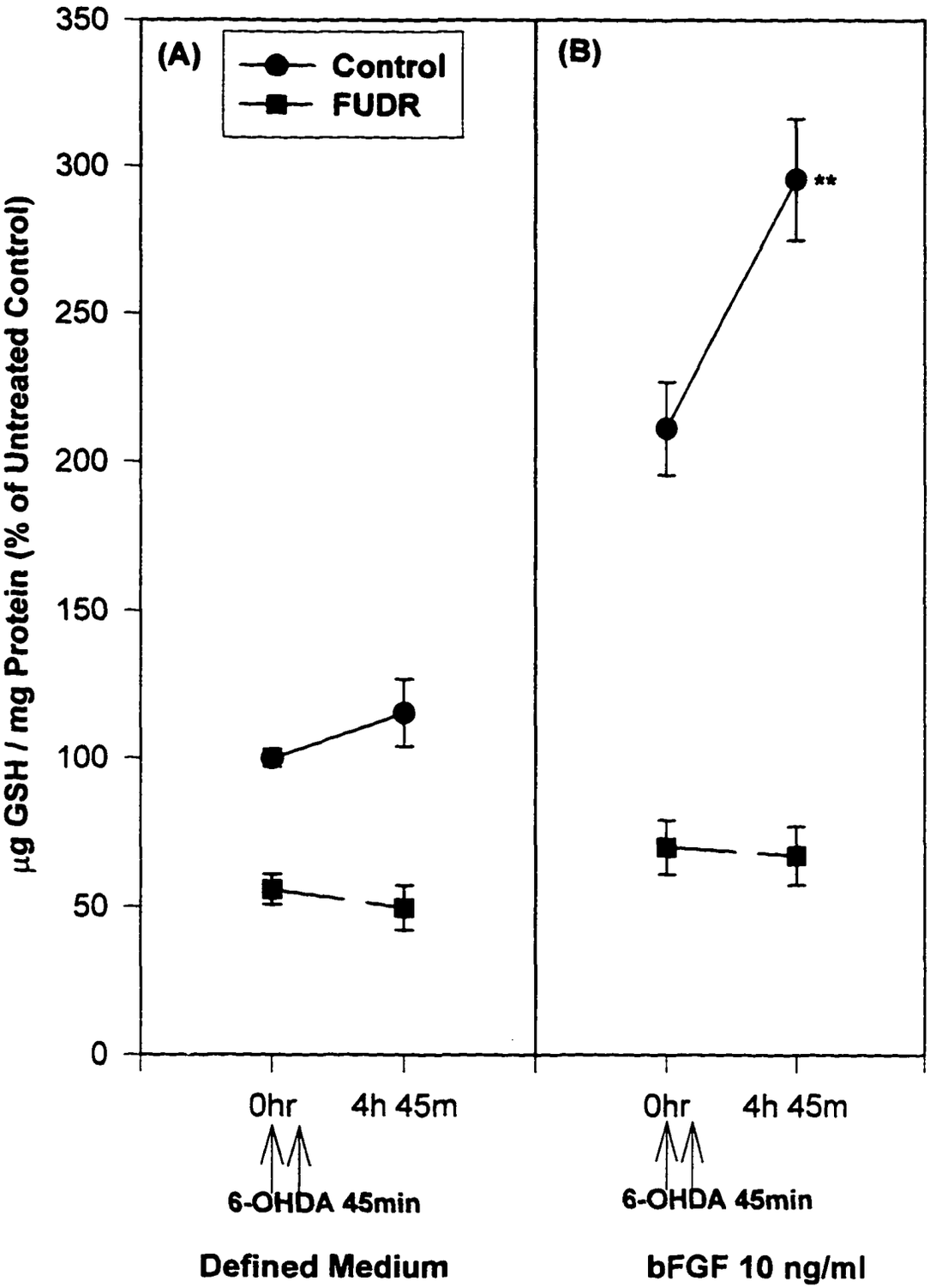


Figure 17

Effect of 6-OHDA treatment on GSH level in mesencephalic cultures treated with bFGF in the presence of FUDR. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. FUDR 8 μM / uridine 20 μM was added to the cultures 24 hours after plating. 6-OHDA 30 μM with 200 μM ascorbic acid was added on DIV 8 for 45 minutes. Medium was then removed and refed with fresh medium containing appropriate concentrations of bFGF and FUDR. GSH level was assayed on (1) before; and (2) 4 hours after 6-OHDA treatment. Data are mean ± SEM values expressed as percentage of the untreated controls (n = 9) based on three experiments. Actual values of untreated controls varied between experiments from 1.14 to 1.72 μg GSH / mg protein. Statistical analysis was by ANOVA followed by Tukey's post hoc test: $^{**}P < 0.01$ versus GSH level before 6-OHDA treatment (0 hour).

Figure 18

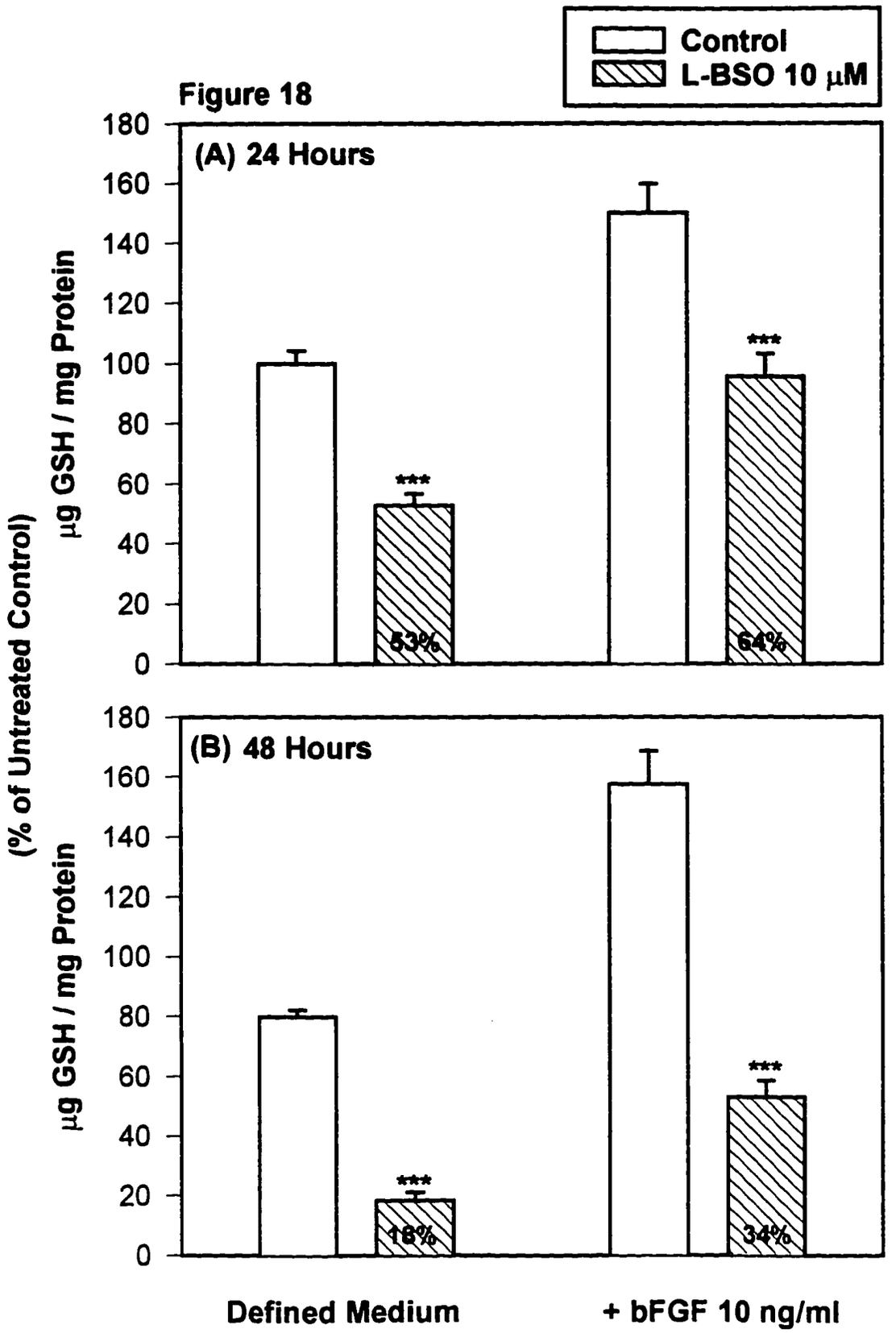


Figure 18

Effect of L-BSO on GSH levels in mesencephalic cultures in the presence of bFGF at (A) day 8 and (B) day 9 in vitro. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. L-BSO 10 μM was added into the cultures on DIV 7. GSH level was assayed 24 (DIV 8) and 48 hours (DIV 9) later. Data are mean ± SEM values expressed as percentage of the untreated control (n = 9) based on three experiments. Actual values of untreated controls varied between experiments from 0.93 to 1.55 μg GSH / mg protein. The % values on the graph represent the remaining GSH level after L-BSO treatment compared to the corresponding non-L-BSO treated control. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***$P < 0.001$ versus corresponding non-L-BSO treated control.

Figure 19

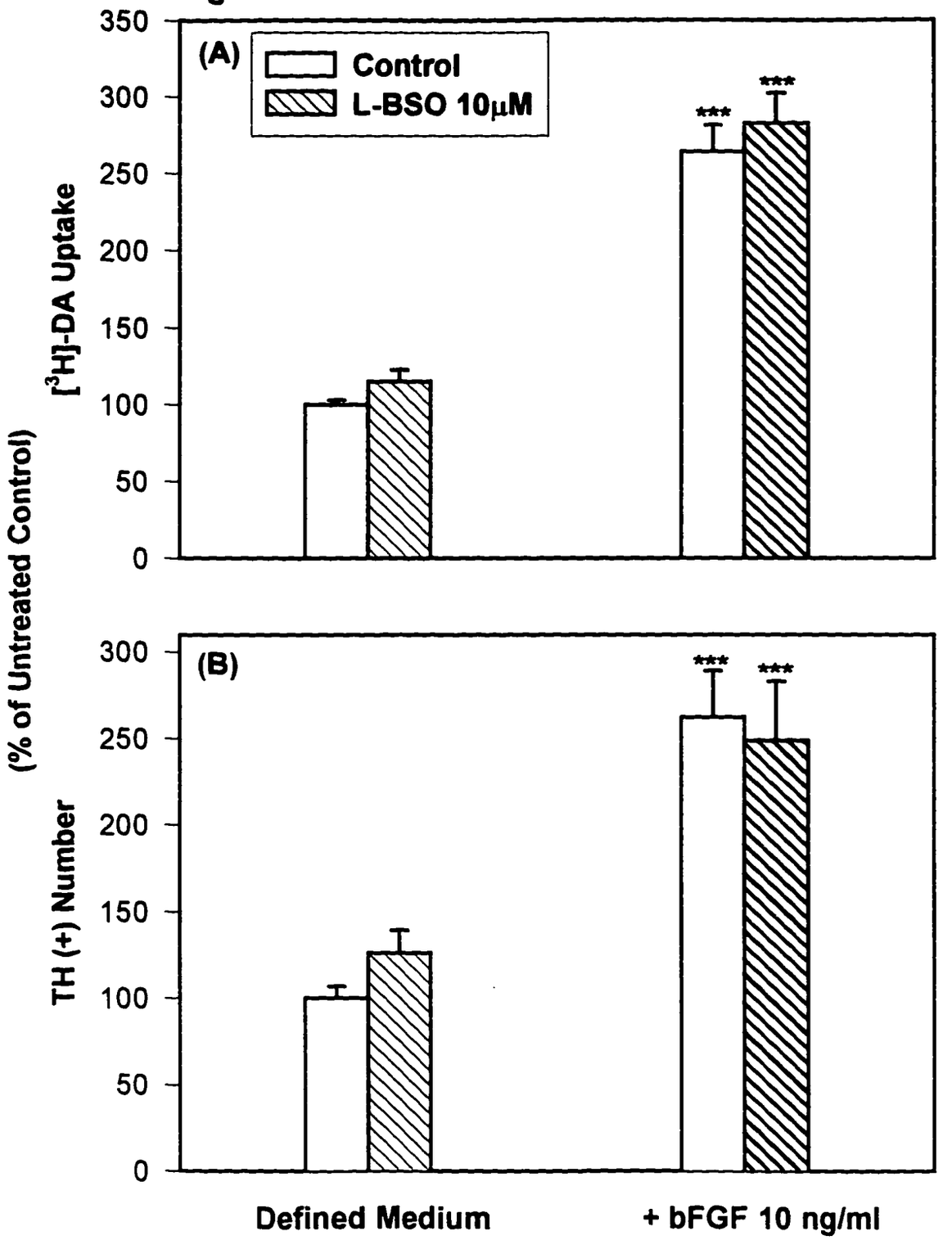


Figure 19

Effect of L-BSO on (A) [^{3}H]DA uptake and (B) TH+ cell number in mesencephalic cultures treated with bFGF. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. L-BSO 10 μM was added into the cultures on DIV 7. DA uptake and TH immunostaining were performed on DIV 9. Data are mean ± SEM values expressed as percentage of the untreated controls (n = 18 in DA uptake and n = 9 in TH staining) based on four experiments. Actual values of untreated controls varied between experiments from 461.2 to 795.8 fmol/10 min per culture in uptake and 157 to 361/cm^2 in TH+ cell numbers. Statistical analysis was by ANOVA followed by Tukey's post hoc test: ***$P < 0.001$ compared with corresponding non-bFGF treated control.

Figure 20

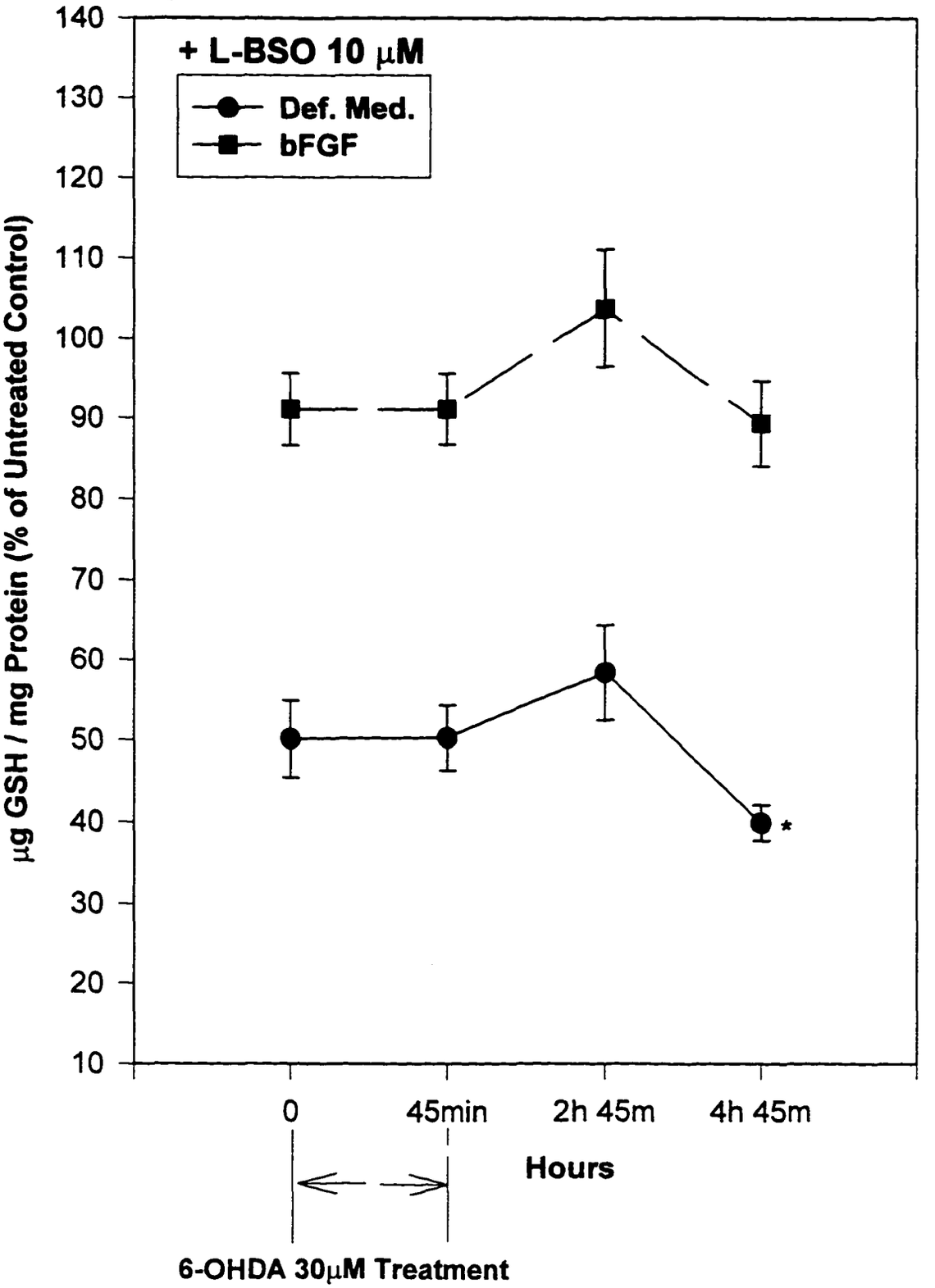


Figure 20

Effect of L-BSO on GSH levels in mesencephalic cultures after treatment of 6-OHDA. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. L-BSO 10 μM was added on DIV 7. 6-OHDA 30 μM with 200 μM ascorbic acid was added on DIV 8 for 45 minutes. Medium was then removed and refed with fresh medium containing appropriate concentrations of bFGF. GSH levels were measured on before (0 hr); immediately after (45 min); 2 hours after (2h 45min); and 4 hours (4h 45min) after 6-OHDA treatment. Data are mean ± SEM values expressed as percentage of the untreated controls (n = 15) based on five experiments. Actual values of untreated controls varied between experiments from 1.39 to 1.72 μg GSH / mg protein. Statistical analysis was by ANOVA followed by Tukey's post hoc test: *P < 0.05 versus the GSH level before 6-OHDA treatment (0 hour).

Figure 21

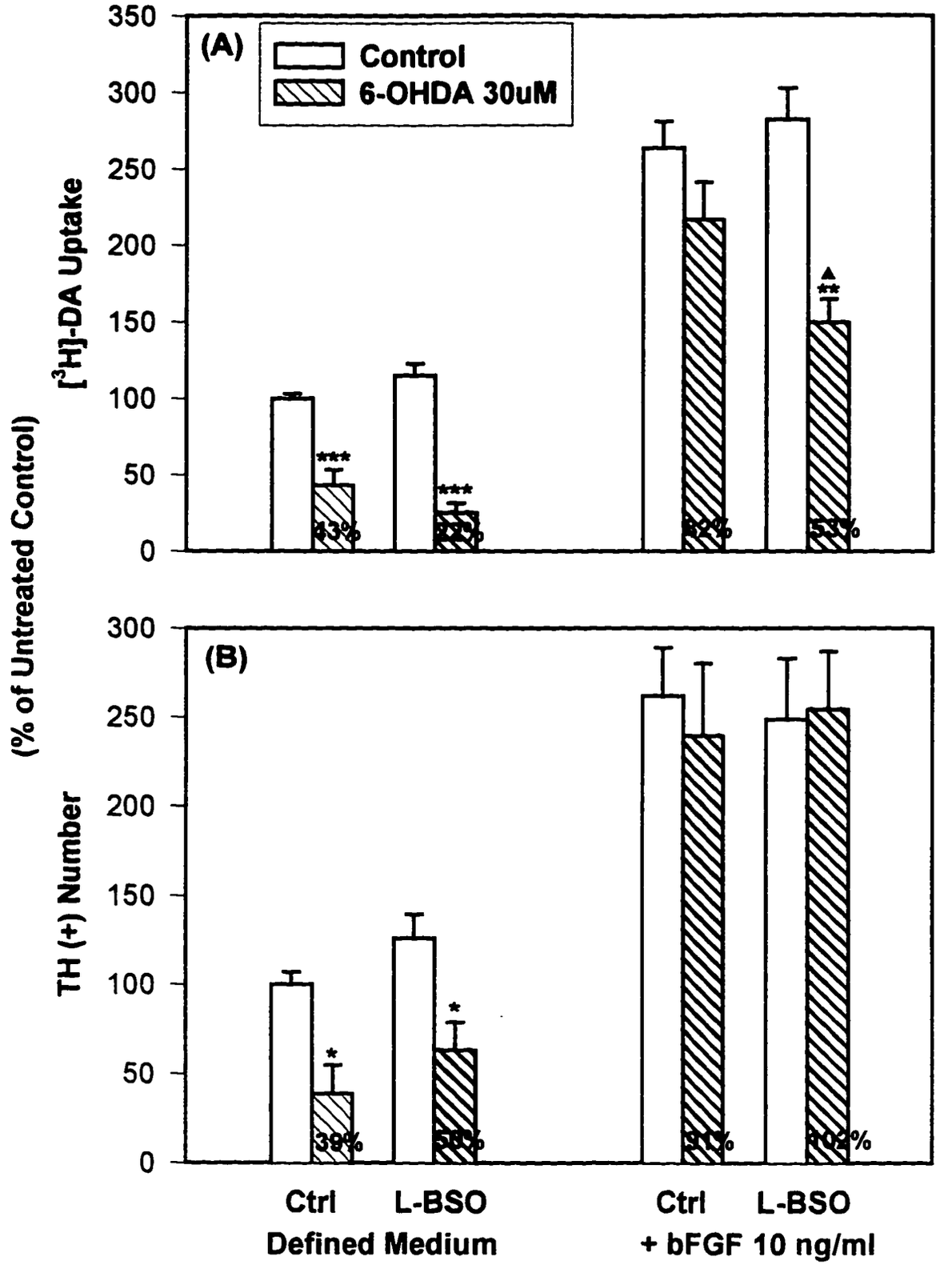


Figure 21

Effect of L-BSO on (A) [^{3}H]DA uptake and (B) TH+ cell number in mesencephalic cultures after the treatment of 6-OHDA. Mesencephalic cultures from embryonic day 14 ± 0.5 rat embryos were maintained in the presence of bFGF 10 ng/ml. L-BSO 10 μM was added on DIV 7. 6-OHDA 30 μM with 200 μM ascorbic acid was added on DIV 8 for 45 minutes. Medium was then removed and refed with fresh medium containing appropriate concentrations of bFGF. DA uptake and TH immunostaining were performed on DIV 9. Data are mean ± SEM values expressed as percentage of the untreated controls (n = 18 in DA uptake and n = 9 in TH staining) based on four experiments. Actual values of untreated controls varied between experiments from 461.2 to 795.8 fmol/10 min per culture in uptake and 157 to 361/cm^2 in TH+ cell numbers. Statistical analysis was by ANOVA followed by Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$, ***$P < 0.001$ compared with the corresponding non-6-OHDA treated group; ▲$p < 0.05$ versus corresponding non-L-BSO treated group.

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