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INTERACTIONS OF GROWTH FACTORS AND NEUROACTIVE AMINO ACIDS IN NORMAL AND ABNORMAL DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

by

ABDESLEM EL IDRISSI

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

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ABDESLEM EL IDRISSI

Advisor: Professor EKKEHART TRENKNER

In the developing nervous system, individual neurons constantly receive inputs from their external environment. The combinatorial effects of these signals determine the structure and function of particular neurons. In this study, we used primary cultures of cerebellar granule cells (CGCs) as a model system for the study of the interactions between growth factors and neuroactive amino acids. The intrinsic properties of this system are very advantageous for such a study, particularly with regard to developmental stages, which take place predominately postnatally.

We found that growth factors and neuroactive amino acids play a very important role in CGC development *in vitro*, through regulation of the survival and function. The survival-promoting effects of these signaling molecules were contingent on their balanced interactions.

Other conclusions that stem from this study is that neurons use alternative signaling pathways to maintain their survival and function depending on their developmental stages, their responsiveness to a particular factor, and the availability of these signaling molecules in the external environment.

CGCs use glutamate as their primary neurotransmitter. The effects of glutamate were concentration-dependent. While low concentrations promoted cell survival, high concentrations induced excitotoxic cell death. Glutamate-induced excitotoxicity was mediated through neuronal energy-depletion and perturbation of calcium homeostasis. Because calcium ions play a pivotal role during development and maintenance of neuronal function, regulation of calcium homeostasis is a crucial event. In this study, we found that one common denominator in the function of bFGF and taurine was the restoration of calcium homeostasis and the regulation of calcium-dependent reactions.

Neuronal energy metabolism is recognized as one of the fundamental determinants of cell survival. All processes central to neuronal function depend on energy. The vulnerability of CGCs to glutamate toxicity was energy-dependent. Under conditions that compromised neuronal energy, glutamate-toxicity was enhanced. Growth factors and taurine increased mitochondrial function and prevented excitotoxicity.

In conclusion, the net cellular effects of glutamate are modulated by the presence of taurine and growth factors. Thus, the cascade of events culminating in a decreased calcium response to glutamate and an increased neuronal energy metabolism, could represent a mechanism by which taurine and growth factors prevent glutamate excitotoxicity.

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ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA	analysis of variance
Ara C	cytosine arabinoside
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
CaBP	calcium-binding proteins
cAMP	cyclic adenosine monophosphate
CGCs	cerebellar granule cells
ChAT	choline-acetyltransferase
CSAD	cysteine-sulfinate decarboxylase
CSF	cerebrospinal fluid
DEPC	diethyl pyrocarbonate
DIV	days in vitro
DNQX	6,7-dinitroquinoxaline-2,3-dione
DTT	dithiothreitol
EGF	epidermal growth factor
EPSP	excitatory post-synaptic potential
FCCP	carbonyl cyanide p-(trifluoromethoxy) phenyl hydrazone
FDA	fluorescein diacetate
GFs	growth factors
IGF	insulin-like growth factor
IP_3	inositol 1,4,5 triphosphate
MEM	minimal essential medium
MK801	dizocilpine maleate
MMLV-RT	moloney murine leukemia virus reverse transcriptase
MTT	3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide
MtECG	mitochondrial electrochemical gradient
NAAs	neuroactive amino acids
NFDM	non-fat dry milk
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
PI	prodidium diiodide
PKA	protein kinase A
PKC	protein kinase C
PNS	peripheral nervous system
SEM	standard error of the mean
SDS-PAGE	sodium dodecyl sulfate-polyacrilamide gel electrophoresis
TNF	tumor necrosis factor

INTRODUCTION:

Throughout development, from early embryonic assembly through the aging process, neurons receive numerous external timed signals that act as cues for particular cellular responses. These signals guide the formation of functional connections between individual neurons, the maintenance of such connections, and often the degradation of these connections. It is well known that the physiological environment *in situ* are composed of a sequence of cues that modulate neuronal survival and function. The combinatorial effects of external signals are integrated to produce a specific cellular response. Here, we have concentrated to elucidate the interaction of factors that are known to play a decisive role in neuronal development, survival and function.

Prominent among these signals are growth factors (GFs) and neuroactive amino acids (NAAs). In addition to their involvement in development and neuronal plasticity, they each have been implicated in the abnormal neurodegeneration associated with central nervous system disorders such as Parkinsonism, Huntington's disease, and Alzheimer's disease. Since it has become more and more evident that in the functioning nervous system neither individual growth factors nor neuroactive amino acids act alone, *we propose to test the hypothesis that the balanced interaction of these molecules in conjunction with the energy status of neurons control and regulate cell survival, function as well as cell vulnerability to environmental signals*.

SPECIFIC AIMS:

The goal of this thesis is to characterize and elucidate the interaction between particular growth factors and neuroactive amino acids that affect the development and function of cerebellar granule cells as well as excitatory and excitotoxic processes (Fig. 1). *We hypothesize that the balance between concentrations of these signaling molecules determines the energy status of neuronal cells. This in turn determines the vulnerability of neurons toward extracellular factors such as excitatory amino acids and growth factors.*

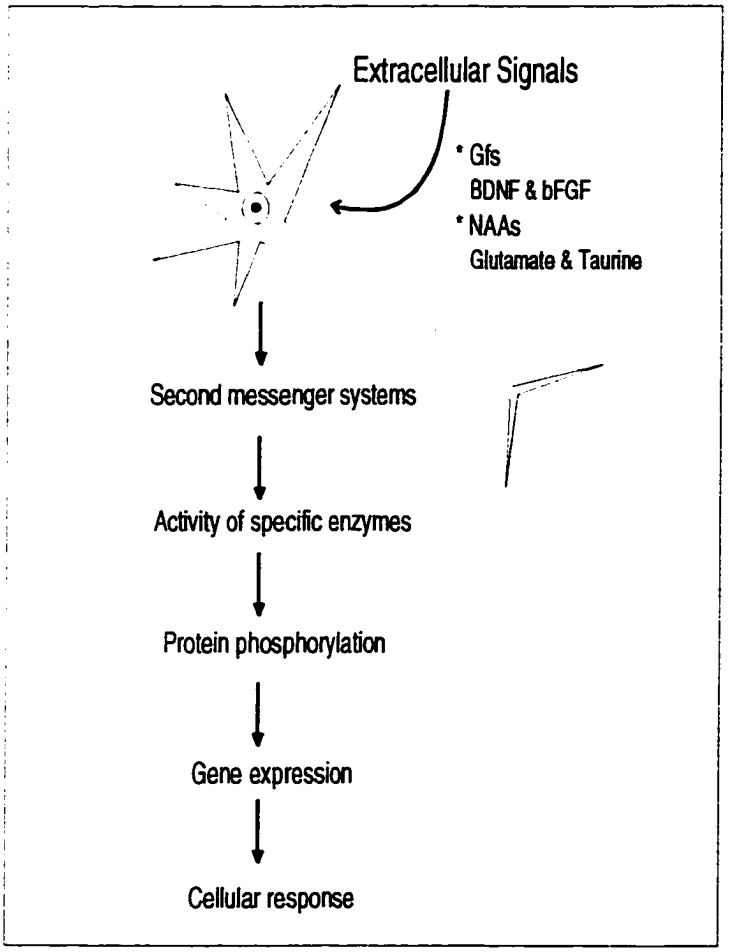


Figure 1. Schematic representation of a neuron interacting with its external environment. Extracellular signals, e.g growth factors (BDNF and bFGF) and NAAs (glutamate and taurine), can induce a variety of intracellular events upon binding to their surface receptors. These include, among others, increased membrane permeability to ions and generation of intracellular second messengers. These in turn activate their dependent enzymes. If these are kinases, they will phosphorylate their specific substrate. These signaling events will be carried on to the nucleus where specific genes are expressed. Finally, the intracellular events induced by different signaling molecules will be integrated to yield a net cellular response that can be expressed by growth, differentiation, metabolic alterations, changes in energy levels, and finally neuronal survival or death.

We propose to test this hypothesis with the following questions:

First, to establish whether or not growth media affect the survival and function of cerebellar granule cells (CGCs), and whether these neuronal cells are capable of responding to more than one neurotrophic factor, and if yes, do these growth factors work in a synergistic or additive way? We have:

✓ Used different culture media and assessed the survival of CGCs.

✓ Titrated different growth factors to establish critical concentrations for cell survival.

✓ Combined different NAAs and GFs and determined the effects of their interactions.

Second, we have asked the question of whether cell survival and cell vulnerability were affected by a complex array of environmental signals. To answer this, we:

✓ Tested the ability of growth factors to rescue neuronal cells from glutamate-induced neurotoxicity.

✓ Exposed the cultures to excitotoxic glutamate concentrations, and evaluated the neuroprotective effects of combinations of growth factors.

✓ Assessed the neuroprotective ability of growth factors in the presence of taurine.

✓ Investigated temporal correlations between decline in energy levels and increase in cell vulnerability during *in vitro* excitotoxic conditions, and how this relationship was affected in the presence of taurine and growth factors together.

Third, what are the signaling mechanisms underlying such cellular responses to different environmental cues? Calcium homeostasis is an important regulatory principal in neuronal development. We have approached this by testing the interaction of compounds known to control the regulation of calcium homeostasis in calcium-

dependent and -independent reactions.

✓ determined how growth conditions differentially modulated the vulnerability of developing cerebellar granule cells to excitatory amino acids *in vitro*.

✓ determined the relationship between the neuroprotective effects of taurine and growth factors and their calcium-modulatory role.

✓ we repeated experiments of others to establish that calcium levels control excitotoxicity.

✓ determined the correlation between added extracellular glutamate concentrations and intracellular calcium accumulation.

Fourth, what are the intracellular events (for example, enzymatic activities, protein phosphorylation, gene expression) that underlie cellular response after the initial ligand binding, receptor-activation, and second messenger-generation? How these transducing mechanisms are affected by the signal-combination of GFs and NAAs? To answer these questions we have:

✓ Analyzed the effects of different treatments on protein kinases activity. We focused on two enzymes: protein kinase C (PKC) and protein kinase A (PKA).

✓ Analyzed the protein-phosphorylation pattern in the presence of growth factors, glutamate and taurine.

✓ Examined the pattern of gene expression under different growth conditions.

BACKGROUND AND SIGNIFICANCE:

Interactions between GFs and NAAs are complex. Abundant evidence suggests however, that cellular development and plasticity, in the brain and other tissues, are influenced

greatly by afferent neuronal activity (Black *et al.*, 1984; Rakic, 1988; O'Leary, 1989). While characterized GFs undoubtedly are involved in these processes, a growing body of data has also implicated NAAs as important trophic agents.

The neuroactive amino acid glutamate is widely distributed throughout the brain, in both local and projection neurons, and is believed to be the principal excitatory neurotransmitter in the central nervous system (Fonnum, 1984). In the adult CNS, glutamate receptors have been implicated in numerous processes in addition to synaptic transmission. These include use-dependent long-term modifications in synaptic efficacy (Collingridge and Singer, 1990) excitation and neurotoxicity (Garthwaite, *et al.*, 1986; Rothman *et al.*, 1987; Choi, 1988; Meldrum and Garthwaite, 1990; Ankarcrona *et al.* 1995). Unlike mature neurons, most immature neurons in culture are insensitive to receptor-mediated glutamate toxicity (Balazs *et al.*, 1988; Murphy *et al.*, 1990). Glutamate appears to exert receptor-mediated trophic effects on various neuronal populations. The trophic actions of glutamate on cells *in vitro* are complex and depend on a number of factors, including cell types, age in culture, and the presence or absence of growth factors (Fig.2). During development, excitatory amino acid receptors are expressed transiently in a number of brain regions (Tremblay *et al.*, 1989; Hafidi and Hillman, 1997; Zhao *et al.*, 1998). Glutamate induced increases in survival of cultured neurons (e.g. hippocampus and cerebellum) as well as an increase in dendritic outgrowth (Mattson *et al.*, 1988b; Balazs and Hack, 1990; Cohen-Cory *et al* ., 1991). Glutamate-induced cell survival appears to be calcium mediated (Balazs and Hack, 1990), whereas promotion of fiber outgrowth is *N*-Methyl-D- Aspartate (NMDA)-mediated and results from activation of protein kinase C (PKC) (Cambray-Deakin and Burgoyne, 1990).

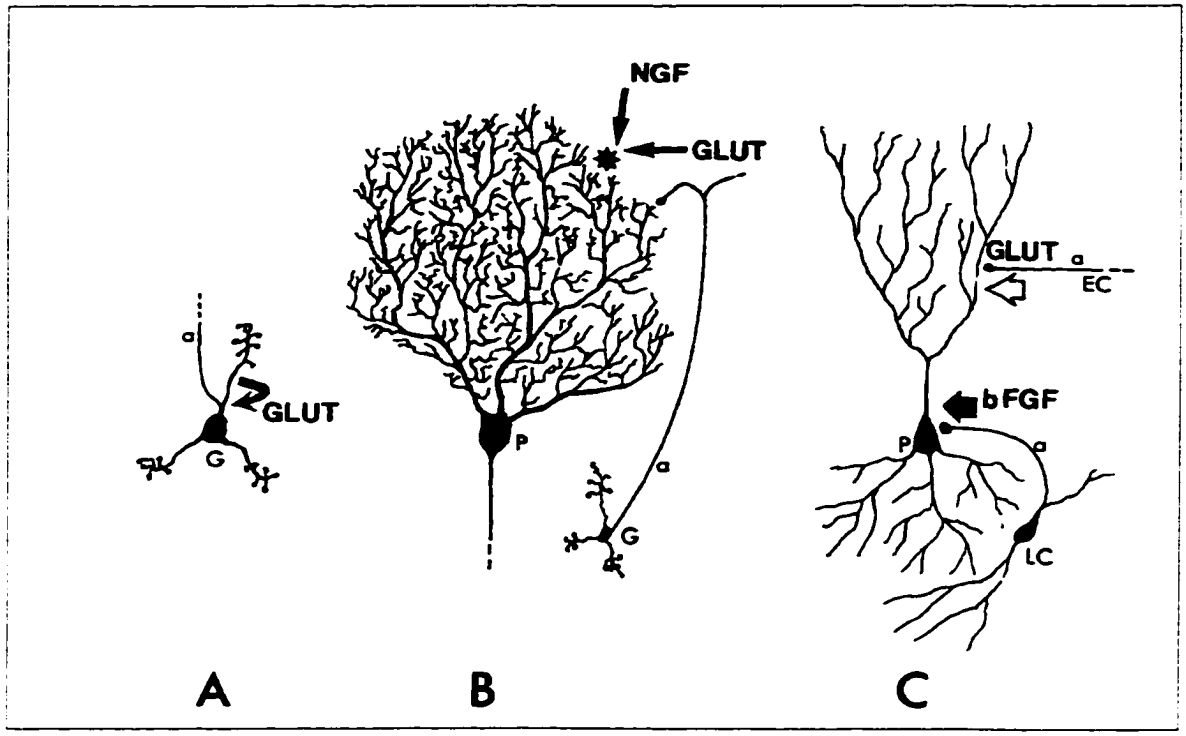


Figure 2. Trophic actions of glutamate on three types of neuronal cells in culture. **A**: Autocrine effects on a cerebellar granule cell (Balazs and Hack, 90; Cohen-Cory *et al.*, 91). Glutamate exerts a positive trophic influence in the absence of GFs. **B**: Paracrine effects of glutamate released from a granule cell on a Purkinje neuron. (Segal, *et al.*,92). The synergy of glutamate and NGF promote neuronal survival and differentiation. Granule neurons are potent regulators of Purkinje cell survival and differentiation. A precise balance between BDNF and glutamate is required for Purkinje cell survival (Morrison and Mason, 1998). **C**: Paracrine effects of glutamate released form the entorhinal cortex terminals on hippocampal pyramidal neurons (Mattson, *et al.*, 1988b). Dendritic pruning effects of glutamate are opposed by bFGF. Abbreviations:(G) cerebellar granule neuron; (P) Purkinje cell in B and Pyramidal neuron in C; (EC) entorhinal cortex; (LC) local circuit neuron; (a) axon.

With increased time in culture, these neuronal populations become increasingly sensitive to the toxic effects of glutamate (Balazs *et al.*, 1988, Brenneman *et al.*, 1990; Schramm *et al.*, 1990). The switch from neurotrophism to neurotoxicity has been suggested to result from an increase in glutamate receptor density (Peterson *et al.*, 1989) and a disturbance of calcium-homeostasis (Kater *et al.*, 1988). Alternatively, it may reflect developmental changes in the binding or signal transduction properties of glutamate receptors (Cambray-Deakin *et al.*, 1990). It is also possible that maturational changes in intracellular calcium buffering activity or in cellular sensitivity to intracellular calcium changes may occur in these cells.

Its is well established that excess extracellular glutamate in the central nervous system can result in over-stimulation of physiological processes that can eventually become neurotoxic. For example, activation of glutamate receptors causes calcium influx through ligand-gated and voltage-dependent channels in the plasma membrane and triggers the mobilization of additional calcium from intracellular stores. Intracellular calcium normally serves physiologically important functions as second messengers regulating neuronal function and plasticity (Lynch, *et at.*, 1983; Kater, *et al.*, 1988). However, excessive and sustained elevation of intracellular calcium results in structural damage to neurons by over-activating proteases and kinases, and by promoting the formation of free radicals (Dykens *et al.*, 1987; Choi, 1988; Siesjo *et al.*, 1988; Mattson, 1992). The cellular and molecular mechanisms that protect neurons against such insults are not completely understood.

It has been established that also growth factors regulate the development, maintenance and survival of neurons (Lindholm *et al.*, 1993a). The potential protective effects of

neurotrophic factors against glutamate neurotoxicity were suggested by their ability to promote cell survival and neurite outgrowth (Fig. 3). However, only recent studies have demonstrated that growth factors can protect neurons against the excitotoxic injuries believed to be involved in some forms of neurodegenerative diseases. Levels of endogenous growth factors are up-regulated in response to excitotoxic insults and free radicals (Pechan, *et al.*, 1992), suggesting a protective function for growth factors. Recent evidence suggests that growth factors are also implicated in the normal functional activity of nerve cells and may play a role in neuronal plasticity (Zafra *et al.*, 1990; Thoenen *et al.*, 1991; Thoenen, 1995; Jarvis *et al.*, 1997; Montcouquiol *et al.*, 1997). This is generally mediated through auto-phosphorylation of cell surface receptors, thereby triggering intracellular signaling cascades that lead to various transcriptional events (Kaplan and Stevens, 1994). The signal transduction cascades, induced by GFs and NAAs are beginning to be elucidated, but how these factors exert their excito-protective effects is not yet fully understood. We have shown in this study that the regulation of intracellular calcium homeostasis and stabilization of mitochondrial function are among the mechanisms by which growth factors protect neurons against glutamate excitotoxicity.

As outlined in figure 3, the ability of growth factors to promote survival of neurons of the peripheral and central nervous system during development and after neuronal damage can happen at various levels. Therefore, this has stimulated the interest in these molecules to better understand development and elucidate their potential therapeutic functions for the treatment of neurodegenerative diseases and nervous system injuries.

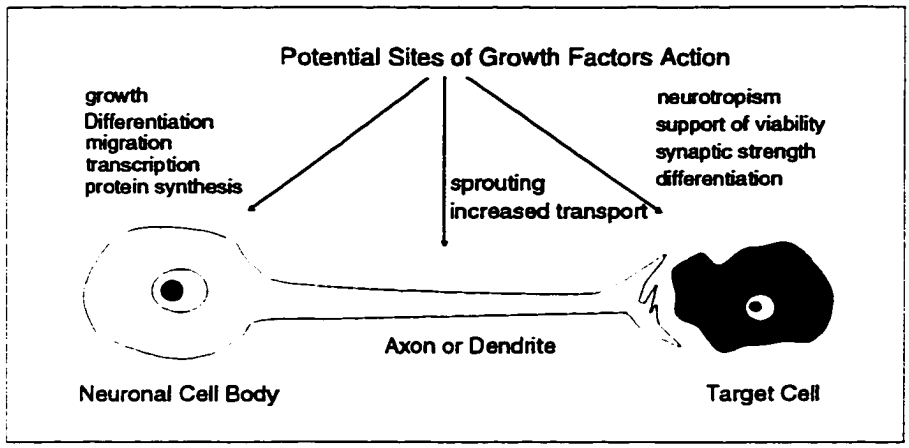


Figure 3. Neurotrophic factors: mediators of growth, survival, and plasticity. A simplified diagram of the multiple sites of action of the neurotrophins on an idealized neuron with an adjacent cell. The adjacent cell, shown here as a target cell, may be an other neuron either projecting to or from the affected neuron, a non-neuronal target cell, or just a surrounding cell mediating a paracrine trophic effect. Adapted from Russell (1995).

Several members of the nerve growth factor (NGF) family, collectively known as neurotrophins, have been cloned. NGF, the best characterized neurotrophic factor, has long served as a prototypical model with which other neurotrophic substances were compared. They have to satisfy certain criteria that were originally set for NGF to be classified as neurotrophic factors. Recently, however, the list of substances that are classified as potential neurotrophic factors has expanded considerably. NGF is a polypeptide growth factor that provides trophic support for sensory and sympathetic neurons of the peripheral nervous system (PNS) (Bottenstein, *et al.*, 1980; Thoenen, 1980; Hamburger, 1993, for review). In the CNS, NGF and NGF mRNA are expressed at high concentration in the hippocampus, where NGF exerts a trophic effect on cholinergic neurons that project from the basal forebrain (Korsching, *et al.*, 1985; Johnston, *et al.*, 1987). Administration of NGF into rat brain results in an increase in the choline acetyltransferase (ChAT) activity levels in the hippocampus,

septum, and striatum (Hefti, *et al.*, 1984; Mobley, *et al.*, 1985). In the developing cerebellum, functional excitatory synaptic connections between granule and Purkinje cells, coincide with the expression of NGF and its receptors (Cohen-Cory *et al.*, 1993), suggesting that NGF plays a role in the maturation of the cerebellum. Other peptide growth factors share some of the characteristics of NGF. Basic fibroblast growth factor (bFGF) is produced at high levels in the developing rat brain and promotes survival of axotomized cholinergic neurons of the basal forebrain after fimbria-fornix transection (Anderson, *et al.*, 1988). bFGF promotes the survival of severed optic axons (Sievers, *et al.*, 1987) and provides trophic support for primary cultures of CNS neurons (Matsuda *et al.*, 1990; Fernandez-Sanchez and Novelli, 1993; Nakao *et al.*, 1996). bFGF has also been shown to promote the survival of cultured cerebral cortical (Morrison, *et al.*, 1986), cerebellar (Hatten, *et al.*, 1988; Trenkner, *et al.*, 1996; El Idrissi *et al.*, 1998) and hippocampal (Walicke, *et al.*, 1986) neurons. bFGF binds with low affinity to a heparin sulfate proteoglycan FGF receptor which then facilitates binding of bFGF monomers to the high affinity bFGF receptor. This interaction leads to receptor autophosphorylation and signal transduction. Epidermal growth factor (EGF) elicits many of the responses common to NGF and bFGF in pheochromocytoma (PC12) cells, including protein phosphorylation, activating second messenger systems and specific enzymes, and inducing immediate-early genes (Greenberg, *et al.*, 1985; Heasley and Johnson, 1989; Tsao *et al.* 1990; Chao, 1992). In PC12 cells, however, EGF is not a differentiating factor but rather a mitogenic factor (Greene and Tischler, 1976; Huff, *et al.*, 1981), suggesting selective functions for growth factors on particular cell types. EGF can protect primary hippocampal cultures from glutamate toxicity (Pauwels, *et al.*, 1989), and cortical cultures from anoxia

(Kinoshita, *et al.*, 1990). Brain-derived neurotrophic factor (BDNF) is predominantly expressed in neurons of the CNS. The highest BDNF mRNA levels were found in the hippocampus and cortex (Hoffer, *et al.*, 1990; Ballarin, *et al.*, 1991; Ernfors, *et al.*, 1991; Isackson, *et al.*, 1991; Phillips, *et al.*, 1990). Similar to NGF, BDNF supported the survival of cholinergic neurons in culture (Alderson, *et al.*, 1990; Knusel, *et al.*, 1991). Moreover, BDNF, in contrast to NGF, also supports the survival and differentiation of mesencephalic dopaminergic neurons in culture (Hyman, *et al.*, 1991). BDNF is highly expressed also in cerebellar granule cells (Wetmore, *et al.*, 1990; Rocamora, *et al.*, 1993; Lindholm *et al.*, 1993b; Leingartner *et al.*, 1994; Gao *et al.*, 1995; Zirrgiebel *et al.*, 1995; Neveu and Arenas, 1996; Baxter *et al.*, 1997), and both granule and Purkinje cells express the BDNF receptor TrkB (Klein *et al.*, 1990; Alvarez-Dolado, *et al.*, 1994; Gao, *et al.*, 1995; Segal *et al.*, 1995; Morrison and Mason, 1998). BDNF protein is present in the adult cerebellum, particularly in the Purkinje cells (Kawamoto, *et al.*, 1996; Morrison and Mason, 1998). Tissue culture studies indicated that exogenous BDNF acted directly on granule cells to promote survival and axonal elongation (Lindholm *et al.*, 1993; Goa *et al.*, 1995; Segal *et al.*, 1995), and exogenous neurotrophins promoted dendritic arborization of Purkinje cells (Cohen-Cory *et al.*, 1991; Larkfors *et al.*, 1996; Morrison and Mason, 1998). Thus BDNF is directly involved in cerebellar morphogenesis.

Insulin-like growth factors (IGF-I and IGF-II) protected cultured rat hippocampal and septal neurons against glucose deprivation-induced damage (Cheng and Mattson, 1992). Relatively high levels of IGF-I were found both in the developing and adult cerebellum (Calissano *et al.*, 1993; Folli *et al.*, 1994; D'Costa *et al.*, 1995; Fernandez-Sanchez *et al.*,

1996). IGF-I was described as neuromodulator on glutamatergic transmission in the cerebellum (Castro-Alamancos and Torres-Aleman, 1993). Taken together, these data are consistent with roles for growth factors in the CNS development. On the other hand, growth factors may also be important in adult plasticity as well as in aging and disease. Furthermore, data suggest that growth factors are involved in adaptive mechanisms, such as sprouting that occur in response to alterations in activity or injury to the adult CNS (Crutcher, 1987). Roles for growth factors in age-related neuronal losses and in specific neurodegenerative disorders, including Alzheimer's disease, Parkinsonism, and amyotrophic lateral sclerosis, have been suggested (Appel, 1986; Korsching, 1986). Thus, growth factors may be involved not only in promoting cell survival but also cell death when their concentrations are out of balance, or when they act in combination with other molecules such as neuroactive amino acids.

Neuroactive amino acids (NAAs) constitute another class of signaling molecules which are important during neuronal development as well as neurodegeneration. NAAs have developmental roles as regulators of cell survival, neurite outgrowth and synaptogenesis. For example, glutamate, the principal excitatory amino acid transmitter in the brain, exerts important trophic effects on cerebellar granule cells through activation of the NMDA subtype of glutamate receptors (Pearce *et al.*, 1987; Balazs *et al.*, 1988; Trenkner, 1990; Burgoyne *et al.*, 1993). The effects of glutamate are concentration-dependent, since at very low concentrations, glutamate selectively inhibited dendritic outgrowth in isolated hippocampal pyramidal neurons (Mattson, *et al.*, 1988b), or can act in the opposite way, *in vitro* by promoting the formation of synapses with target hippocampal pyramidal neurons (Mattson *et al.*, 1988a). Blockade of NMDA receptors in the vertebrate visual system disrupts synapse

formation in the tectum (Cline *et al.*, 1987) and blocks neurite outgrowth *in vitro* (Baird *et al.*, 1992, 1996). In addition, there is very strong evidence that glutamate mediates the synaptic plasticity associated with long-term potentiation (LTP) in the hippocampus (Chang 1983; Desmond 1983; Collingridge and Singer, 1990). Finally, excessive glutamate utilization may result in neurodegeneration. Indeed, there is now convincing data showing that excess activation of glutamate receptor-channel complexes, particularly the NMDA receptor type, results in the death of neurons (Butcher *et al.*, 1987; Gill *et al.*, 1987; Choi *et al.*, 1988; Foster *et al.*, 1988; Trenkner, 1990). These effects may be common denominator of many types of brain damage (Garthwaite *et al.*, 1986; Dykens *et al.*, 1987; Choi, 1988; Hahn *et al.*, 1988; Anegawa *et al.*, 1995). At least two sequential mechanisms are probably involved in glutamate-induced neurotoxicity. The first one is an osmotic lysis of the cells as a result of massive ion influx through activation of NMDA and/or non-NMDA receptors (Rothman *et al.*, 1985; Choi, 1987). The second process, a relatively slow one, involves the increase in the intracellular calcium concentration mainly resulting from the opening of NMDA channels following glutamate-induced depolarization (Choi, 1987). A third mechanism has been shown to involve the formation of free radicals (Dykens *et al.*, 1987; Choi, 1988; Dykens, 1994; Yang *et al.*, 1996; Nicotera *et al.* 1997), formed in excess of extracellular glutamate. Glutamate has also been shown to play a role in many neurological disorders ranging from acute insults such as stroke, hypoglycemia, trauma, and epilepsy to chronic neurodegenerative states such as Huntington's, the acquired immune-deficiency syndrome (AIDS) dementia complex, amyotrophic lateral sclerosis, and perhaps Alzheimer's diseases (Coyle, 1976; Maragos *et al.*, 1987; Mattson, 1988a; Greenamyre and Young, 1989; Meldrum and

Garthwaite, 1990; Lipton, 1992).

Another important neuroactive amino acid is taurine, 2-aminoethansulfonic acid. Taurine has been shown to play an essential role during development in a number of systems, including the visual system of the cat (Sturman *et al.*, 1981; Sturman *et al.*, 1984), the reproductive system (Imaki *et al.*, 1986; Sturman *et al.*, 1985; Sturman *et al.*, 1987; Sturman and Messing, 1991), the immune system of cat and mouse (Schuller-Levis and Sturman, 1990), the cardiovascular system (Pion *et al.*, 1987; Pion *et al.*, 1990) and the central nervous system of rodents (Huxtable *et al.*, 1989; Trenkner, 1990; for reviews see, Huxtable, 1992; Sturman, 1993). The nutritional requirements for taurine in animals and humans are met partly by dietary sources and partly by biosynthesis. The biosynthetic capacity is developmentally regulated. The activity of cysteine-sulfinate decarboxylase (CSAD), the rate-limiting enzyme for taurine biosynthesis, is almost negligible in fetuses and newborns but increases with age to reach the adult levels. The CSAD activity can be induced *in vitro* with excitotoxic concentrations of glutamate, suggesting that taurine synthesis is regulated upon requirement (Trenkner *et al.*, 1992). In contrast, the tissue concentrations of taurine, which are very high in some tissue during development, including the cerebellum, decrease with age to the adult levels of about one-third of the concentrations found in neonates (Sturman and Gaull, 1985). Taurine is most abundant in tissues that are excitable, and its concentration in the mammalian brain is second to that of glutamate (Sturman, 1993; Huxtable, 1992). During development, taurine was described as membrane stabilizer, antioxidant, and osmoregulator (Sturman, 1993; Huxtable, 1992, for reviews). Taurine has been shown to prevent kainate and quinolinate excitotoxicity *in vitro* (Trenkner and Dykens 1986; Trenkner, 1990). Taurine also

has been found to modulate neuroactivity by inhibiting the phosphorylation of specific proteins regulated by the signal transduction system in the brain (Li and Lombardini, 1991). This study shows that taurine can reduce the level of phosphorylation induced by glutamate and growth factors. Existing evidence (Lehman *et al.*, 1988; Trenkner, 1990; Trenkner *et al.*, 1998; El Idrissi *et al.*, 1998) points to a modulating role of taurine in the regulation of the ionic/sequestered Ca^{2+} homeostasis in neuronal cells. Consistent with this, we have found that taurine reduces $^{45}Ca^{2+}$ accumulation in cerebellar cultures after exposure to glutamate (Trenkner *et al.*, 1996; El Idrissi *et al.*, 1997, 1998).

Thus it appears that levels of input from different NAAs and GFs can determine whether a given neuron will survive and function or die. Therefore, it is important to understand how different environmental signals interact to yield a net effect on neuronal function and on neuroarchitecture. Our findings have shown that neuroactive amino acids (glutamate and taurine) and growth factors (BDNF and bFGF) interact to regulate cell survival and cell vulnerability. Furthermore, we have shown that these interactions were dependent on mitochondrial energy. When these signaling molecules are out of balance, they promote cell death rather than cell survival.

GROWTH FACTORS AND THEIR RECEPTORS:

Neurons employ numerous routes for incorporating growth factors (Fig. 4). Many polypeptide growth factors mediate their biological responses by binding to and activating cell surface receptors with intrinsic tyrosine kinase activity. Upon ligand binding, these receptors become autophosphorylated on multiple tyrosine residues and subsequently induce signal

transduction cascades (Rowland *et al.*, 1987; Rowland-Gagne and Greene, 1990; Tsao *et al.*, 1990; Ullrich and Schlessinger, 1990; Volonté and Greene, 1992; Ip *et al.*, 1993; Pawlowska *et al.*, 1993; Cunningham *et al.*, 1997).

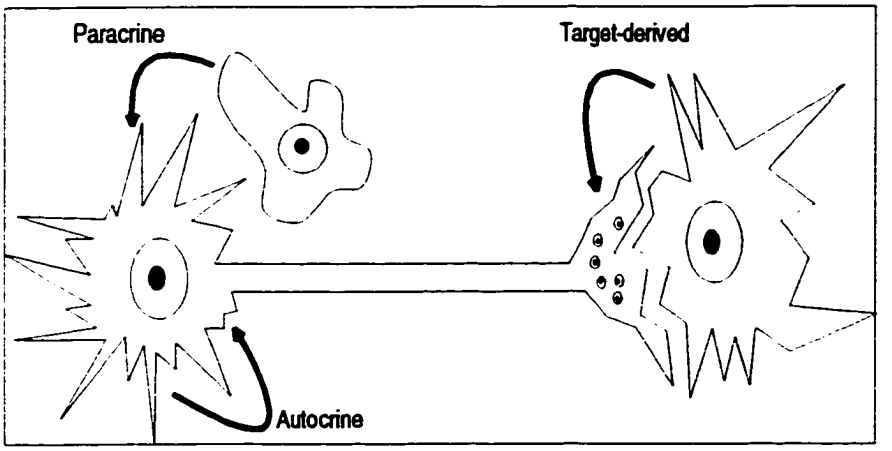


Figure 4. Modes of action of neurotrophic factors. *Paracrine* effects seem to be most common for neurotrophic factors under physiological conditions. These are local effects mediated by soluble or extracellular matrix-associated factors. *Juxtacrine* stimulation is mediated by membrane-anchored growth factors and receptors on adjacent cells. *Endocrine* effects are actions at a distance or systemic effects of soluble factors. Relatively few neurotrophic factor effects have been documented to be produced by this mechanism under physiological conditions. *Autocrine* refers to the production of a neurotrophic factor by its target cell population and acting on the target cell itself.

Most of our knowledge of neurotrophic factors comes from the pioneering work on NGF, which belongs to a family of growth factors collectively known as neurotrophins. These include, BDNF (Barde *et al.*, 1982; Leibrock *et al.*, 1989), neurotrophin-3 (NT-3; Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990) and neurotrophin-4 (NT-4; Ip *et al.*, 1992) also named neurotrophin-5 (Berkemeier *et al.*, 1991). The neurotrophins show approximately 50 % amino acid sequence homologies and display both overlapping and specific sets of neurotrophic activities on peripheral and central neurons. A detailed understanding of the

receptors that are involved in eliciting signal transduction by neurotrophins has begun to emerge. NGF has been known to bind to a transmembrane glycoprotein of approximately 75kDa with a K_d of 10^{-9}M (Johnson *et al.*, 1986; Radeke *et al.*, 1987) known as the low-affinity NGF receptor or $p75^{LNGFR}$. This receptor is a member of the tumor necrosis factor (TNF) cytokine receptor superfamily. Activation of this receptor leads to cerimide production, NFκB, and c-Jun N-terminal Kinase (JNK) activation (Greene and Kaplan, 1995; Bothwell, 1996; Casaccia-Bonnefil *et al*, 1996; Park *et al.*, 1996). Both BDNF (Rodriguez-Tebar *et al.*, 1990) and NT-3 (Squinto *et al.*, 1991) have the capacity to compete with NGF for binding to this receptor. However, in order to mediate a biological response, high-affinity binding ($K_d = 10^{-11}$M) is required (Richardson *et al.*, 1986). TrkA, a membrane-spanning receptor tyrosine kinase, the protein product of the *trk* proto-oncogene, has been identified as a functional high-affinity receptor for NGF (Kaplan *et al.*, 1991; Klein *et al.*, 1991; Meakin and Shooter, 1991; Nebreda *et al.*, 1991). A related molecule, TrkB, has been found to be a functional receptor for BDNF, NT-4/5, and to a lesser extend, NT-3 (Soppet *et al.*, 1991; Squinto *et al.*, 1991). A third family member, TrkC, functions as the preferred high-affinity receptor for NT-3 (Lamballe *et al.*, 1991) (Fig. 5). One model of the molecular components constituting high-affinity (and thus signal-transducing) binding sites for NGF suggests the involvement of both TrkA and $p75^{LNGFR}$ (Hemstead *et al.*, 1991; Kaplan *et al.*, 1991; Ip *et al.*, 1993). Alternatively, there is considerable evidence *in vitro* suggesting that the Trk proteins alone constitute high-affinity neurotrophin receptors (Klein *et al.*, 1991; Squinto *et al.*, 1991).

The control of cell survival by neurotrophins is mediated by the selective interplay of both Trk and $p75^{LNGFR}$. Several studies have shown a role for $p75^{LNGFR}$ as a positive or

negative inducer of cell viability depending on the levels of the two receptors during different stages of neuronal development. This functional antagonism between TrkA and $p75^{LNGFR}$ have been proposed as a model for active neuronal selection during naturally occurring neuronal death (Volenté *et al.*, 1993; Miller, 1998; Yoon *et al.*, 1998).

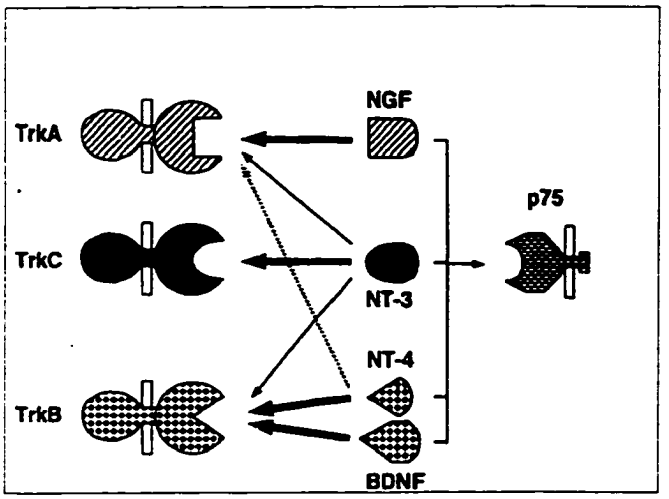


Figure 5. Relationship between neurotrophins and their receptors. Interactions between ligands and their preferred Trk receptors are indicated by thick arrows. Interactions with the p75 low affinity NGF receptor are grouped by a bracketed arrow. From Klein, (1994).

The binding of neurotrophins to members of the Trk receptor family initiates a signaling cascade involving the regulation of several different protein kinases (Rowland *et al.*, 1987; Rowland-Gagne and Greene, 1990; Tsao *et al.*, 1990; Volonté and Greene, 1992; Pawlowska *et al.*, 1993) and rapid phosphorylation of intracellular proteins on tyrosine residues (Kaplan *et al.*, 1991). These proteins include phospholipase -γ1 (PLC-γ1), the 85 KDa/PI-3 kinase subunit, Nck, SHC, Erk 1 and 2, and SNT (Boulton *et al.*, 1991; Kim *et al.*, 1991; Miyasaka *et al.*, 1991; Knusel *et al.*, 1992; Raffioni and Bradshaw, 1992; Rabin *et al.*, 1993). In this study, we have examined the tyrosine phosphorylation pattern induced by growth factors and how glutamate and taurine affect signaling mechanisms initiated by growth

factors. We found that taurine is an important regulator of growth factors- and glutamate-induced protein phos-phorylation and therefore might play an important role in signal transduction mechanisms mediated by growth factors and glutamate.

TYPES OF EXCITATORY AMINO ACID RECEPTORS:

Excitatory amino acids (glutamate and aspartate) are neurotransmitters in the CNS. Five subtypes of glutamate receptors have been recognized on the basis of their molecular cloning, electrophysiological and pharmacological properties. At least 20 separate genes encode the subunits of these receptors, and many combinations of subunits and alternative splice products occur (Nakanishi, 1992). There are two main divisions of glutamate receptors: ionotropic and metabotropic. The ionotropic receptors are coupled directly to membrane ion channels, the metabotropic receptors are coupled to G proteins and modulate intracellular second messengers such as inositol triphosphate, calcium and cyclic nucleotides. Three major types of ionotropic receptors have been identified based on their selective agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), and kainate. These selective receptor agonists resemble glutamate or aspartate but do not occur naturally. The NMDA receptor is widely distributed in mammalian CNS, especially enriched in hippocampus, cerebellum and cerebral cortex (Daggett *et al*, 1998), and plays an important role in synaptic plasticity as well as in excitatory transmission and excitotoxic reactions. AMPA receptors are widespread in the CNS. They parallel the distribution of NMDA receptors and are involved in the generation of fast component of excitatory post-synaptic potentials (EPSPs) in many central excitatory pathways. Kainate receptors, concentrated in

a few specific areas of CNS, are complementary to the distribution of NMDA/AMPA receptors. In addition to the NMDA, kainate and AMPA types of central excitatory amino acid receptor, two further types have been recognized. These, however, are not directly linked to ion channels. One is the receptor activated by the δ-phosphono analogue of glutamate (L-AP4). These receptors are likely to be located presynaptically and possibly function as autoreceptors, controlling the release of transmitter glutamate. The other receptor is activated by quisqualate, ibotenate and *trans*-ACPD and termed "metabotropic receptor". The activation of this receptor produces a delayed second messenger response mediated by receptor interaction with GTP binding proteins, leading to the generation of inositol 1,4,5 triphosphate (IP_3) and diacylglycerol, the mobilization of intracellular Ca^{2+}, and the activation of protein kinase C (Sladeczek *et al.*, 1985). Among these receptors, the best defined is the NMDA receptor. The NMDA receptor is an ion channel formed by distinct subunits. The obligatory subunit, termed NR1, contains the binding site for ligands (Moriyoshi *et al.*, 1991). The NR2 regulatory subunit consists of four homologous isoforms (NR2A-D) (Kutsuwada *et al.*, 1992; Monyer *et al.*, 1992). When co-expressed with NR1, each of the NR2 subunits can form an ion channel (Meguro *et al.*, 1992; Ishii *et al.*, 1993) characterized by high calcium conductances (MacDermott *et al.*, 1986; Nicoll *et al.*, 1988; Nakanishi, 1992). This receptor can be distinguished from other glutamate receptors by the action of several selective agonists and antagonists and also by its unique properties, including modulation by glycine, a voltage-dependent channel block by Mg^{2+}, polyamine activation, and Zn^{2+} inhibition (Johnson and Asher, 1987; Watkins *et al.*, 1990; Nakanishi, 1992). In this study, we have examined the pharmacological characteristics of the different type of glutamate receptors expressed in our

culture system, and how the functional properties of these receptors are affected in the presence of taurine and growth factors.

Stimulation of any of the ionotropic glutamate receptors results in membrane depolarization because of the influx of positively charged ions, and thus indirectly activates voltage-gated calcium channels. The influx of extracellular Ca^{2+}, together with any Ca^{2+} release triggered from intracellular stores, would elevate cytosolic free Ca^{2+}. Such an elevation, if sustained, would likely be cytotoxic because of the over-stimulation of normal processes, thus damaging neurons. Ca^{2+} can activate a series of enzymes, including protein kinases, proteases, protein phosphatases, and nitric oxide synthase (Choi, 1988; Dawson and Snyder, 1994; Huang *et al.*, 1994; Nichol *et al.*, 1995; Strijbos *et al.*, 1996; Wei and Quast, 1998). Elevated cytosolic Ca^{2+} also activates phospholipases, capable of breaking down the cell membrane and liberating arachidonic acid. Metabolism of arachidonic acid by oxidases leads to the production of oxygen free radicals, that can trigger peroxidative degradation of lipid membranes and other destructive events. Superoxide radicals may also be generated by the enzymatic action of xanthine oxidase, generated from xanthine dehydrogenase by the action of a Ca^{2+}-activated protease (Dykens *et al.*, 1987; Dykens, 1994).

In cerebellar granule cell cultures, the expression of different glutamate receptor subtypes is regulated by growth conditions, electrical activity and growth factors. We have shown that the presence of horse serum in culture medium reduced the sensitivity to NMDA in cultured cerebellar granule cells (Trenkner *et al.*, 1996; El Idrissi *et al.*, 1998).

Growth factors have been shown to modify neuronal response to glutamate. Pretreatment of cultured embryonic hippocampal neurons with bFGF counteracted the

dendrite outgrowth-inhibiting action of subtoxic levels of glutamate by affecting the expression of glutamate receptors (Mattson *et al.*, 1993; Brandoli *et al.*, 1998). In addition, electrical activity itself may modulate sensitivity to glutamate. K^+-induced depolarization or NMDA treatment promoted the survival of cerebellar granule cells and led to an increase in functional NMDA receptor (Gallo *et al.*, 1987; Balazs *et al.*, 1988, 1992). This was shown by an increase in NMDA-induced ion conductance in voltage clamped granule cells (Van der Valk *et al.*, 1991), and by a progressive rise during development in the NMDA-induced $^{45}Ca^{2+}$ influx (Balazs *et al.*, 1991). These effects seem to be mediated though Ca^{2+} influx and are thought to mimic the influence of innervation received from the mossy fibers of immature post-migratory granule cells during cerebellar development (Gallo *et al.*, 1987; Balazs *et al.*, 1988). In addition to the effect of high $K^+$and NMDA on the survival of granule neurons, these treatments exert an influence on the biochemical maturation of the cells, as indicated by the promotion of the developmental increase of stimulus-coupled glutamate release and glutaminase activity (Levi *et al.* 1989; Moran and Patel, 1989). Furthermore, NMDA added to granule cells after prolonged depolarization is toxic and causes granule cell death probably through excess stimulation of NMDA receptors (Cox *et al.*, 1990). Here, we have analyzed the functional properties of the different glutamate receptor subtypes and how they are affected by different growth conditions. Consistent with previous findings, we found that among the glutamate receptor subtypes, the NMDA were the most permeable to calcium, and this property was regulated by growth factors and taurine.

CALCIUM AND CELL SURVIVAL:

A large number of neurotrophic factors with the potential to protect against excitotoxicity and related insults have been identified. An even larger number of receptors, including splice variants, are known to mediate the effects of these neurotrophic factors. Despite this variety, there appears to be significant degeneracy in the second messengers used by activated neurotrophic factor receptors (Saltiel and Decker, 1994; Russell, 1995). Interestingly, similar second messenger systems are activated by excitotoxins and are thought to be important in mediating neuronal death in response to these agents.

A variety of effects are mediated by the auto-phosphorylation of tyrosine kinases intrinsic to neurotrophic factors. Although the details of each pathway are only beginning to be defined, it is also apparent that activation of these second messenger pathways ultimately leads via phosphorylation of transcription factors to induction of similar sets of immediate-early genes (Ghosh *et al.*, 1994; Russell, 1995). In addition, of course, neurtrophic factors also induce protective mechanisms that are factor- and neuronal-phenotype specific. Again, many of these same mechanisms are also induced in neurons by excitotoxins. The presence of common second messenger systems among different extracellular signals suggest that neurons have developed alternative pathways that can be a activated by different factors at one time or an other to regulate cellular functions and promote cell survival. Furthermore, when several factors are present in the extracellular environment, which likely represent the *in vivo* situation, these signals are integrated to produce a specific cellular response.

The most commonly regulated second messenger among the factors we examined is calcium. As these experiments show, calcium represents a common denominator between

growth factors and neuroactive amino acids. Therefore, we have focused on the intraneuronal regulation of calcium homeostasis and the downstream reactions that are triggered by calcium.

Calcium serves an important intermediary role in the two main recognized patterns of neuronal death: necrosis and apoptosis (Buja *et al.*, 1993). Necrosis follows irreversible neuronal injury and is typically characterized by cellular and mitochondrial swelling, nuclear pyknosis, and karyolysis. DNA cleavage is random, although fragmentation can occur (Bicknell and Cohen, 1995). Neuronal necrosis commonly follows a variety of brain insult such as ischemia, trauma and hypoglycemia. Experimentally induced neuronal death from ischemia or hypoglycemia can be dramatically reduced by removing extracellular calcium, by adding intracellular calcium chelators or buffers, or by antagonizing excess calcium entry through ion channels, especially the NMDA subtype of glutamate receptor (Dubinsky, 1993). Calcium overload causes necrosis by damaging mitochondria and activating proteases and phospholipases that disrupt membrane integrity and cause irreversible cell injury (Nicotera, 1992). The second main pattern of neuronal death, apoptosis, results from a genetically controlled suicide program that produces cellular and nuclear shrinkage, nuclear envelope loss, and endonuclease-mediated DNA cleavage (William and Smith, 1993). Calcium activates endonucleases and topoisomerases that irreversibly fragment cellular DNA in many examples of apoptotic death (McCabe *et al.*, 1992). Although apoptosis is important during nervous system development, it may also contribute to certain neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Eide *et al.*, 1993).

The dynamics of calcium regulation in central neurons is complex (Miller, 1991;

Hoyer, 1993; Simpsom, *et al.*, 1995). The level of calcium can rise in the neuronal cytoplasm through a variety of mechanisms, but nerve cells can regulate extensive calcium entry and can dispose of large calcium loads. Extracellular calcium can enter neurons through voltage-gated calcium channels, transmitter-gated channels permeable to calcium, or through ion exchangers. Regardless of the mechanism by which calcium enters the cells and to which level it transiently rises, under physiological conditions intraneuronal calcium homeostasis is responsible for maintaining calcium concentrations at resting levels consistent with cell survival. If the calcium challenge is within the physiological range, the excess calcium will be eventually removed from cells (Mattson, *et al.*, 1989).

In certain situations, nerve cells face calcium concentrations outside the physiological range. Regardless of the route through which calcium rises inside a neuron, an excessive elevation of intracellular calcium levels is usually an antecedent of neuronal death (Garthwaite *et al.*, 1986; Choi, 1988; Garthwaite and Garthwaite, 1989; Beal, 1992; Brorson *et al.*, 1994; Tymianski, *et al.*, 1994; Budd, Nicholls, 1996). Excitotoxicity, the term introduced to describe the "excitation to death" of neurons (Olney, *et al.*, 1971), mainly results from increases in intracellular calcium subsequent to the over-excitation of neurons, most commonly though excessive stimulation of ionotropic excitatory amino acid receptors.

Since calcium channel blockers and antagonists at both NMDA and non-NMDA excitatory amino acid receptors appear to protect neurons effectively against excitotoxicity (Choi, 1988; Weiss *et al.*, 1990; Bleakman *et al.*, 1991; Beal, 1992), it is clear that the first and foremost step necessary for excitotoxic cell damage is the calcium entry (Garthwaite *et al.*, 1986; Choi, 1988, 1990; Tymianski *et al.*, 1994). Consistent with this, we found that

increases in intracellular calcium are responsible for the excitotoxic reactions associated with excessive activation of the glutamate receptors. Taurine and bFGF attenuated glutamate-induced excitotoxicity through calcium modulation.

ROLE OF ENERGY METABOLISM IN NEURONAL CELL FUNCTIONS AND EXCITOTOXICITY:

The functional and structural integrity of neurons is guaranteed by an adequate formation and turnover rate of ATP, which in neuronal tissues, derives from glucose only (Hoyer, 1993; Leino *et al.*, 1997). All processes central to neuronal function, such as exocytosis, protein phosphorylation and maintenance of membrane potential, directly or indirectly require ATP. During normal synaptic neuro-transmission, most of the ATP generated in brain tissues is used to restore the ionic gradients that have been run down. To meet these demands, mitochondria and glycolytic enzymes are concentrated in dendritic and axonal terminals (Wong-Riely, 1989; Leino *et al.*, 1997). With sustained glutamate depolarization, the demand for ATP becomes even greater. The restoration of the resting potential after excitatory events, which are associated with increased levels of Na^{+} and Ca^{2+}, requires ATP as energy source to pump out these ions against their electrochemical gradients. Moreover, it is believed that the enhancement of excitotoxicity by impaired metabolism may be a ubiquitous mechanism of neuronal death in neurological diseases (Greene and Greenamyre, 1996; Beal, 1993; Hoyer, 1993). In this study, we provide evidence that growth conditions (with or without serum and astrocytes) and external stimuli (GFs and NAAs) significantly alter neuronal energy states. Furthermore, we show that compromised neuronal

energy metabolism enhances the deleterious effects of glutamate excitotoxicity.

CHOOSING THE SYSTEM:

Prior to testing our hypothesis, we needed to establish a system in which interactive mechanisms between GFs and NAAs could be studied. Such system requires that the cells respond to NAAs and to GFs. We chose the cerebellum as the system in which to investigate the role of GFs and NAAs in the development of CNS neurons. The cerebellum offers several advantages for this analysis. The general organization and the pattern of neuronal interconnections of the mature cerebellum have been known for a considerable time (Cajal, 1894) and the detailed ultra-structure has been well documented (Sidman and Rakic, 1973; Rakic 1976). The limited number of cell types, their organization, and the distinctive architecture of the cerebellum have aided these studies. Within the cerebellar cortex, there are a small number of clearly distinguishable neuronal cell types with well defined connections (Altman, 1972; Rakic, 1975, 1976). The cerebellar granule cells, located in the inner most layer of the cerebellum, form synapses on Purkinje cells, which are located in a single row in the middle layer of the cerebellum; the Purkinje cell layer. The outer most layer, the molecular layer, consist predominantly of granule cell axons synapsing on Purkinje cell dendrites (Fig.6).

In addition, cerebellar cells from immature rodents as well as from cats can be readily grown in culture (Trenkner and Sidman, 1977; Trenkner, 1990; Trenkner and Sturman, 1991). These cultures provide a dynamic *in vitro* system for the study of neuronal developmental as well as the interaction between neurons and different glial cell types (Trenkner and Sidman, 1977; Trenkner *et al.*, 1984; Trenkner, 1990). In this system, neurons

behave *in vitro* as they would *in vivo.*

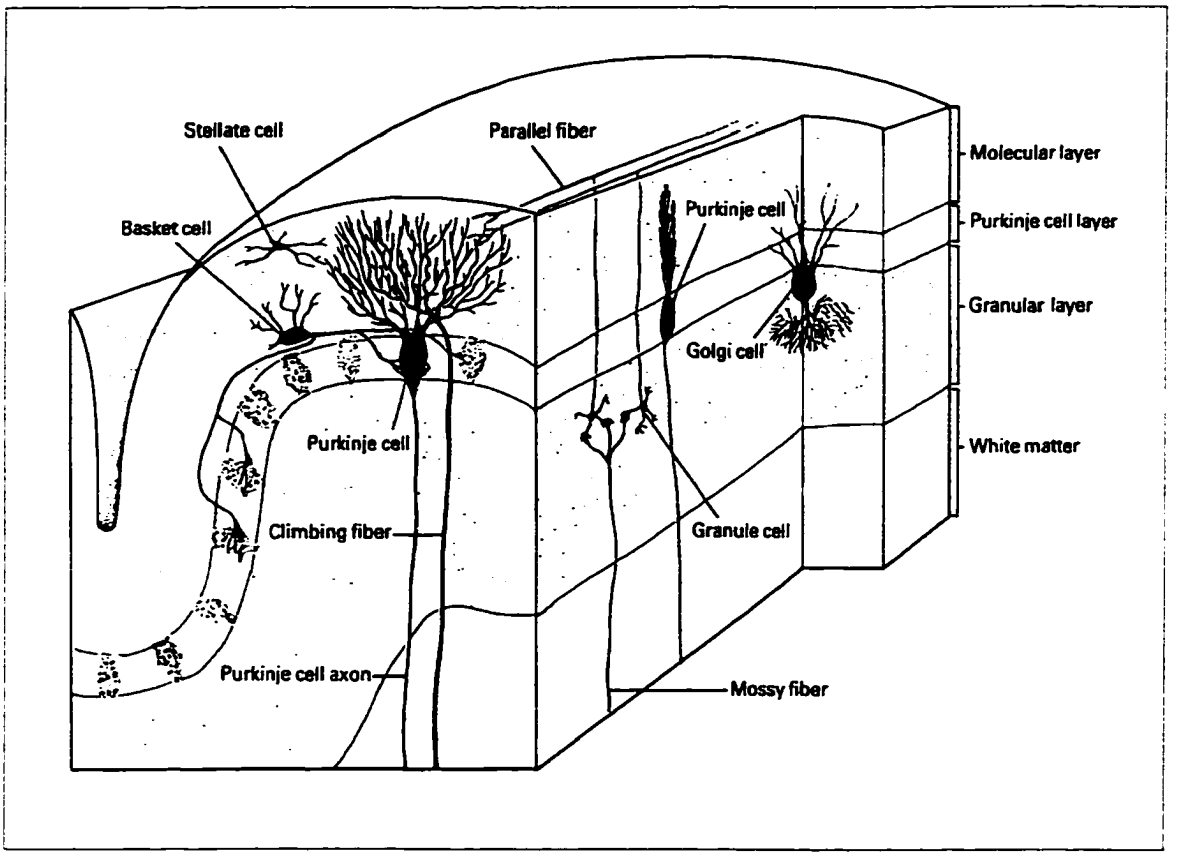


Figure 6: Organization of the cerebellar cortex. The cerebellar cortex consists of three layers that contain five types of neurons: stellate, basket, Purkinje, Golgi, and granule cells. The cerebellar granule cells, located in the inner most layer of the cerebellum, form synapses on Mossy fibers terminals and on Purkinje cells, which are located in a single row in the middle layer of the cerebellum; the Purkinje cell layer. The outer most layer, the molecular layer, consists predominantly of granule cells axons, known as parallel fibers, synapsing on Purkinje cell dendrites. It also contains scattered local interneurons, the stellate and basket cells, as well as the dendrites of neurons lying in deeper layers (From Ghez and Fahn, 1985).

Cerebellar granule cells use glutamate as their primary neurotransmitters and express a number of glutamate receptor subtypes on their dendrites and perikarya (Gallo *et al.*, 1982; Drejer *et al.*, 1986; Burgoyne and Cambray-Deakin, 1988). Consequently, cerebellar granule neurons have been used extensively to characterize the mechanism (s) of excitatory and excitotoxic actions of glutamate receptor subtypes such as NMDA (Novelli *et al.*, 1988; Henneberry *et al.*, 1989; Weller *et al.*, 1993) and non-NMDA receptors (Balazs *et al.*, 1990; Kovalchuk *et al.*, 1994), as well as for examining the neuroprotective actions of glutamate antagonists and agonists (Boje *et al.*, 1993; Pizzi *et al.*, 1993; Weller *et al*, 1993). Furthermore, the mechanisms of excitotoxins on cerebellar neurons provide an experimental model of potential relevance to neurological diseases characterized by selective neuronal death in the cerebellum, including olivopontocerebellar degeneration and Friedrich's ataxia. Links between the clinical findings of these disorders and excitotoxic models are not yet as well established as for other neurodegenerative diseases (Tatter *et al.*, 1995).

Numerous neurotrophic factors have been shown to protect cerebellar neurons against excitotoxicity in culture. These include EGF (Abe and Saito, 1992), bFGF (Fernandez-Sanchez and Novelli, 1993; Trenkner, *et al.*, 1996; El Idrissi, *et al.*, 1998), and BDNF in granule cell cultures (Lindholm *et al.*, 1993b) and NGF in Purkinje cell cultures (Cohen-Cory *et al.*, 1991). Conversely, IGF-I increases the susceptibility of cerebellar granule cell cultures to excitotoxicity (Calissano *et al.*, 1993; D'Mello *et al.* 1993; Ciotti *et al.*, 1996), concluding that the effects or growth factors are both cell- and factor-specific.

As described in the methods section, we have used several protocols for the preparation of cerebellar primary cell cultures. The culture conditions and the population of

cells obtained are different among these protocols, which in turn may affect cell-cell interaction and communication. The four culture conditions that we have established included, *mixed cultures*, these were characterized by the presence of granule neurons, glial cells (mainly astroglial) and fibroblasts. The advantage of these cultures is the ability of neurons to migrate along the bundle of fibers of astroglial processes that form between reaggregates, in an analogous fashion to the *in vivo* process (Trenkner and Sidman, 1977), providing us therefore with a functional model system to study cell migration (Fig. 7). On the other hand, the presence of non-neuronal cells in the cultures may alter the overall response of neurons to external signals.

Figure 7. Functional assay for the study of cell migration. Time lapse photograph depicting migrating granule cells along fiber bundles. Mixed cerebellar cells were prepared from P7 mice and plated at high density into a non-PDL coated microwell dish, conditions that favor cell-to-cell rather than cell-to-substrate interaction. Cerebellar granule cells migrate along cables according to their developmental stage in an analogous fashion to the *in vivo* environment (Trenkner and Sidman, 1977; Trenkner *et al*, 1984). The number of granule cells per fiber bundle reflects migratory activity. Migrating cells are distinguished by measuring the distance of granule cells from both ends of these fiber bundles over a period of 2 h. Arrows indicate migrating granule cells.

The second system we have used consisted of *enriched neuronal cultures*, these are characterized by the abundance of neurons over glial cells, which, in turn, changed the requirements of neuronal cell function in development. However, this method allowed us to study the response of neurons in the absence (less than 5 %) of non-neuronal cell types, mainly astroglial cells and fibroblasts. The two other culture conditions consisted of *purified neurons in serum* and *purified neurons in serum-free medium*. These two culture conditions enabled us to study the response of neurons without glial cells, and to assess the supportive effects of glial-derived factors, as well as NAAs in defined medium (serum-free). However, although *in vitro* tissue culture systems enable detailed examination and manipulation of GFs and NAAs actions under controlled experimental conditions, the non-physiological nature of such model must be considered, since physiologic responses in an artificial environment may differ form those observed *in situ*. In cultures, neurons may express different combinations of cell surface proteins, or receptors with an altered functional properties than those *in vivo*. However, similarly *in vivo* studies are fraught with interpretational problems, mainly due to the complexity of the *in vivo* environment. Therefore, comparison of cellular behavior or reactions *in vitro* and *in vivo* is essential in order to understand mechanisms of normal and abnormal cell behavior during development and maintenance of neuronal functions.

RESULTS:

CELL SURVIVAL AND TROPHIC FACTORS:

The primary goal of this thesis is to better understand the function of GFs and NAAs that affect survival, maintenance and function of cerebellar granule cells. Using different culture conditions, we examined the trophic effects of taurine, BDNF, bFGF and the

combination thereof, since these factors have a well documented role in cerebellar development and during glutamate depolarization. We used specific glutamate agonists (NMDA and kainate) and antagonists (MK801 and DNQX) to pharmacologically characterize the receptor subtypes through which glutamate elicits its excitotoxic effects. Finally, we examined the neuroprotective effects of taurine and growth factors under glutamate-mediated excitotoxicity, alone and together. And this is what we found.

Excitatory amino acids-induced neurotoxicity in cultured cerebellar cells

To determine the pharmacological profiles of glutamate toxicity, we used specific agonists (NMDA, kainate) and antagonists (MK801, DNQX) of glutamate. This allowed us to identify members of the glutamate receptor family through which the toxic effects of the glutamate are mediated. Mixed cultures of cerebellar cell were exposed for 30 min to the glutamate agonists or antagonists as indicated in figure 8. The neurotoxicity of glutamate or its selective agonists NMDA and kainate was morphologically apparent within several hours after their application. The extent of cell damage was assessed 24 hr after glutamate application. Glutamate at 1mM was toxic to cultured cerebellar neurons in these mixed cultures, as over 75% of neurons died (Fig. 8). A similar effect was observed when cerebellar cultures were treated with NMDA (1mM) or kainate (1mM). This toxicity was eliminated by five minutes pre-incubation with the NMDA receptor antagonists MK801 (10 μM) and AP5 (10 μM), or the kainate receptor antagonist DNQX (10 μM). Glia and fibroblasts remained intact and were not subject to glutamate toxicity. When the cultures were treated with neurotoxic

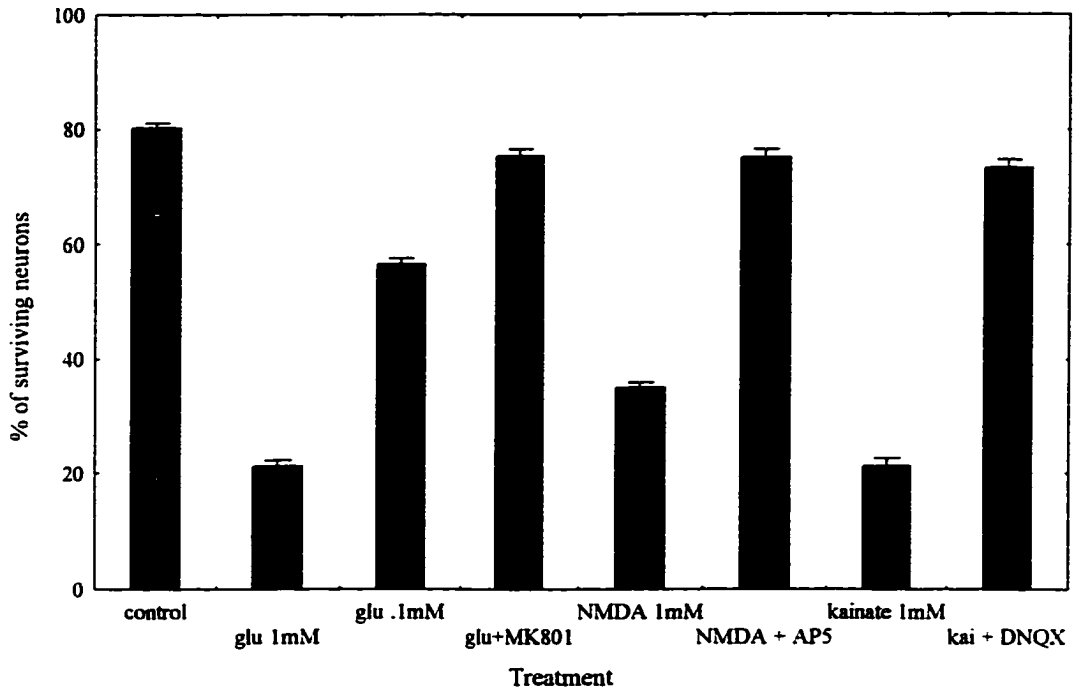


Figure 8. Glutamate-induced neurotoxicity in cultured cerebellar cells. Cerebellar granule cells were grown in serum-free medium for 4 days. Excitotoxicity was induced by treatment with 1 mM kainate, NMDA, or glutamate for 30 min. All these treatments caused a significant cell death ($p < .001$). Kainate and glutamate induced the same extent of cell death. Whereas treatment with NMDA resulted in less cell death than glutamate and kainate, but the difference was not significant. Glutamate-induced cell death was dose-dependent, as 100 μM caused significantly ($p < .005$) less cell death than 1 mM. Pretreatment for 5 min with receptor-specific antagonists (MK801, AP5 and DNQX at 10 μM) completely blocked cell death.

concentrations of glutamate (1mM), NMDA (1mM) or kainate (1mM), neuronal response or degeneration did not include all neuronal population, as about 25% of neurons survived in the presence of glutamate and kainate and 40% with NMDA treatment. This discrepancy in the response to glutamate is most likely based on selective expression of different glutamate receptors by the responsive granule neurons (Didier *et al.*, 1994; Hack and Balazs, 1995; Hack *et al.*, 1995; Resink *et al.*, 1995), or to different maturational states of these neurons.

These data indicate that cerebellar granule cells in our culture system express different glutamate receptor subtypes, and that these receptors are functional. Furthermore, excessive stimulation of any of these receptors elicited excitotoxicity, confirming results of others (Garthwaite *et al.*, 1986; Trenkner *et al.*, 1994; Budd and Nicholls, 1996).

bFGF and taurine protected cerebellar neurons from glutamate excitotoxicity

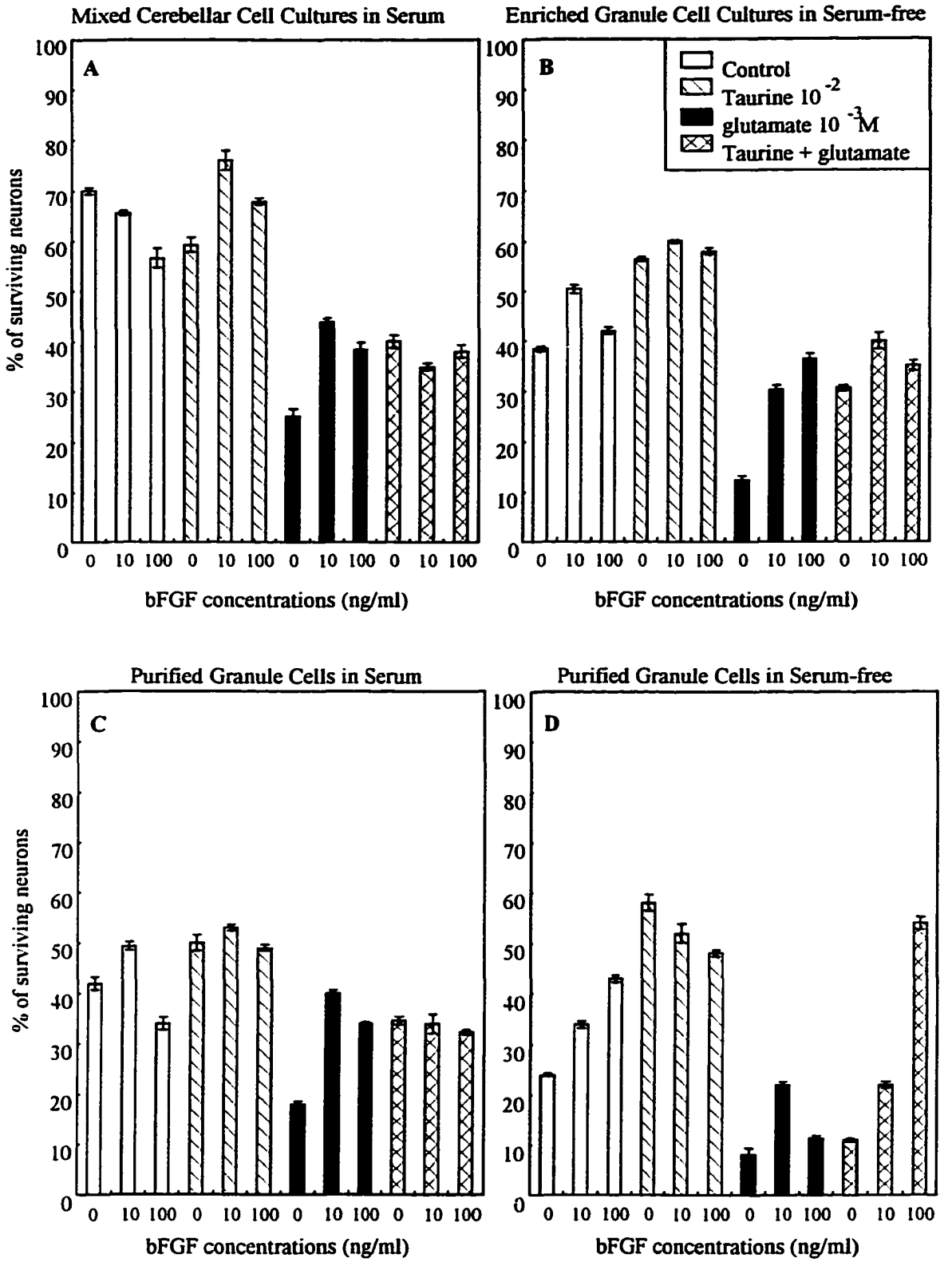
The neuroprotective functions of bFGF and taurine against glutamate excitotoxicity were analyzed and expressed as survival of cerebellar granule cells grown under four different culture conditions (see methods, choosing the system and figure 9). We confirmed that neuronal survival was dependent on astroglial cells (Trenkner and Sidman, 1977; Hatten *et al.*, 1988; Trenkner, 1991; Rosenberg, 1991), since the highest degree of neuronal survival, when compared to cultures without astroglia cells, was reached in serum-supplemented medium containing neurons and glia cells (Fig. 9A). In these cultures bFGF, with or without taurine, had little measurable effect on the survival of neurons.

One mM glutamate significantly reduced the number of surviving granule neurons (Fig. 9A-D). This excitotoxic effect of glutamate was more pronounced in cultures of purified cerebellar neurons than in mixed cultures, suggesting a protective role of astroglia during glutamate excitotoxicity (Rosenberg and Li, 1995). bFGF and/or taurine had little or no effect on mixed cerebellar cultures under excitotoxic conditions. However, purified granule cells did survive glutamate-excitotoxicity without glial cells but in the presence of taurine and/or bFGF, in numbers similar to controls (Fig. 9C), but significantly less than in the presence of astrocytes and serum (Fig. 9A), suggesting that these factors can replace the role of astrocytes. In serum-free medium, on the other hand, the highest neuroprotection against

glutamate excitotoxicity was observed only when taurine and low concentrations of bFGF were combined, whereas both factors alone could not prevent glutamate-induced cell death (Fig. 9D). Thus taurine can support bFGF function under certain conditions.

Taurine and bFGF did not act synergistically nor additively but rather both factors were capable of providing optimal conditions for cell survival. However, under serum-free conditions, in the presence of glutamate, maximal cell survival was obtained only by the collaboration of taurine and bFGF. Since both factors act through different mechanisms (Ca^{2+}-regulation, activation of second messenger systems), we suggest that a variety of alternative mechanisms might be activated to control cell survival.

Figure 9. bFGF and taurine protected cerebellar neurons from glutamate excitotoxicity. The survival of cerebellar granule neurons, isolated form early postnatal (P6-8) C57Bl/J mice, was determined *in vitro* as a function of bFGF (10 and100 ng/ml), taurine (10 mM) and glutamate (1mM). Four conditions were compared: A. mixed cerebellar cell populations including astrocytes in MEM containing 10 % HS and 5% FCS. B. enriched neuronal population (5% glia 95 % neurons) in serum-free medium +N2 supplement. C. purified cerebellar neurons maintained in MEM with serum (10 % HS and 5% FCS). D. purified cerebellar neurons maintained in serum-free medium (MEM + 15 % N-2 supplement). Cells were initially plated in serum-containing MEM (MEM + 10 % HS + 5% FCS). After 24 h, the culture medium was replaced with the growth medium as indicated. Cells were pre-incubated with bFGF (0, 10 or100 ng/ml) and/or taurine for 24 h before glutamate was added. The number of living cells was determined after 6-10 h. A three-way ANOVA showed a statistically significant interaction effect among NAAs x bFGF concentrations x culture condition [$F(18,96) = 19.24$, $p < .0001$]. There were significant main effects of NAAs [$F(3,96) = 612.45$, $p < .0001$], bFGF concentrations [$F(2,96) = 81.08$, $p < .0001$] and culture condition [$F(3,96) = 208.97$, $p < .0001$]. Post hoc tests showed the cell survival of controls in mixed cultures was significantly ($p < .001$) higher than that of controls in all other cultures. Cell survival of controls from purified neurons in serum-free was significantly ($p < .001$) lower than that of controls from all other cultures. Glutamate caused a significant ($p < .001$) reduction in cell viability in all culture conditions as compared to the corresponding controls. Under all culture conditions, taurine was neuroprotective against glutamate excitotoxicity, except in cultures of purified neurons in serum-free medium where taurine had no significant effect on cell viability as compared to glutamate-treated cultures.

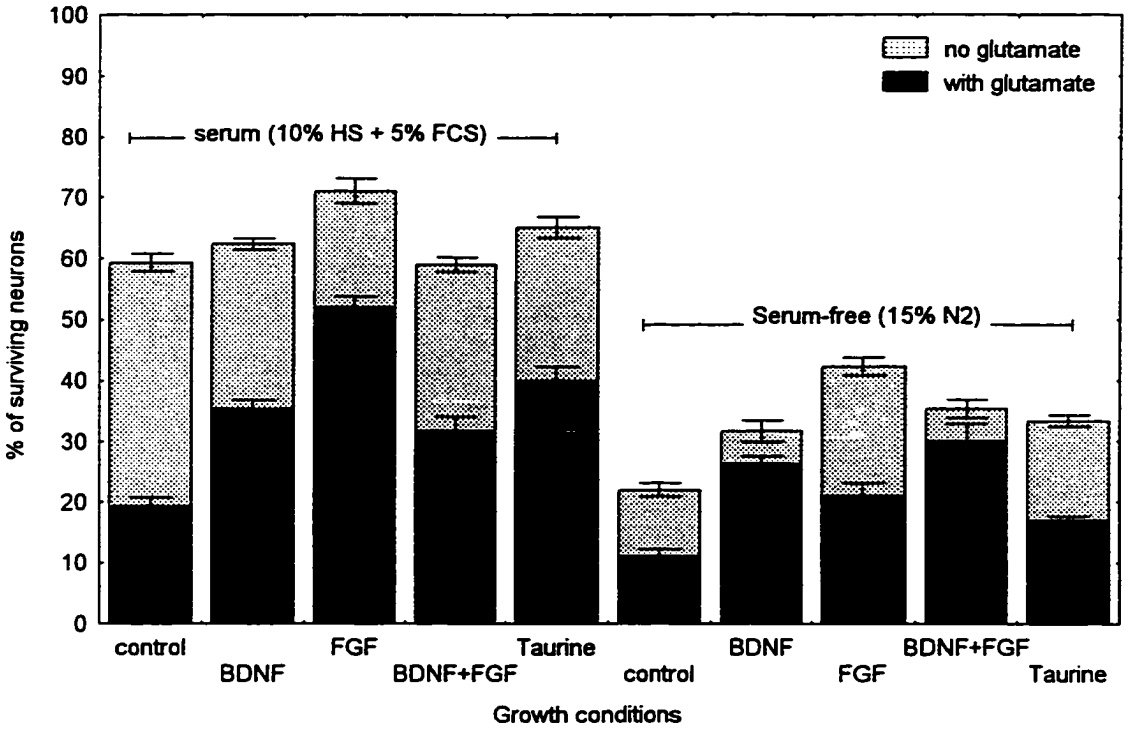


Trophic and neuroprotective effects of BDNF

Similarly, we have tested the trophic and neuroprotective role of BDNF on cerebellar granule cells. When grown in serum-containing medium, cerebellar granule cells responded poorly to BDNF, bFGF or the combination of the two factors (Fig. 10a). On the other hand, in serum-free medium, the effects of BDNF and bFGF were more pronounced (Fig 10b). This finding further indicates that serum may contain a factor (s) that promote neuronal survival. Glutamate was more neurotoxic under serum-free conditions. Taurine, BDNF, bFGF and the combination thereof, rescued more neurons from glutamate excitotoxicity in the presence of serum than in serum-free medium, suggesting that the effects of these factors are context specific.

Figure 10. Trophic and neuroprotective effects of BDNF. The survival of purified cerebellar granule cells was analyzed in the presence or absence of serum, BDNF, bFGF, taurine and glutamate. Growth conditions significantly affected the survival, as the removal of serum resulted in a significant reduction in cell number. A. Purified cerebellar neurons maintained in MEM with serum (10 % HS and 5% FCS). B). Purified cerebellar neurons maintained in serum-free medium (MEM + 15 % N-2 supplement). Cells were initially plated with serum conditions (MEM + 10 % HS + 5% FCS). After 24 h, the culture medium was replaced with the indicated growth medium. Cells were pre-incubated with BDNF (10 ng/ml), bFGF (10 ng/ml) and taurine (10 mM) for 24 h before glutamate was added. Cells were treated with glutamate (1mM) for 30 min, then washed with growth medium and returned to the incubator overnight. The number of living cells was determined after 24 h. A three-way ANOVA showed a statistically significant interaction among growth factors x growth condition x glutamate [$F(4,40) = 8.82$, $p < .0001$]. There was a significant main effect of growth conditions (serum or N2) [$F(1,40) = 331.05$, $p < .0001$], growth factors (BDNF, bFGF or BDNF +bFGF) [$F(4,40) = 22.21$, $p < .0001$] and glutamate [$F(1,40) = 278.79$, $p < .0001$]. Post hoc tests showed that cell survival of controls grown in serum was significantly ($p < .001$) higher than controls in serum-free cultures. In serum containing medium, additions of BDNF, bFGF, or taurine did not cause a significant increase in cell survival, whereas, in serum-free cultures, bFGF induced a significant ($p < .005$) increase in cell survival above controls. Glutamate treatment (1 mM) initiated a significant cell death in serum ($p < .001$) or

in serum-free ($p < .05$) medium. Under serum conditions, significant protection from glutamate toxicity was achieved by pretreatment with BDNF ($p < .05$), bFGF ($p < .001$) and taurine ($p < .005$).



CONCLUSIONS

The main finding of these experiments was that growth conditions, mainly the presence of serum and astrocytes, profoundly affected the survival of cerebellar granule cells *in vitro*. Additionally, the vulnerability of cerebellar granule cells to glutamate showed characteristic differences depending on culture conditions, as neurons were more vulnerable to glutamate excitotoxicity in the absence of serum and astrocytes. Taurine, bFGF and BDNF all showed neurotrophic and neuroprotective effects. Such effects were more noticeable under

certain culture conditions (in serum-free medium). The differential effects of growth conditions on cerebellar granule cells could be due, in part, to differences in the maturation of these cells, which can be enhanced or delayed under specific growth media. Primarily, the expression of cell surface receptors (e.g. glutamate and growth factors), which have been shown to be subject to such regulation (Didier *et al.*, 1994; Balazs *et al.*, 1990; Lindholm *et al.*, 1993b; Burgoyne *et al.*, 1993; Resink *et al.*, 1994; Didier *et al.*, 1994; Hack and Balazs, 1995; Morrison and Mason, 1998). However, the possibility that manipulation of growth conditions may lead to a selection for certain sub-populations of granule cells with characteristic properties, responsible for the differences in sensitivity to glutamate stimulation, cannot be excluded. Finally, these results are consistent with the idea that CNS neurons, in general, may require a menu of signals to differentiate, survive and function under given conditions (Meyer-Franke *et at.*, 1995, Wood *et al.*, 1997; Morrisson and Mason, 1998). As shown here, *in vitro*, some of these factors substitute for each other depending on their availability and the responsiveness of cells.

In our culture systems, glutamate or its specific agonists NMDA and kainate caused significant cell death, indicating that these cells expressed different glutamate receptor-subtypes. As described later, glutamate neurotoxicity induced excessive influx of calcium resulting in a loss of intracellular calcium homeostasis (Mattson and Kater, 1988; Choi, 1988; Milani *et al.*, 1991; Manev *et al.*, 1993; Mattson *et al.*, 1995; Pizzi *et al.*, 1996; Budd and Nicholls, 1996). In order to elucidate the possible neuroprotective mechanism of growth factors and taurine, we determined whether the neuroprotective effects of taurine, BDNF and bFGF were mediated through the regulation of intracellular calcium homeostasis.

Note: for the rest of these experiments, we have used the enriched granule cell cultures in serum-free medium, only when otherwise specified.

CALCIUM UPTAKE STUDIES

The cytoplasmic concentration of calcium is maintained at levels approximately 1000 times less than those outside the cells (Hoyer, 1993). It is established that during depolarization calcium enters neurons through voltage-gated calcium channels, transmitter-gated channels permeable to calcium, or through ion exchangers. Neuronal cells are equipped with a multitude of ion pumps, which produce and maintain critical voltage and ion gradients across neuronal membranes, and help restore the resting membrane potential. Excess glutamate receptor stimulation results in disturbances of intracellular ion concentrations. This would likely mediate auto-destructive processes linked to excitotoxic injury and cell death.

In the following experiments, we have tried to monitor variations in the intracellular calcium concentrations, and characterize the routes of calcium entry in cells under excitotoxic conditions. Additionally, we have examined the neuroprotective effects of taurine and growth factors based on the regulation of calcium homeostasis. It should be emphasized that these experiments have measured steady state levels of calcium accumulation in a dynamic equilibrium and therefore do not distinguish between free and bound calcium ions, nor do they indicate the rate of exchange between labeled and unlabeled calcium. They do indicate however, the net intracellular accumulation of $^{45}Ca^{2+}$.

Calcium uptake as function of glutamate-depolarization

Since increases in intracellular calcium have been implicated in excitotoxic cell death in several different paradigms (Dykens *et al.*, 1987; Mattson *et al.*, 1988a; Choi, 1988; Siesjo *et al.*, 1988; Mattson, 1992), and because elevation of intracellular calcium is known to mediate the glutamate-induced excitotoxicity, we performed experiments designed to determine the intracellular calcium accumulation in response to increasing extracellular glutamate stimuli (Fig. 11). We found that intracellular calcium concentrations were directly related to extracellular glutamate concentrations. Using the enriched granule cell cultures in serum free medium, depolarization with 1mM glutamate for 15 min resulted in a significant intracellular $^{45}Ca^{2+}$ accumulation. Glutamate concentrations higher than 1mM caused immediate cell death. On the other hand, using 1 μM glutamate, a concentration not sufficient enough to depolarize neurons in our system, $^{45}Ca^{2+}$ uptake was not different from untreated controls. Theses data show a dose-dependent function of glutamate, and confirm previous findings that excessive calcium influx is the reason for glutamate-induced cellular damage (Mattson and Kater, 1988; Choi, 1988; Milani *et al.*, 1991; Manev *et al.*, 1993; Mattson *et al.*, 1995; Pizzi *et al.*, 1996; Budd and Nicholls, 1996).

NMDA receptors are the main mediators of glutamate-induced $^{45}Ca^{2+}$ uptake

It is well established that among the glutamate receptor subtypes, the NMDA receptors are the most permeable to calcium (Nicoll *et al.*, 1988; Lysko and Feuerstein, 1990; Milani *et al.*, 1991; Nakanishi, 1992; Bading *et al.*, 1995; Sucher *et al.*, 1996; Scherzer *et al.*, 1997; Lipton *et al.*, 1997). Furthermore, at least *in vitro*, glutamate toxicity is primarily

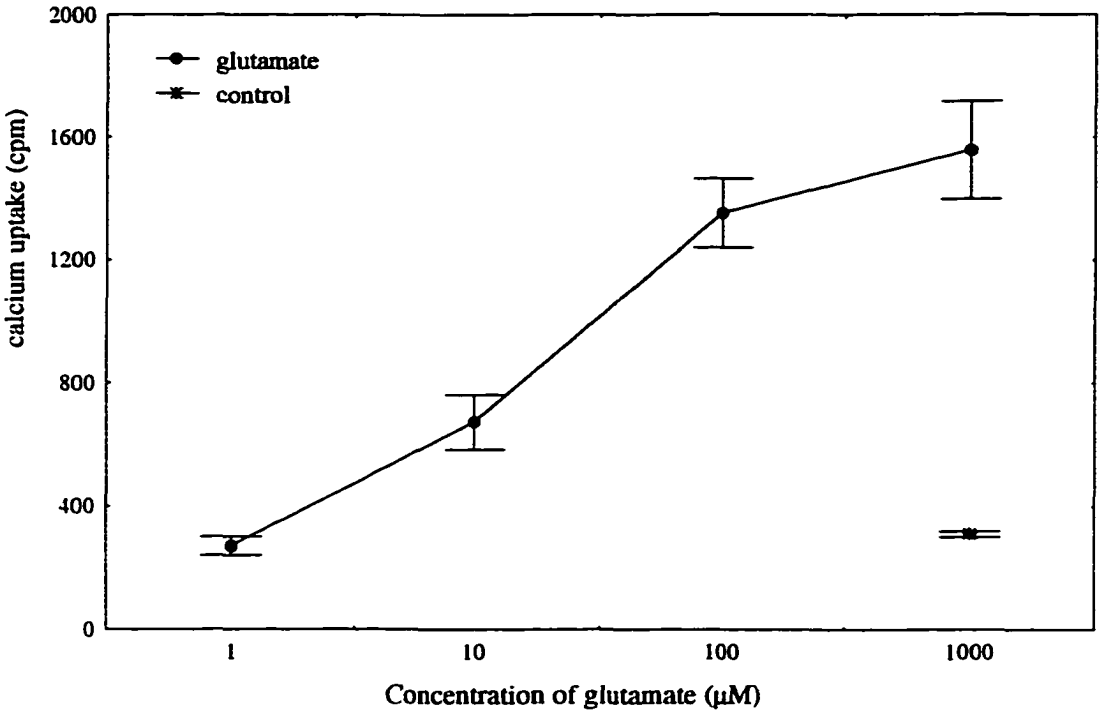


Figure 11. Glutamate-induced calcium accumulation is concentration-dependent. Calcium accumulation was measured in cerebellar granule cells grown in serum-free medium for 5 DIV. On day 5, cultures were depolarized with glutamate at different concentrations as indicated. $^{45}Ca^{2+}$ accumulation was determined 15 min after depolarization. Each data point represents mean ± SEM from three experiments. ANOVA showed a significant main effect of glutamate treatment [$F(3,8) = 30.16$, $p < .001$]. Post hoc tests indicated that 100 and 1000 μM glutamate caused a significant ($p < .001$) increase in $^{45}Ca^{2+}$ uptake as compared to controls, whereas the increase in $^{45}Ca^{2+}$ uptake induced by 10 μM was not significantly different from baseline.

mediated though NMDA receptor activation (Favaron *et al.*, 1988; Resink *et al.*, 1994; Bading *et al.*, 1995). To determine whether this was the case in our system, we measured $^{45}Ca^{2+}$ uptake induced by glutamate or NMDA. Figure 12 shows that stimulation with 1 mM glutamate or NMDA resulted in a significant increase in $^{45}Ca^{2+}$ uptake. This was completely suppressed by the NMDA specific antagonist MK801 (10 μM). The blockade of the

glutamate response by the NMDA antagonist indicated that the NMDA-gated ion channels were the main routes of calcium entry into cells following glutamate depolarization.

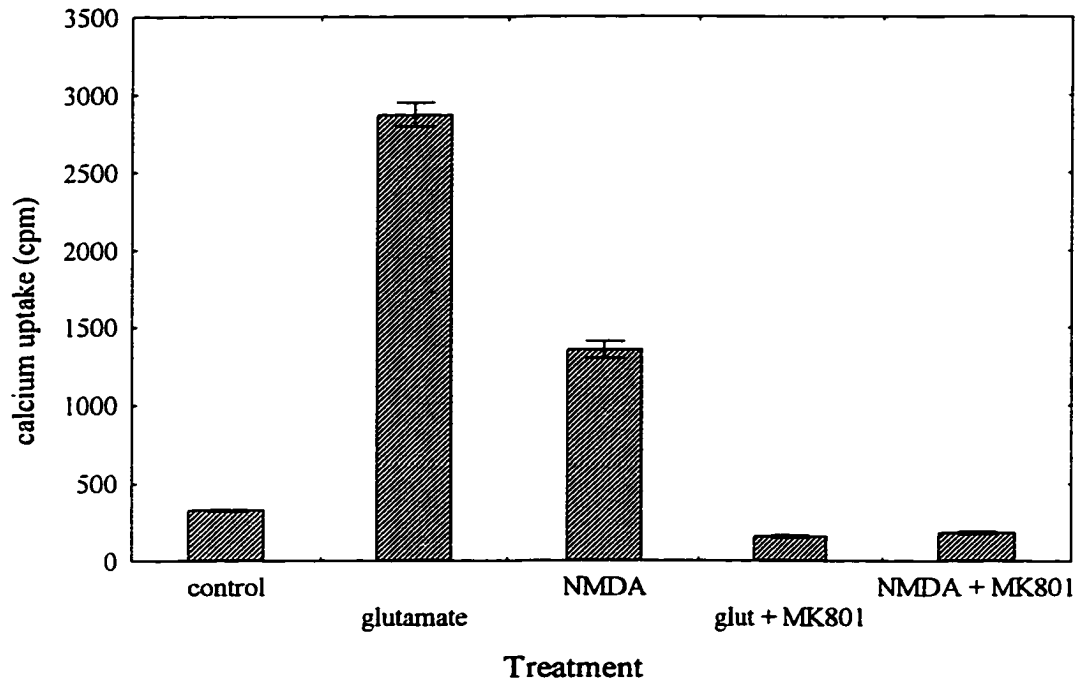


Figure 12. NMDA receptors are the main mediators of glutamate-induced $^{45}Ca^{2+}$ uptake. To determine the relative contribution of the NMDA receptors to glutamate-induced $^{45}Ca^{2+}$ uptake, cerebellar granule cells were grown in serum-free medium and stimulated with glutamate or NMDA (1mM) in the presence or absence of MK801 (10 μM), a specific blocker of the NMDA receptor channels. Glutamate depolarization resulted in a significant ($p < .005$) $^{45}Ca^{2+}$ uptake as compared to control. Likewise, NMDA receptor stimulation resulted in a significant $^{45}Ca^{2+}$ uptake, but the induction with NMDA was significantly ($p < .01$) lower than with glutamate. Under these conditions, about 50 % of the glutamate-induced $^{45}Ca^{2+}$ uptake is mediated via NMDA receptors. Using MK801 prior to glutamate or NMDA depolarization resulted in a complete inhibition of $^{45}Ca^{2+}$ uptake, indicating that the activation of the NMDA receptors is an essential component of glutamate-induced $^{45}Ca^{2+}$ uptake.

Contribution of non-NMDA receptors to glutamate-induced $^{45}Ca^{2+}$ uptake

Binding of glutamate to non-NMDA receptors leads to membrane depolarization which in turn removes the Mg^{2+} block from the NMDA channels resulting in the activation of these receptors. As expected, glutamate induced more $^{45}Ca^{2+}$ uptake than NMDA, indicating the presence of other channels than the NMDA through which calcium finds its way into the cytoplasm under glutamate depolarization. Voltage-sensitive calcium channels (VSCCs), as indicated in figure 13, are one example. About 10 % of the glutamate-induced $^{45}Ca^{2+}$ uptake was mediated through VSCCs.

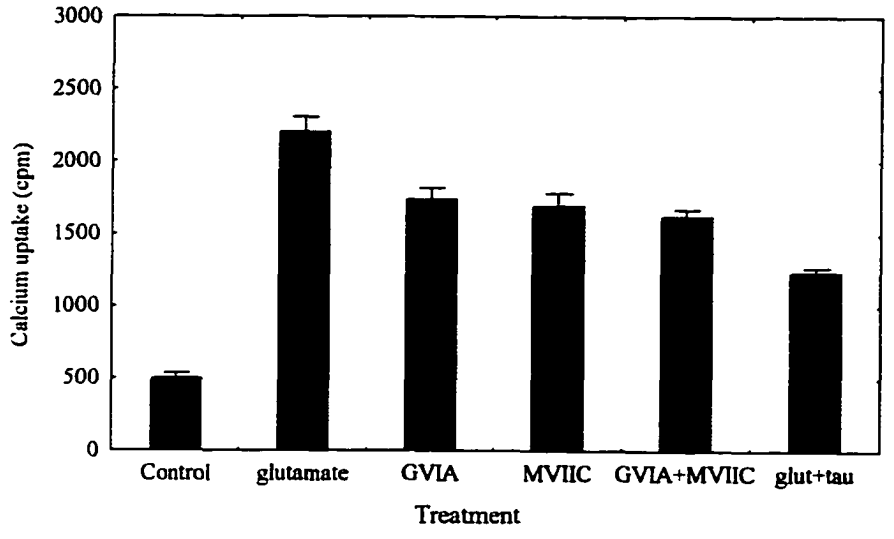


Figure 13. Activation of voltage-sensitive calcium channels (VSCCs) by glutamate. Cerebellar granule cells, maintained in serum-free medium for 5DIV. were depolarized with glutamate in the presence or absence of VSCCs blockers. Glutamate depolarization resulted in a significant ($p < .005$) $^{45}Ca^{2+}$ uptake as compared to untreated controls. Pretreatment of cells with 5μM ω-conotoxin GVIA, a specific blocker of the N-type VSCCs in neurons, resulted in a significant ($p < .05$) decrease in the glutamate-induced $^{45}Ca^{2+}$ uptake. Similarly, pretreatment with 5μM ω-conotoxin MVIIC, that inhibits Q-type calcium channels and VSCCs resistant to GVIA, resulted in a significant ($p < .05$) decrease in the glutamate-induced $^{45}Ca^{2+}$ uptake. However, combination of GVIA and MVIIC (5μM), did not result in a reduction significantly different from individual blocker. Taurine reduced glutamate-induced $^{45}Ca^{2+}$ uptake significantly ($p < .05$) lower than the specific calcium-channels blockers.

To determine the contribution of non-NMDA receptors to glutamate stimulation, cells were depolarized with 1 mM kainate, a non-NMDA receptor agonist. Figure 14 shows that depolarization with kainate induced a substantial accumulation of $^{45}Ca^{2+}$. This was completely blocked by a 2 min pre-incubation with the specific kainate antagonist DNQX (10 μM). Interestingly, MK801 significantly reduced the kainate-induced $^{45}Ca^{2+}$ uptake by about 75 %, suggesting that the NMDA receptors contribute a significant part to the kainate response. The remaining 25% of the kainate response could be due to a direct receptor-gated component and/or the VSCCs.

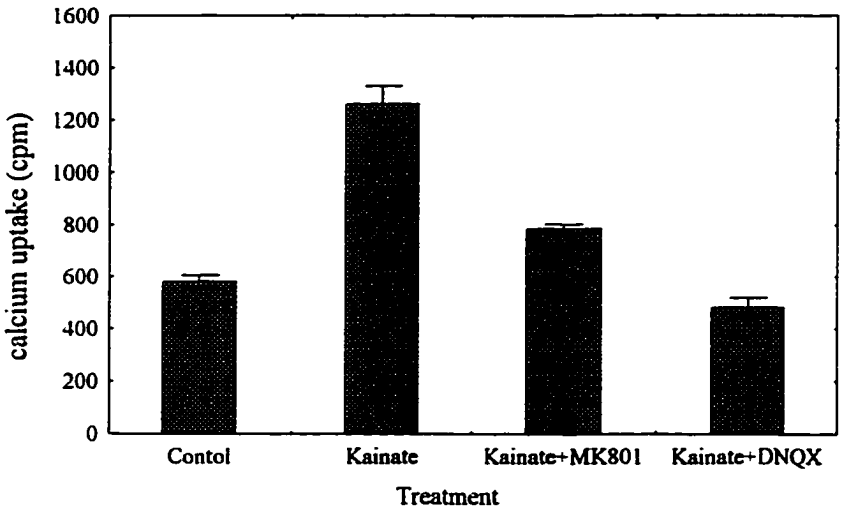


Figure 14. Co-activation of NMDA and non-NMDA receptors by kainate. Kainate receptors are known to contribute to the fast EPSPs, and their activation results in the subsequent activation of NMDA receptors through the removal of the Mg^{2+}. To determine the relative contribution of the NMDA receptors to the kainate-induced $^{45}Ca^{2+}$ uptake, cerebellar granule cells grown in serum-free medium for 5DIV were depolarized with kainate (1 mM) in the absence or presence of MK801 (10 μM) or DNQX (10 μM). Although kainate receptors are only slightly permeable to calcium, depolarization with kainate resulted in a significant ($p< .005$) $^{45}Ca^{2+}$ uptake. This $^{45}Ca^{2+}$ induction was completely blocked with DNQX, a specific kainate receptor blocker. MK801, the specific NMDA receptor antagonist, significantly ($p< .05$) reduced the kainate-induced $^{45}Ca^{2+}$ uptake, by approximately 75 %. The other 25 % of the kainate-induced $^{45}Ca^{2+}$ uptake, not blocked by MK801 could be mediated through kainate receptors or VSCCs.

Taurine as well as heterologous serum down-regulated glutamate-induced calcium influx.

We have proposed that the mechanism by which taurine prevents excitotoxic cell death could function through regulating calcium influx into neuronal cells during glutamate receptor-mediated depolarization. As shown in figure 15, glutamate induced a significant increase in $^{45}Ca^{2+}$ uptake into cerebellar granule cells grown in serum-free medium. This glutamate-induced $^{45}Ca^{2+}$ uptake was significantly reduced when the cells were pre-treated for 24h with 10 mM taurine. Taurine alone without glutamate had no effect on $^{45}Ca^{2+}$ uptake as compared to baseline. Glutamate-induced $^{45}Ca^{2+}$ uptake was significantly reduced when 2% horse serum was added to the culture medium (+13% N2 supplement) and it was completely countered when the culture medium was supplemented with 10 % (+ 5% N2) horse serum. These data indicate that the content of the growth medium significantly affect the cellular response to external stimuli, and suggest that horse serum contains a factor (s) that suppresses the glutamate-induced calcium uptake.

Growth conditions differentially modulated glutamate receptor function

The previous experiment clearly suggests that growth conditions (presence or absence of serum), significantly affects the outcome of glutamate depolarization. To further examine this, we pharmacologically characterized the receptor subtypes that are subject to this regulation. Under serum-free conditions, depolarization with NMDA, kainate or glutamate all induced a significant increase in $^{45}Ca^{2+}$ uptake (Fig. 16). As expected, glutamate was more active, since glutamate activates both NMDA and kainate receptors. Kainate-induced $^{45}Ca^{2+}$ uptake was measured in the presence of the NMDA antagonist MK801 to eliminate the

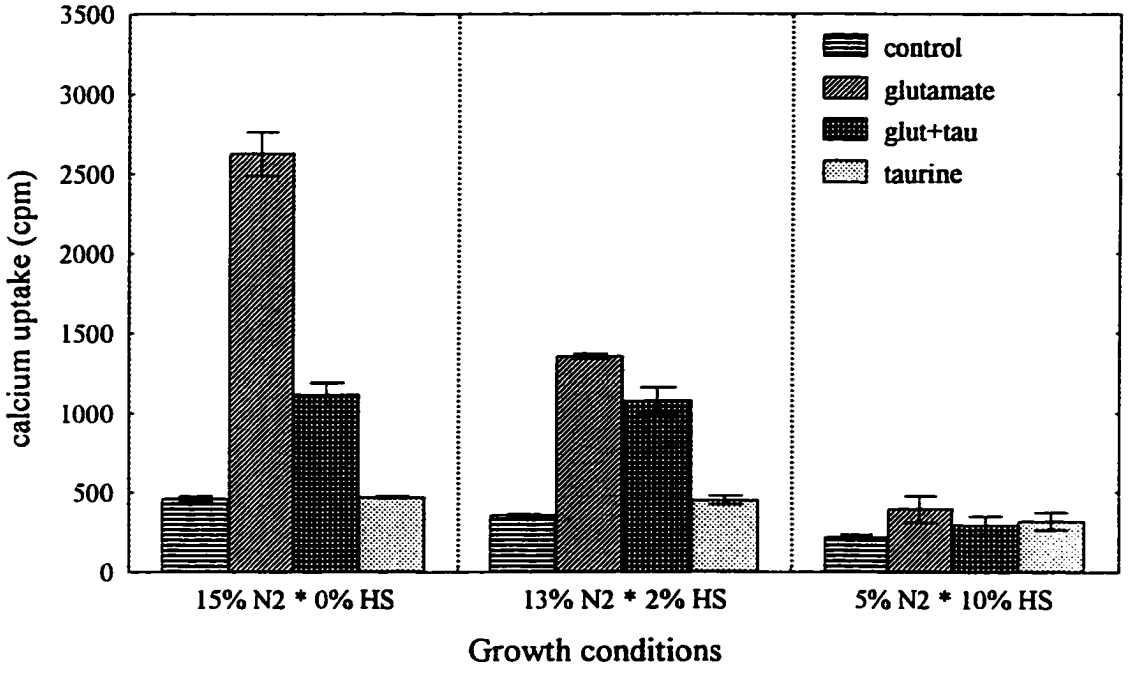


Figure 15. Taurine and horse serum down-regulate glutamate-induced calcium uptake. Taurine modulation of glutamate-induced $^{45}Ca^{2+}$ uptake was analyzed in early-postnatal cerebellar granule cells cultured in serum-free medium and supplemented with different concentrations of horse serum (HS; 0%, 2% and 10% respectively). Cells were initially plated under serum conditions (10% HS+ 5% FCS). After 24h in vitro, cultures were switched to serum-free conditions (15%N2 supplement). At 3 DIV, 0.25 ml of culture medium was removed and replaced with fresh medium supplemented with HS to give the desired final concentration (O%, 2%, 10%). Ten mM taurine was added at this time. Twenty four h later, $^{45}Ca^{2+}$ uptake was measured after depolarization with 1mM glutamate for 30 min. Data represent the means ± SEM from at least 3 separate experiments. A two-way ANOVA showed a statistically significant interaction effect between horse serum content and NAA treatment [$F(6,24) = 65.82$, $p < .0001$]. There were significant main effects of horse serum content [$F(2,24) = 200$, $p < .0001$] and NAA treatment [$F(3,24) = 200$, $p < .0001$]. Post hoc tests showed that glutamate-induced $^{45}Ca^{2+}$ uptake was significantly ($p < .001$) higher than control under 0 % and 2 % HS. However, under 10 % HS glutamate-induced $^{45}Ca^{2+}$ uptake was not statistically different from controls. Taurine significantly ($p < .001$) reduced glutamate-induced $^{45}Ca^{2+}$ uptake but only under serum-free conditions.

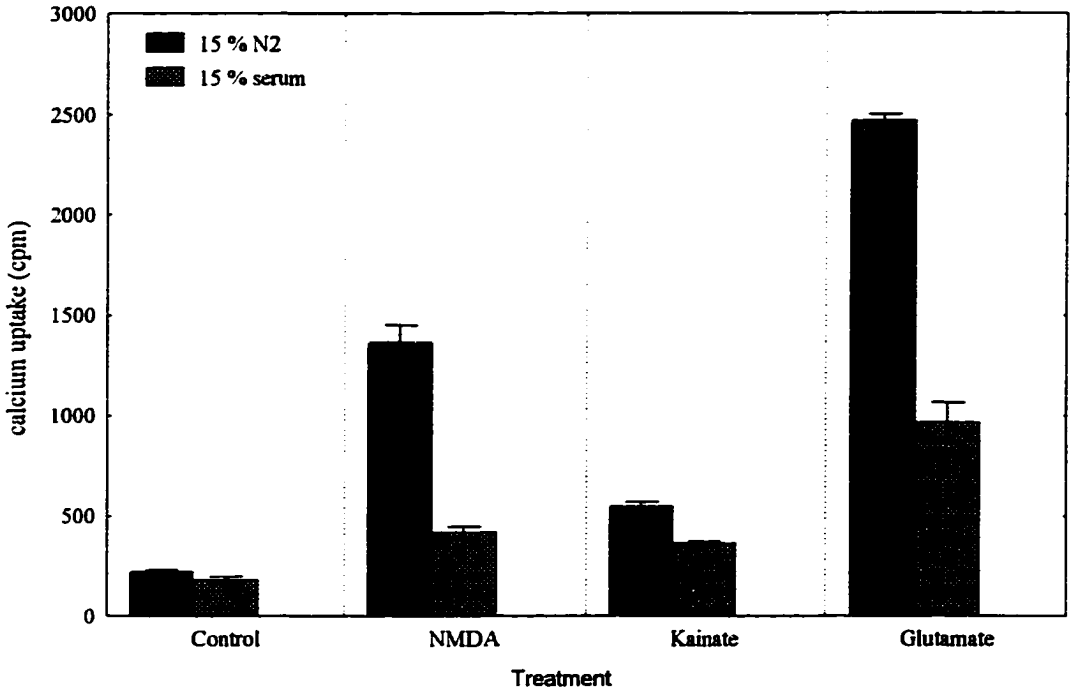


Figure 16. Growth conditions differentially modulate glutamate receptor function. The function of different glutamate receptor subtypes was compared in cerebellar granule cells grown in serum-containing or serum-free medium for 5 DIV. $^{45}Ca^{2+}$ uptake was assessed after 30 min depolarization with NMDA, kainate or glutamate at 1 mM. Data represent mean ± SEM from at least 3 separate experiments. A two-way ANOVA showed a statistically significant interaction effect between growth conditions and excitatory amino acid agonists treatment [$F(9,32) = 93.42$, $p < .0001$]. There were significant main effects of serum presence or absence [$F(3,32) = 251.86$, $p < .0001$] and EAA-agonist treatment [$F(3,32) = 567$, $p < .0001$]. Post hoc tests showed that, under serum-free conditions, NMDA-, kainate- and glutamate-induced $^{45}Ca^{2+}$ uptake were significantly ($p < .001$) higher than control, whereas with serum, only NMDA and glutamate caused a significant increase in $^{45}Ca^{2+}$ uptake ($p < .05$, $p < .001$ respectively). Horse serum significantly reduced the NMDA- and glutamate-induced $^{45}Ca^{2+}$ uptake ($p < .001$), kainate response was not significantly affected. These data support the idea that horse serum differentially regulates NMDA receptor function.

NMDA-mediated component of the $^{45}Ca^{2+}$ uptake. We found that when cultures were grown in serum-containing medium, the induction of $^{45}Ca^{2+}$ uptake by NMDA, kainate and glutamate was significantly reduced compared to serum-free conditions (Fig. 16). The most prominent reduction was observed with NMDA- and glutamate-depolarization. The reduction of the glutamate-induced $^{45}Ca^{2+}$ uptake could be due to the reduced function of the NMDA receptor subtypes, as these receptors are the most permeable to calcium among the glutamate receptor subtypes. We interpret these data to show that growth conditions significantly affect NMDA receptor activity. A similar regulation of other subtypes of glutamate receptors by growth conditions could not be excluded. This is in agreement with previous electro-physiological findings (Resink *et al.*, 1994; Hack and Balazs, 1995), concluding that the activity of non-NMDA receptors is also regulated by growth conditions.

Excitatory and inhibitory receptor subtypes interacting with taurine *in vitro*

In order to identify the possible interaction of taurine with different subtypes of glutamate receptors, we measured the modulatory effects of taurine on the NMDA-, kainate- as well as glutamate-induced calcium uptake. In order to assure that NMDA receptors were expressed, we tested whether magnesium would act as specific block for NMDA receptor function (Nowak *et al.*, 1984). We found that 1 mM Mg^{2+} added to the medium reduced $^{45}Ca^{2+}$ accumulation induced by 1 mM NMDA and glutamate (65 % and 42 %, respectively), indicating again that functional NMDA receptors were expressed in our system (Fig. 17). When Mg^{2+} was omitted from the assay however, NMDA as well as glutamate induced a significant increase in $^{45}Ca^{2+}$ uptake when compared to untreated controls or cultures treated

with NMDA in the presence of Mg^{2+}. As expected, Mg^{2+} did not inhibit kainate-induced $^{45}Ca^{2+}$ uptake. Although the NMDA receptors are the most permeable to calcium among glutamate receptor subtypes the NMDA-induced $^{45}Ca^{2+}$ uptake in the absence of Mg^{2+} was not significantly different from that of kainate under these experimental conditions (Fig. 17).

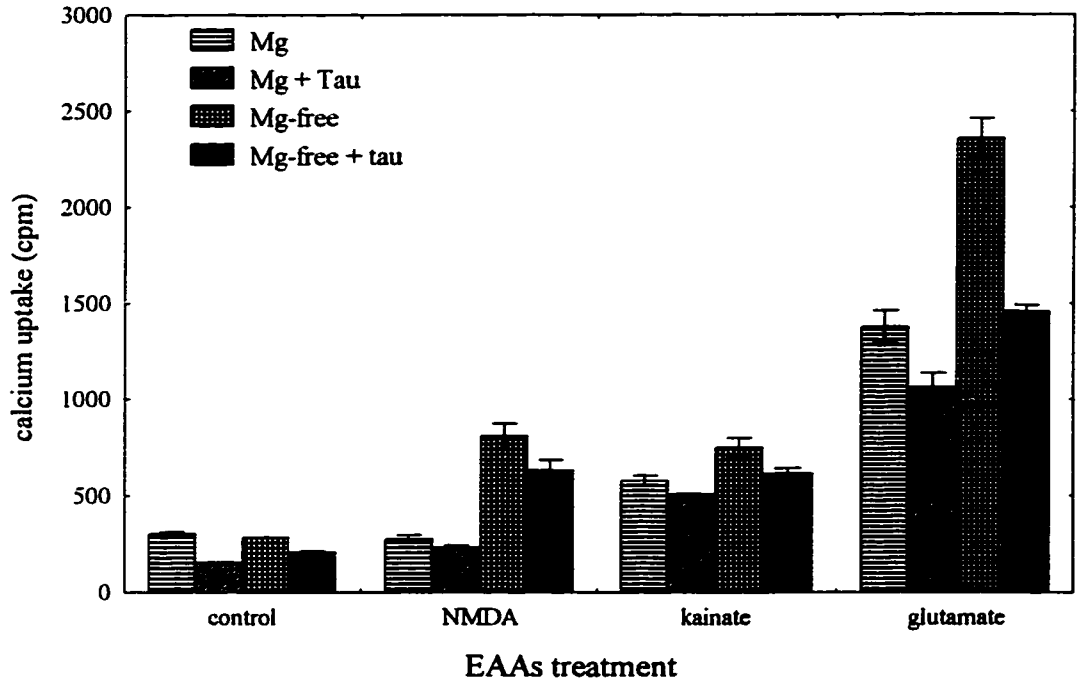


Figure 17. Interactions of taurine with different glutamate-receptor subtypes in culture. Glutamate receptor agonist-induced $^{45}Ca^{2+}$ uptake was measured in cultures grown under serum-free condition. $^{45}Ca^{2+}$ uptake assay was performed in the presence or absence of 1mM $MgCl_2$ to assess the magnesium block of the NMDA receptor. Cultures were treated with NMDA, kainate or glutamate (1mM) for 30 min and $^{45}Ca^{2+}$ uptake was measured as described. Data represent mean ± SEM from 3 separate experiments. A three-way ANOVA showed statistically significant interaction effects among taurine x magnesium x excitatory amino acids [$F(3,80) = 7.89$, $p < .001$]. There was significant main effects of taurine [$F(1,80) = 86.97$, $p < .0001$], magnesium [$F(1,80) = 197.78$, $p < .0001$] and excitatory amino acids [$F(3,80) = 588.5$, $p < .0001$]. Post hoc tests showed that Mg^{2+}caused a significant reduction in NMDA- and glutamate-induced $^{45}Ca^{2+}$ uptake ($p < .001$). The reduction of kainate response by Mg^{2+} was not significant. The reduction of NMDA- and kainate induced $^{45}Ca^{2+}$ uptake by taurine was not significant, whereas glutamate-induced $^{45}Ca^{2+}$ uptake was significantly ($p < .001$) reduced by taurine.

Consistent with previous findings of others, we found that the full activation of the NMDA receptor requires the occupation of both the agonist glutamate or NMDA and the co-agonist glycine. Low concentrations of glycine (10^{-6} - 10^{-5} M) enhanced the NMDA-induced $^{45}Ca^{2+}$ uptake, whereas high concentrations (1mM) inhibited calcium uptake (Fig. 18), indicating a concentration-dependent function of glycine (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Moriyoshi, *et al.*, 1991; Newell *et al.*, 1997). As expected, the induction of calcium uptake with kainate was independent of glycine (Fig. 19). Therefore both kainate and NMDA receptors were expressed and functional in our tissue system.

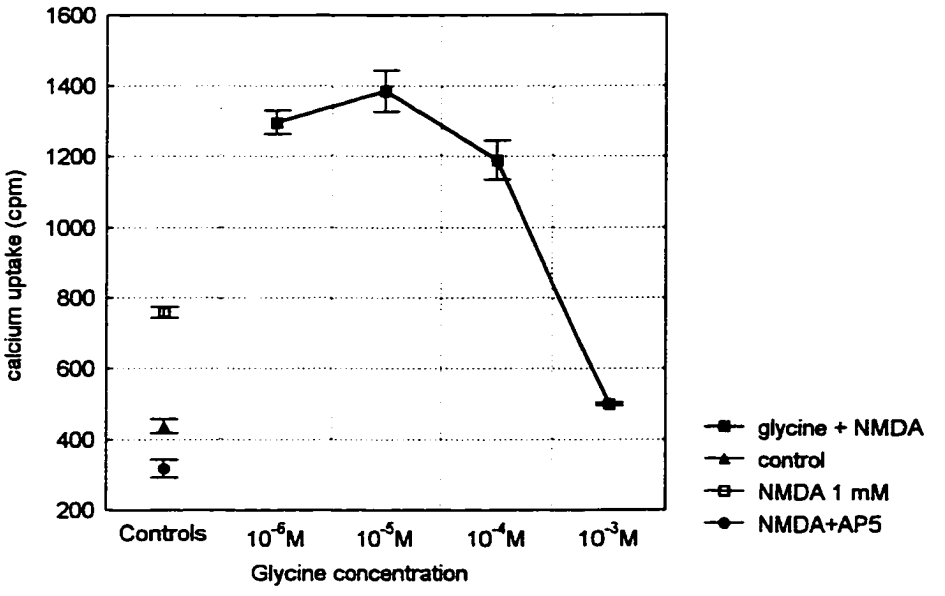


Figure 18. Modulation of NMDA-induced $^{45}Ca^{2+}$ uptake by glycine. NMDA-induced $^{45}Ca^{2+}$ uptake was analyzed in the presence of different concentrations of glycine as indicated. NMDA (1 mM) caused a significant ($p < .001$) increase in $^{45}Ca^{2+}$ uptake over control, which was significantly ($p < .001$) reduced by 10 μM of AP5. Glycine at 10^{-6}, 10^{-5} and 10^{-4} M significantly enhanced ($p < .01$; $p < .02$; $p < .03$, respectively), whereas 10^{-3} M significantly ($p < .005$) reduced the NMDA-induced $^{45}Ca^{2+}$ uptake. Each data point represents mean ± SEM from 3 separate experiments.

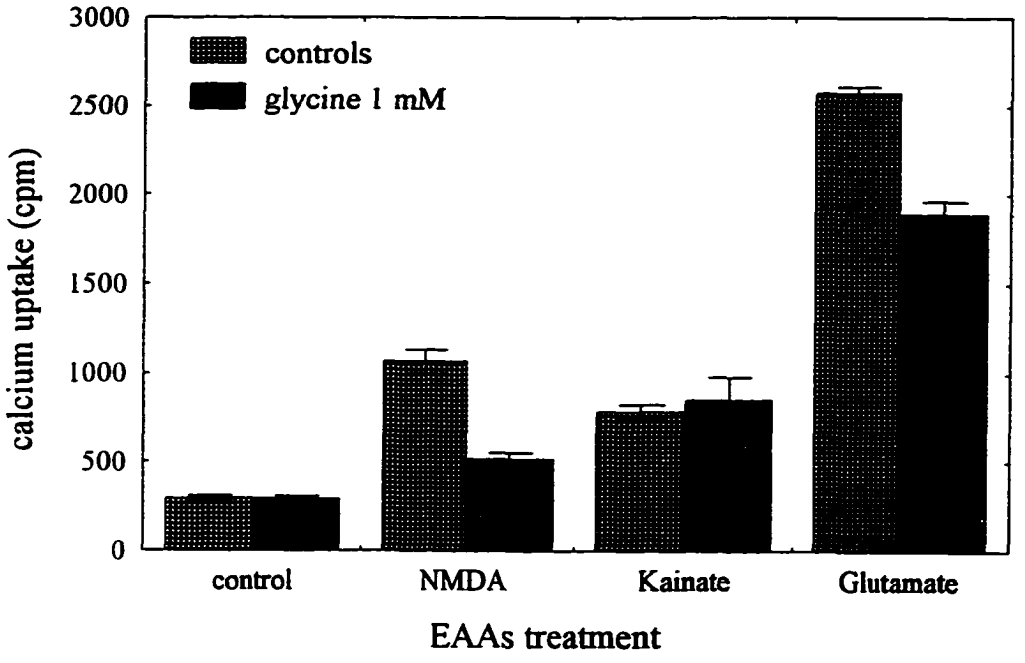


Figure 19. The function of kainate receptors is not modulated by glycine. The effects of glycine on the function of different glutamate receptor subtypes were analyzed in cerebellar granule cells grown in serum-free medium for 5 DIV. $^{45}Ca^{2+}$ uptake was assessed 30 min after adding kainate, NMDA or glutamate (1 mM) with or without 1 mM glycine. Data represent mean ± SEM from at least 3 separate experiments. A two-way ANOVA showed statistically significant interaction effects between glycine and excitatory amino acid agonist treatment [$F(3,16) = 18.18$, $p < .0001$]. There were significant main effects in the presence or absence of glycine [$F(1,16) = 43.55$, $p < .0001$] and EAA-agonist treatment [$F(3,16) = 353$, $p < .0001$]. Post hoc tests showed that, with the exception of NMDA in the presence of glycine, NMDA-, kainate- and glutamate-induced $^{45}Ca^{2+}$ uptake were significantly ($p < .001$) higher than control. Addition of glycine significantly ($p < .001$) reduced the NMDA- and glutamate-induced $^{45}Ca^{2+}$ uptake. The kainate response was not affected by glycine. These data indicate that glycine specifically regulates NMDA receptor function.

In contrast, taurine did not inhibit or interact with NMDA or kainate receptors, but taurine's modulatory role became apparent only after depolarization. The inhibitory neurotransmitter GABA did not interfere with taurine- modulation of calcium uptake, although it was suggested that taurine and GABA shared binding sites (Varga *et al.*, 1994; Saransaari and

Oja, 1994; Wahl *et al.*, 1994; Quinn and Harris, 1995). Therefore, we conclude that taurine did not recognize the GABA binding site, but the inhibition of calcium uptake was mediated through another inhibitory mechanism (Fig. 20).

One possible mechanism could reflect taurine's ability to osmoregulate and thus balance calcium concentrations through ion exchange. This will be subject to further investigations. Taurine has been suggested to function as an organic osmoregulator in the brain (Schurr and Rigor, 1987; Huxtable, 1989; 1992; Pasantes-Morales, 1993; Vitarella *et al*, 1994; Martinez *et al.*, 1994; Pasantes-Morales *et al.*, 1996; Sanchez-Olea *et al.*,1996; Moran *et al.*, 1997).

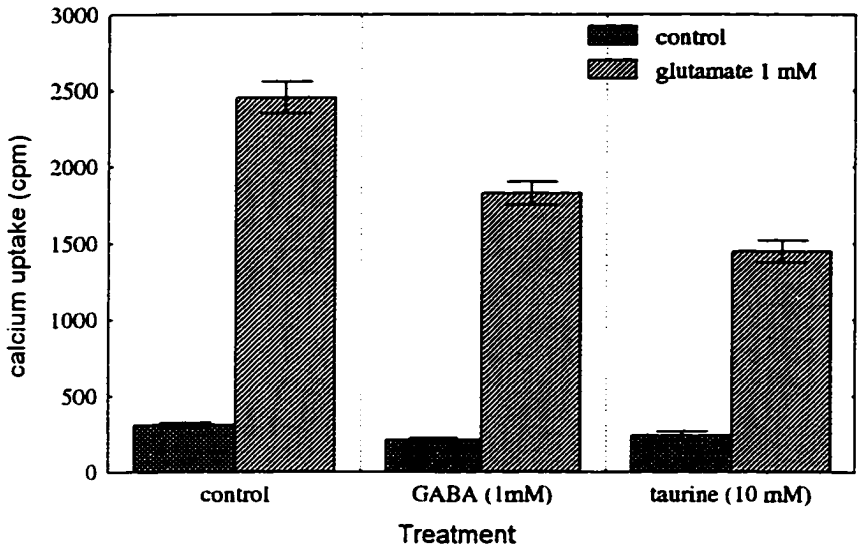


Figure 20. Taurine and GABA modulation of glutamate-induced calcium uptake. $^{45}Ca^{2+}$ uptake was measured in cerebellar granule cells grown in serum-free for 5 DIV, in the presence of 10 mM taurine or 1 mM GABA. Taurine was added 24 h before glutamate depolarization, whereas GABA was simultaneously added with glutamate. In the absence of glutamate, GABA- and taurine-induced $^{45}Ca^{2+}$ uptake was not different from that of untreated controls. Data represent mean ± SEM from 3 separate experiments. Glutamate caused a significant ($p < .001$) increase in $^{45}Ca^{2+}$ uptake, which was significantly reduced by taurine ($p < .001$) and GABA ($p < .01$).

Taurine modulation of glutamate-induced calcium uptake is time-dependent

The possibility of an osmotic exchange mechanism for the reduction of calcium uptake led us to measure the efficiency of taurine over time. Time course studies of glutamate-induced $^{45}Ca^{2+}$ uptake showed that depolarization with 1 mM glutamate caused a significant increase in $^{45}Ca^{2+}$ uptake already beginning after a 2 min stimulation. The linear increase of intracellular $^{45}Ca^{2+}$ uptake continued over time up to 30 min (Fig. 21 A+B).

Taurine interfered with $^{45}Ca^{2+}$ accumulation depending on the time it was added to the cultures. We measured $^{45}Ca^{2+}$ uptake in cultures treated with taurine and glutamate simultaneously (Fig. 21A), and in cultures pre-treated with taurine for 24 h prior to the addition of glutamate (Fig. 21B). At each time point examined, taurine significantly reduced glutamate-induced $^{45}Ca^{2+}$ uptake (Fig. 21B). However, when cultures were treated with taurine at the same time of glutamate depolarization, it took 20 min for taurine to reduce $^{45}Ca^{2+}$ uptake. In contrast, pretreatment of cultures with taurine for 24 h reduced calcium levels at 2 min and up to 30 min of depolarization (Fig. 21B), suggesting that taurine modulation of the glutamate-induced $^{45}Ca^{2+}$ uptake is not mediated at the level of the receptors (GABA, glutamate, glycine), but rather takes place in the cytoplasm, agreeable with an osmoregulatory function. Furthermore, a minimum of 20 min required for taurine to elicit its calcium-modulatory effects, is consistent with a transporter-mediated uptake system rather than a taurine receptor on the cell surface. At this time no taurine specific cell surface receptors have been identified.

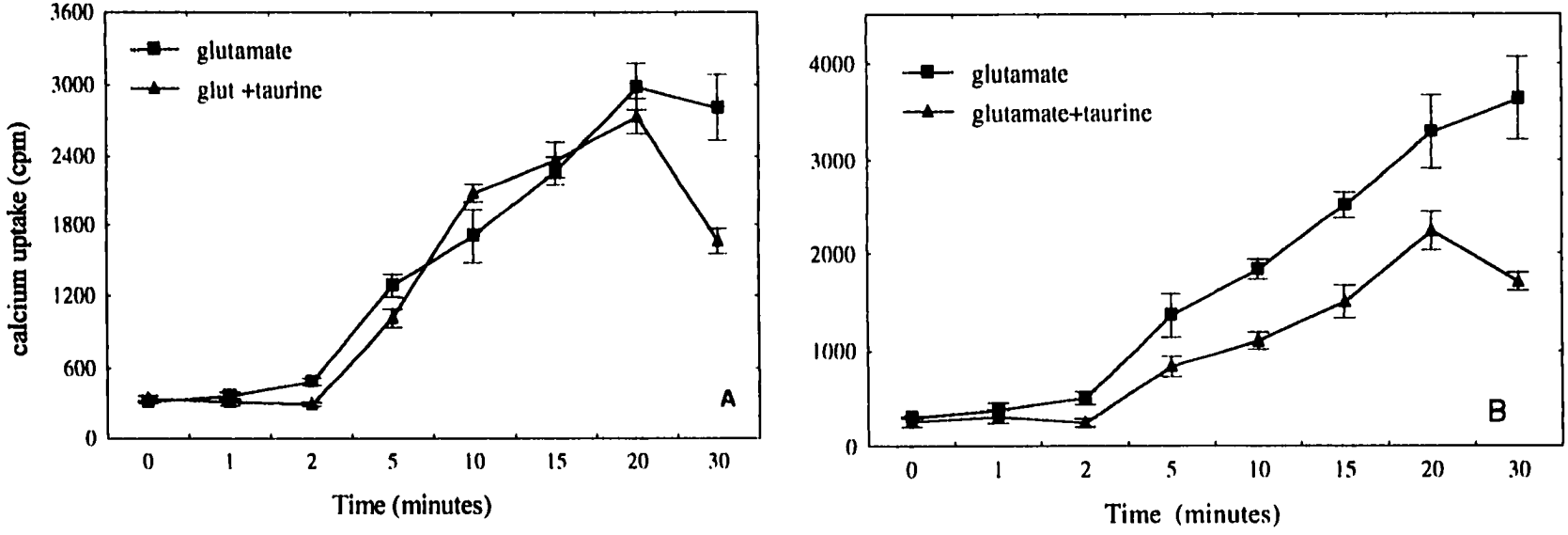


Figure 21. Time course of glutamate-induced calcium accumulation. Calcium uptake was determined over time in enriched cerebellar cultures grown in serum-free medium for 5 DIV. In (A), taurine was added simultaneously with glutamate. In (B), cultures were pretreated with taurine (10 mM) for 24 h before exposure to glutamate. $^{45}Ca^{2+}$ accumulation was determined after depolarization with 1 mM glutamate for the indicated time. Each data point represent the mean ± SEM of three sets of separate experiments. A two-way ANOVA showed statistically significant main effects of taurine treatment [$F(2,48) = 33.88$, $p < .0001$] and time of depolarization [$F(7,48) = 103.92$, $p < .0001$]. The interaction between taurine and time was also significant [$F(14,48) = 5.53$, $p < .0001$]. Post hoc tests indicated that glutamate significantly ($p < .05$) increase in $^{45}Ca^{2+}$ uptake at 5 min, and thereafter ($p < .0001$). When taurine was added simultaneously with glutamate, only after 30 min taurine caused a significant ($p < .001$) reduction in glutamate-induced $^{45}Ca^{2+}$ uptake. However, when cultures were pretreated with taurine, a significant ($p < .05$) reduction was observed after 15 min depolarization.

bFGF and taurine protect against excitotoxicity through modulation of calcium uptake.

Basic FGF was described to prevent excitotoxicity (Mattson and Cheng, 1993). Therefore, we compared the ability bFGF and taurine to control calcium uptake. Basic FGF, similar to taurine, modulated calcium influx in response to glutamate. As shown in figure 22A, bFGF at 10 ng/ml significantly reduced the glutamate-induced $^{45}Ca^{2+}$ uptake, suggesting that bFGF like taurine, possibly protected neurons from glutamate excitotoxicity through regulation of intracellular calcium concentrations. BDNF at 10 ng/ml, on the other hand, had no significant effect on glutamate-induced $^{45}Ca^{2+}$ uptake (Fig.22A), suggesting that BDNF's neuro-protection may be mediated through other mechanisms. However, the combination of BDNF and bFGF (10 ng/ml) significantly reduced glutamate-induced $^{45}Ca^{2+}$ uptake to levels ranging between those of BDNF and bFGF (Fig.22A). Thus it appears that BDNF can modulate bFGF's activity during maintenance of calcium homeostasis. Therefore not only do GFs and NAAs interact, but GFs also interact. Thus, we characterized the role of taurine in the presence of both BDNF and bFGF during glutamate-induced $^{45}Ca^{2+}$ uptake in cultures pretreated with these factors for 24 h. As expected, glutamate-induced $^{45}Ca^{2+}$ accumulation was reduced significantly when cultures were pre-treated with 10^{-2} M taurine or with bFGF (Fig. 22A). Furthermore, taurine was more efficient in reducing the glutamate-induced calcium uptake when cells were pre-treated with either BDNF or bFGF (10 ng/ml). When cells were pre-treated with all three factors however, glutamate-induced $^{45}Ca^{2+}$ uptake significantly exceeded the levels seen with taurine alone or in combination with either growth factor. Under non-depolarizing conditions, on the other hand, $^{45}Ca^{2+}$ uptake with added factors was similar to that of controls.

Simultaneous treatment of cultures with growth factors did not affect the glutamate stimulated $^{45}Ca^{2+}$ uptake, as shown in figure 22B. Growth factors had no effect on the accumulation of intracellular $^{45}Ca^{2+}$ unless the cultures were exposed to these factors prior to glutamate, indicating that growth factors act through an intracellular pathway involving probably post-transcriptional modifications and gene expression as key regulatory elements. We have not pursued this possibility. However, there is extensive data on this subject (Chao, 1992; Barbacid, 1994; Kaplan and Stevens, 1994; Greene and Kaplan, 1995; Levine *et al.*, 1995; Kang and Schumann, 1996; Lauterborn *et al.*, 1996; Bothwell, 1996), pointing to post-transcriptional modifications and gene expression as mediators of growth factor-mediated mechanisms.

Interaction of taurine and bFGF during the modulation of calcium homeostasis.

After realizing that taurine and bFGF activities resulted in the same inhibition of calcium uptake, we raised the question whether these activities were synergistic or additive. First, we examined the synergistic effect of taurine and bFGF on glutamate-induced $^{45}Ca^{2+}$ uptake, as these factors were shown to enhance each other's function (Fig. 22B). Depolarization with 1 mM glutamate resulted in a time-dependent increase in $^{45}Ca^{2+}$ uptake (Fig. 23). This increase was characterized by a fast phase (from 1 to 10 min) and a slow phase (from 10 to 45 min). The slow phase of the curve appears to be characteristic of receptor-desensitization (Brorson *et al.*, 1995; Kyrizis *et al.*, 1995; Jones *et al.*, 1997; Wood and Bristow, 1998). Pretreatment of cultures with taurine alone significantly reduced the glutamate-induced $^{45}Ca^{2+}$ uptake. Interesting is the phase past 20 min. Previously, we found

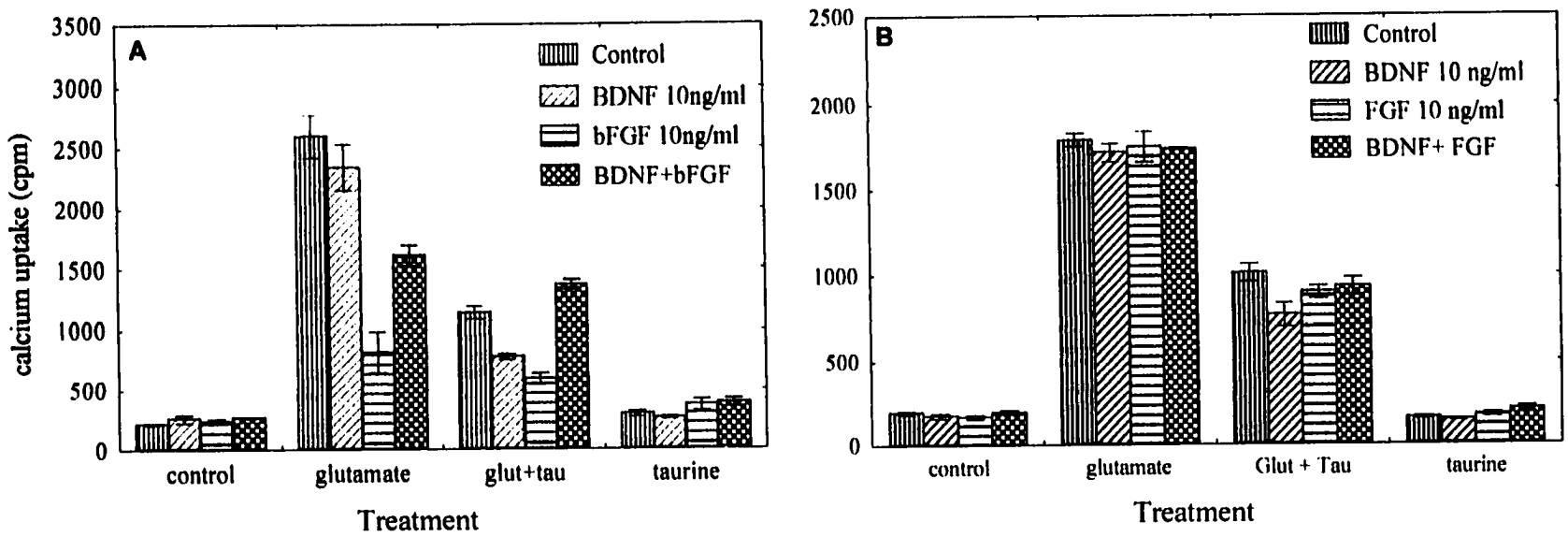


Figure 22. bFGF and taurine protect against excitotoxicity through calcium modulation. The role of bFGF, BDNF and taurine in regulating $^{45}Ca^{2+}$ uptake was determined in neuron- enriched cultures grown in serum-free medium. In (A), Cells were treated with bFGF and BDNF (10 ng/ml) and with taurine (10 mM) or a combination thereof, for 24h. In (B), cultures were pre-treated with taurine for 24 h, but growth factors were added simultaneously with glutamate. Glutamate was added for 30 min with $^{45}Ca^{2+}$ and accumulation was measured as described. Data represent mean ± SEM from 3 separate experiments. In (A), two-way ANOVA showed statistically significant interaction effects between GFs and NAAs [$F(9,32) = 25.10$, $p < .0001$]. There was also a significant main effect of growth factors [$F(3,32) = 32.09$, $p < .0001$] and NAAs [$F(3,32) = 304.52$, $p < .0001$]. Post hoc tests indicated that glutamate caused a significant ($p < .005$) increase in $^{45}Ca^{2+}$ under all conditions examined. bFGF or bFGF and BDNF caused a significant ($p < .001$) reduction in glutamate induced $^{45}Ca^{2+}$ uptake. Taurine alone, or combined with the growth factors significantly ($p < .0005$) reduced glutamate-induced $^{45}Ca^{2+}$ uptake. In (B), the interaction between growth factors and NAAs was not significant [$F(9,32) = 1.38$, $p = .234$]. There was a significant main effect of growth factors [$F(3,32) = 3.7$, $p < .05$] and NAAs [$F(3,32) = 1452$, $p < .0001$]. Post hoc tests indicated that glutamate caused a significant increase in $^{45}Ca^{2+}$ under all conditions examined ($p < .005$). BDNF, bFGF or the combination of the two did not affect glutamate-induced $^{45}Ca^{2+}$ uptake. Taurine on the other hand caused a significant ($p < .005$) reduction in the glutamate response, which was not potentiated by the addition of growth factors.

that when taurine was added simultaneously with glutamate, a significant reduction in glutamate-induced $^{45}Ca^{2+}$ uptake was seen only after 20 min (Fig. 21A). Although we treated the cells with taurine for 24 h, the reduction was more pronounced after 20 min. These data are consistent with a transporter-mediated uptake mechanisms (Moran *et al.*, 1994; Lu *et al.*, 1996).

Treatment of cultures with both taurine and bFGF 24 h prior to glutamate depolarization achieved more reduction in calcium uptake than taurine alone. The kinetics of glutamate-induced increase in $^{45}Ca^{2+}$ uptake with taurine and bFGF showed several phases (Fig. 23). Mainly, a slow increasing phase lasting up to 10 min., a fast increasing phase (from 10 to 20 min.) and a fast decreasing phase (from 20 to 45 min). Whereas glutamate induced a significant $^{45}Ca^{2+}$ increase already after 1 min, the delay of the fast component of the curve in the presence of bFGF and taurine could be interpreted as an allosteric modulation of the channels gating calcium influx. Such a regulation was not seen with taurine alone, indicating that bFGF alone or in combination with taurine, are responsible for the observed effects. Alternatively, bFGF may have caused a functional decrease in the calcium-permeable channels, which lead to longer time for full depolarization to take place. Assuming this was the case, once full depolarization occurs, it will remove the magnesium block from the NMDA channels and activate voltage-dependent calcium channels. Activation of these two channels will lead to the fast component of the $^{45}Ca^{2+}$ uptake. Support for this hypothesis comes from experiments where calcium uptake was analyzed in the presence of bFGF. In cultured hippocampal neurons, bFGF regulated the expression of specific glutamate-receptor subunits. bFGF selectively increased levels of the GluR1 subunit of the AMPA receptor

(Cheng *et al.*, 1995) and suppressed the expression of a 71KDa NMDA receptor protein (Mattson, *et al.*, 1993). This prevented glutamate toxicity (Mattson, *et al.*, 1993).

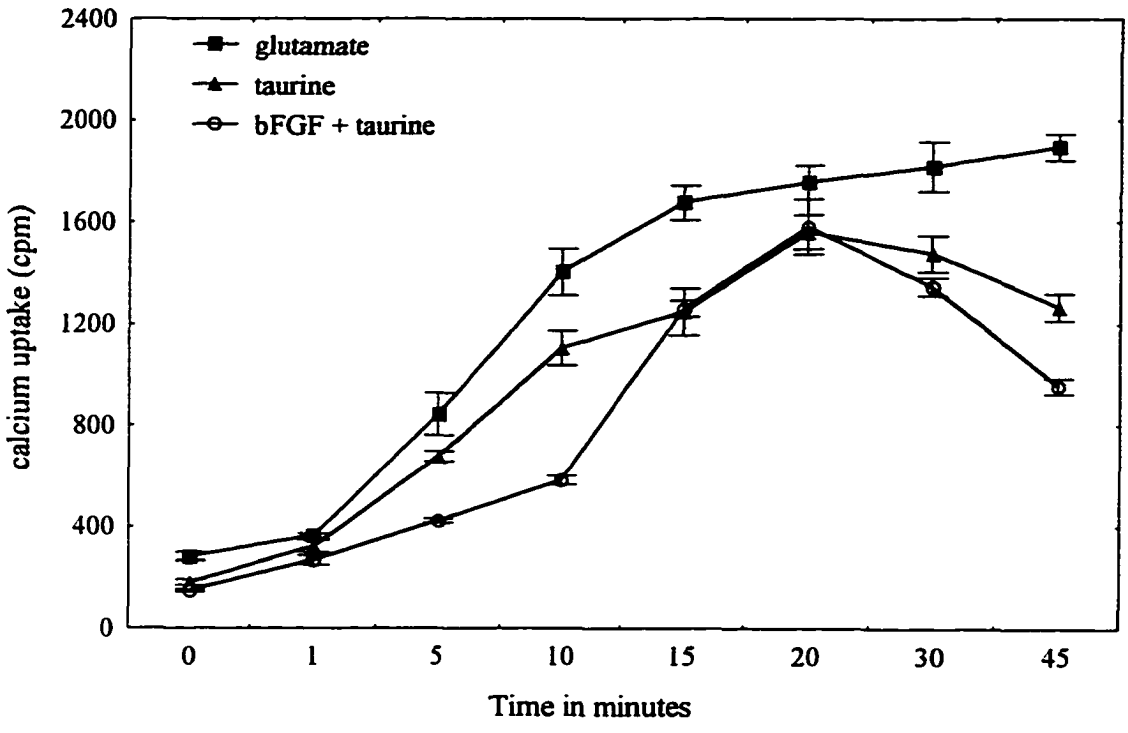


Figure 23. Kinetics of taurine and bFGF synergy during the modulation of calcium homeostasis. The kinetic of glutamate-induced $^{45}Ca^{2+}$ uptake was analyzed in cultures pre-treated with both taurine and bFGF together for 24h before glutamate depolarization. Enriched neuronal cultures of cerebellar granule cells were prepared and treated with taurine and bFGF as described. At 5 DIV, cultures were depolarized with glutamate for the indicated time, up to 45 min. Each data point represents the mean ± SEM of two sets of experiments. A two-way ANOVA showed statistically significant main effects of glutamate treatment [$F(2,120) = 117.63$, $p < .0001$] and time of depolarization [$F(7,120) = 290.54$, $p < .0001$]. The interaction between glutamate and time was also significant [$F(14,120) = 8.62$, $p < .0001$]. Post hoc tests indicated that glutamate, alone or in the presence of taurine, induced a significant ($p < .001$) increase in $^{45}Ca^{2+}$ uptake beginning 1 min after addition, and thereafter ($p < .0001$) up to 45 min. However, with taurine and bFGF, a significant increase was observed only after 10 min. After 5, 10 and 15 min of glutamate addition, in the presence of bFGF and taurine, a significant ($p < .001$) reduction in $^{45}Ca^{2+}$ uptake was observed. However, at 20 min taurine and bFGF+ taurine did not significantly reduce $^{45}Ca^{2+}$ uptake. At 30 and 45 min both taurine and taurine + bFGF caused a significant ($p < .01$). reduction in $^{45}Ca^{2+}$ uptake. Interestingly, at 45 min, the decrease in $^{45}Ca^{2+}$ uptake induced by bFGF and taurine combined was significantly ($p < .05$) lower than that induced by taurine alone.

bFGF selectively down-regulate the function of NMDA receptors

To determine if the calcium-modulatory role of bFGF was mediated through the regulation of the NMDA receptor function, we depolarized the cell with glutamate, NMDA or kainate and measured the $^{45}Ca^{2+}$ uptake (Fig. 24). In cultures treated with bFGF for 24 h prior to depolarization, the $^{45}Ca^{2+}$ uptake was lower than non-treated cultures. However, this reduction was significant only when cells were depolarized with NMDA and glutamate; bFGF had no significant effect on kainate-induced $^{45}Ca^{2+}$ uptake. We conclude from these data that bFGF modulates NMDA receptor function and provides a mechanism for the neuroprotective actions of bFGF during glutamate excitotoxicity. These data agree with findings of Mattson, *et al.*, (1993). Whereas taurine, although producing the same reduction of glutamate-induced $^{45}Ca^{2+}$ uptake as bFGF, functions through a different mechanism. Therefore we conclude that numerous mechanisms exist that regulate calcium homeostasis that may compensate for each other in the event of defects in any single pathway.

Taurine specificity

In order to assure that the effects described were taurine specific, we used taurine analogues as taurine specific uptake blockers. Both β-alanine and guanidinoethan sulfonate (GES) have been used as taurine specific uptake-blockers or competitors (Quesada *et al.*, 1984; Moran and Pasantes-Morales, 1991). We have used both analogues in most of the experiments described, in order to demonstrate taurine's specificity. As shown in figure 25, β-alanine in equal concentrations to taurine (10^{-2}M) did not reduce or inhibit the glutamate induce $^{45}Ca^{2+}$ uptake, as one would have expected if taurine's action was specifically blocked,

neither did β-alanine block taurine's preventive role in excitotoxicity, or interfere with the modulation of $^{45}Ca^{2+}$-uptake, suggesting that taurine has an active role in theses regulatory mechanisms independent of β-alanine and that β-alanine could not be used as a specific uptake blocker for taurine under these experimental conditions (Fig. 25).

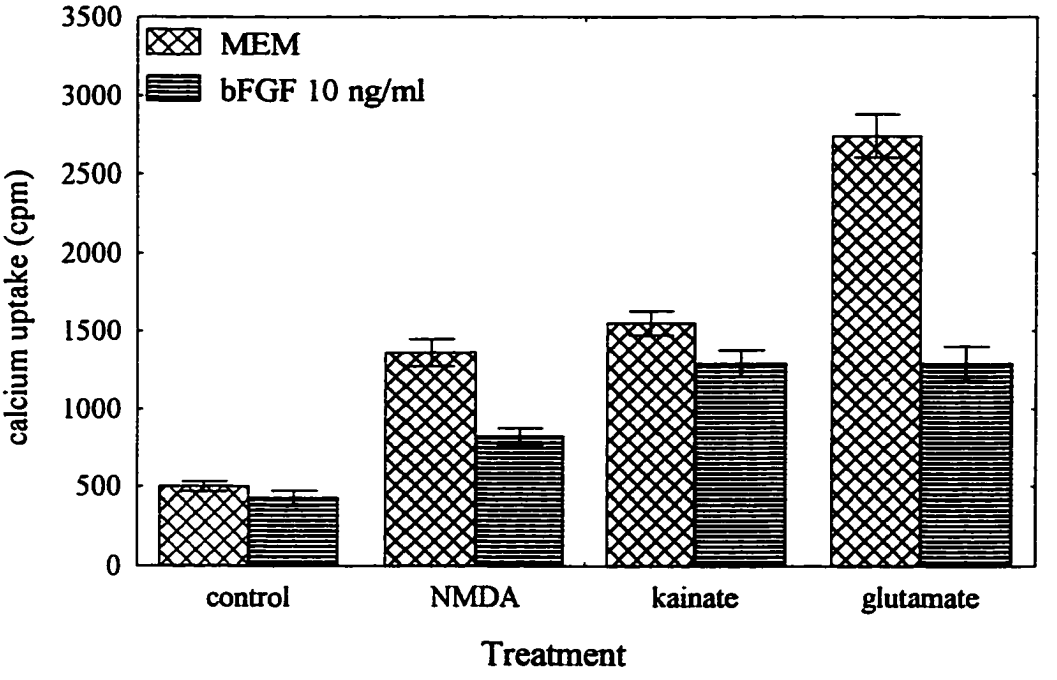


Figure 24. bFGF selectively down-regulates the function of the NMDA receptors. The effects of bFGF on the function of different glutamate receptor subtypes was analyzed in cerebellar granule cells grown in serum-free medium for 5 DIV. $^{45}Ca^{2+}$ uptake was assessed after 30 min depolarization with NMDA, kainate or glutamate at 1 mM. Data represent mean ± SEM from at least 3 separate experiments. A two-way ANOVA showed statistically significant interaction effects between bFGF and excitatory amino acid agonists treatment [$F(3,16) = 25.48$, $p < .0001$]. There was a significant main effect in the presence or absence of bFGF [$F(1,16) = 92.22$, $p < .0001$] and EAA-agonists treatment [$F(3,16) = 115$, $p < .0001$]. Post hoc tests showed that, with the exception of NMDA in the presence of bFGF, NMDA-, kainate- and glutamate-induced $^{45}Ca^{2+}$ uptake were significantly ($p < .001$) higher than control. Pretreatment of cultures with bFGF significantly reduced the NMDA- and glutamate-induced $^{45}Ca^{2+}$ uptake ($p < .01$, $p < .001$ respectively), kainate response was not affected by bFGF. These data indicate that bFGF specifically regulates the NMDA receptor function.

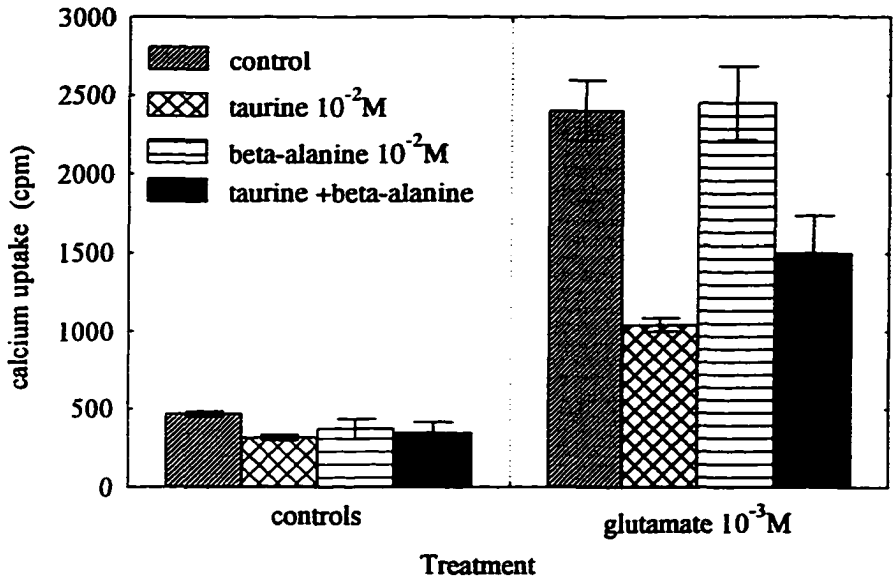


Figure. 25. Effects of taurine and -alanine on glutamate-induced calcium uptake. Glutamate induced-$^{45}Ca^{2+}$ uptake was analyzed in early-postnatal cerebellar granule cells cultured in serum-free medium and pre-treated with taurine, -alanine or a combination of the two at 10 mM. $^{45}Ca^{2+}$ uptake was measured after 30 min depolarization with glutamate. Data represent mean ± SEM from 3 separate experiments. The two-way ANOVA showed a statistically significant interaction effect between glutamate and treatment with taurine and -alanine [$F(3,16) = 10.48$, $p < .0005$]. there was a significant main effects of glutamate [$F(1,16) = 221.88$, $p < .0001$] and taurine and/or -alanine [$F(3,16) = 14.16$, $p < .0001$]. Post hoc tests indicated that glutamate caused a significant ($p < .001$) increase in $^{45}Ca^{2+}$ uptake, which was significantly ($p < .001$) reduced by taurine and by taurine and -alanine. -alanine had no significant effect on glutamate-induced $^{45}Ca^{2+}$uptake.

Secondly, we attempted to measure directly the intracellular concentrations of taurine and -alanine using high pressure ion exchange chromatography (Fig. 26). When cultures were pretreated with taurine (10 mM), the intracellular concentrations of taurine rose approximately 10-fold above untreated controls (from 28 to 254 nmol). Similar levels of intracellular taurine were reached even when cultures were treated with -alanine (before or after treatment with taurine), indicating that the uptake of taurine was independent of -alanine. On the other hand, intracellular concentrations of -alanine reached levels slightly

lower than those of taurine when cultures were treated with β-alanine, indicating that the uptake of β-alanine was not affected by taurine. Intracellular concentrations of β-alanine were undetectable in untreated controls. The concentration of taurine was the highest in untreated cultures compared to other amino acids. Taurine concentration was the highest after glutamate (28 and 24 nmol, respectively). Therefore, taurine must play an important role in the function of cerebellar granule cells. The concentration of the inhibitory neurotransmitter GABA was very low, when compared to glutamate, indicating that most cells in the enriched neuronal cultures are glutamatergic.

We would like to thank Mr. Jeffrey Messing for the technical help in preparing and running the samples on the amino acid analyzer.

Figure 26. Intracellular analysis of amino acids. The intracellular content of amino acids was analyzed in cerebellar granule cells grown under serum-free condition for 5 DIV. B, C, and D are brought to the same scale, A is plotted at a lower scale to accentuate the differences in amino acid concentrations. With the exception of taurine and β-alanine, the concentrations of individual amino acids are not different when compared between different treatments. In (A), untreated cultures, taurine concentration was the highest as compared to other amino acids. Second to taurine, was glutamate, suggesting an important role for taurine in the cerebellum. GABA concentration, was very low as compared to glutamate, indicating that most of the cells in the enriched neuronal cultures are glutamatergic. β-alanine was not detected in these cells. In (B), treatment with taurine resulted in ten folds increase in the intracellular concentration of taurine, with no effects on the concentration of other amino acids, indicating the presence of a high affinity uptake system for taurine. In (C), cells were pretreated with taurine for 1 h followed by β-alanine for 15 min. The intracellular concentration of taurine was not affected by the addition of β-alanine, indicating that β-alanine is not a blocker of taurine uptake. When cells were pretreated first with β-alanine for 1 h followed by taurine for 15 min (D), the concentration of β-alanine was higher than in (C) while taurine's concentration remained unaffected. These data suggest that the uptake of taurine is mediated via a high affinity uptake transporter independent of β-alanine. β-alanine may or may not share the same transporter with taurine, but if so, the affinity is higher for taurine.

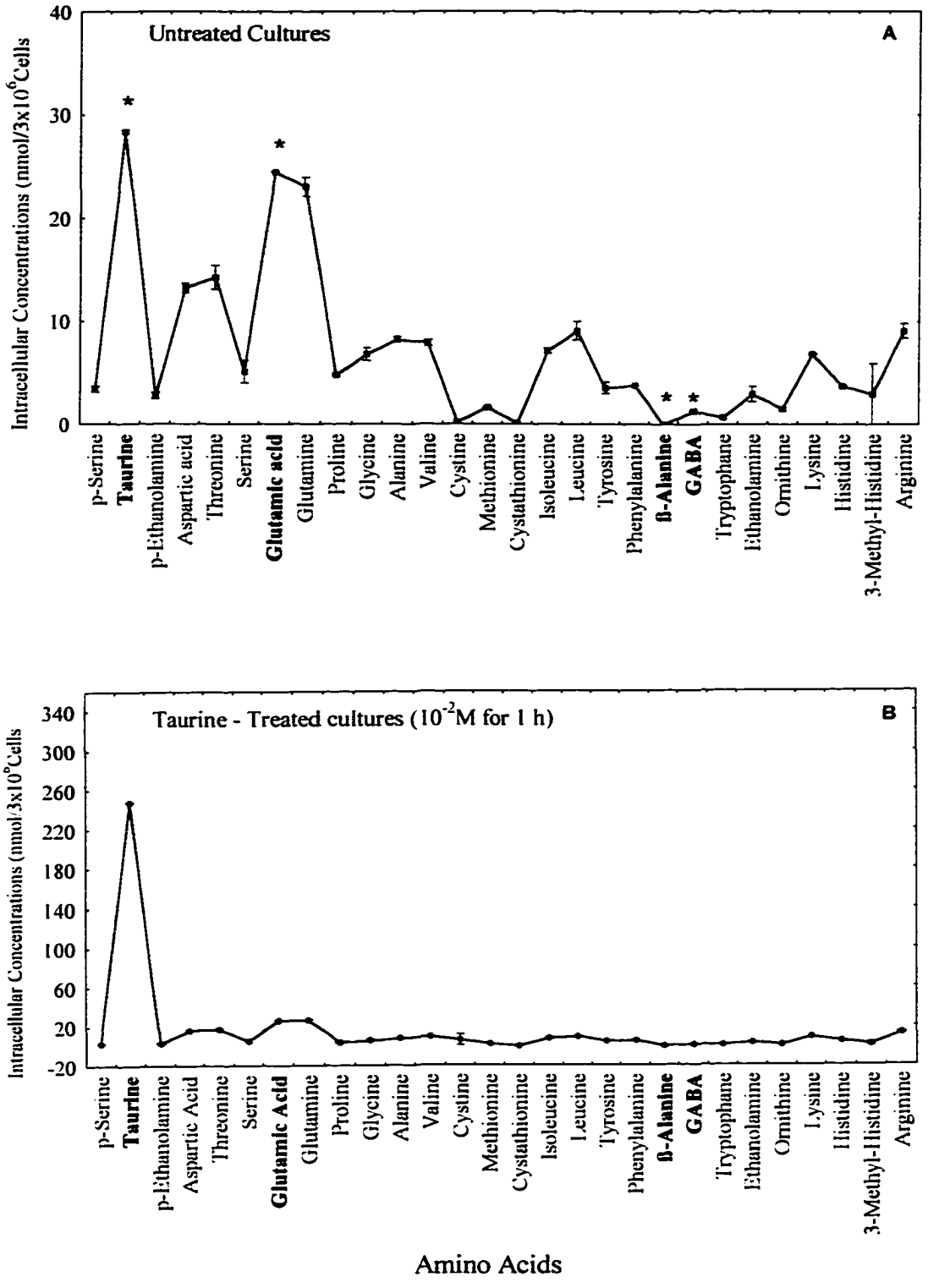


Figure 26 A & B.

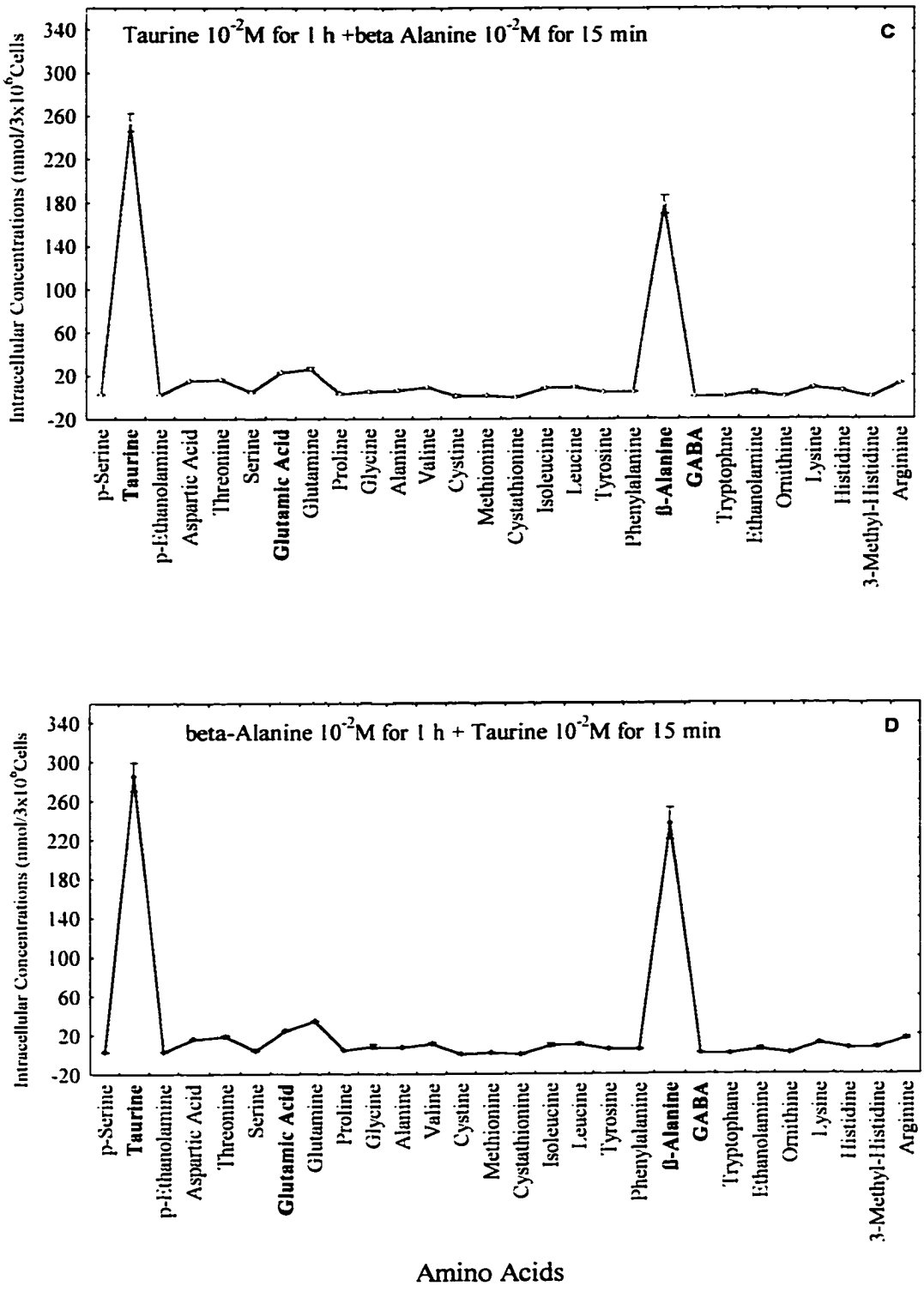


Figure 26 C & D.

CONCLUSIONS

These experiments clearly demonstrate the common use of calcium as a second messenger in response to both GFs and NAAs, and illuminate the complexity of the dynamics of calcium regulation. Maybe because of this, calcium homeostasis is regulated in neuronal cells by several factors. In cerebellar cells, calcium can enter neurons through several routes. These include NMDA receptors, non-NMDA receptors and the VSCCs. Among these channels, the NMDA receptors were the most permeable to calcium. Because of their high permeability to calcium, the function of these subtypes of receptors is extensively regulated. We found that the inclusion of serum in the growth medium significantly down regulated glutamate-induced $^{45}Ca^{2+}$ uptake, through regulation of NMDA receptor function. Furthermore, bFGF also down-regulated the function of the NMDA receptors, probably through down-regulation of receptor expression, as has been suggested for hippocampal and cerebellar cells *in vitro* (Mattson *et al.*, 1993; Brandoli *et al.*, 1998). It is possible that serum contains a factor that mimics bFGF function. In addition to what we have found, NMDA receptor functions are controlled by feedback inhibition (Kyrozis *et al.*, 1995; Rosenmund *et al.*, 1995). There is considerable electrophysiological evidence for a cytoplasmic calcium-mediated feedback inhibition of NMDA receptors in various systems, including cultured neurons, consistent with calcium binding to an inhibitory site on the cytoplasmic face of the receptor or a closely associated protein (Kyrozis *et al.*, 1995; Rosenmund *et al.*, 1995; Krupp and Westbrook, 1995).

Taurine on the other hand, did not seem to affect NMDA receptor function (Fig. 17). Taurine's regulation of calcium homeostasis was dependent on depolarization, regardless of

the depolarizing agent or activated receptors (glutamate, NMDA or kainate). Taurine is highly abundant in the brain, and while its role as neuromodulator is still not clear, its function as an organic osmoregulator in the brain has been firmly established (Huxtable, 1989; 1992; Pasantes-Morales, 1993; Vitarella *et al*, 1994). Our findings showed that taurine did not interact with the NMDA receptors at the glycine site, nor did it interact with GABA receptors. It is possible that taurine balances intracellular calcium homeostasis through its osmo-regulatory function, as was previousely suggested (Schurr and Rigor, 1987; Martinez *et al.*, 1994; Sanchez-Olea *et al.*, 1996; Pasantes-Morales *et al.*, 1996; Moran *et al.*, 1997). This will be subject to further investigation.

The finding that taurine and bFGF both regulated calcium homeostasis and enhanced each other's function in this regulation, suggest to us that neuronal cells possess a variety of defense mechanisms against neuronal cell death and calcium overload. However, one of the important outcomes of this study is that cells may use alternative pathways depending on the availability of these factors in the external environment.

Unlike taurine and bFGF, BDNF did not directly affect glutamate-induced $^{45}Ca^{2+}$ uptake. However, all these factors protected neurons from excitotoxicity. The calcium modulatory role of both taurine and bFGF seems to be a plausible explanation for their excitoprotective role but suggests a different mechanism for BDNF, since BDNF did not regulate calcium homeostasis. However, several reports have shown that BDNF regulates the expression of calcium-binding proteins in neurons. For example, kainic acid-induced seizures increased BDNF and calbindin-D28K mRNAs in hippocampus (Williamson *et al.*, 1997), which were suggested as early responses to maintaining neuronal viability. Or in cultured

striatal neurons, BDNF treatment caused an increase in calbindin-positive neurons under hypoglycemic conditions (Nakao *et al.*, 1995), and BDNF regulated the expression of calcium-binding proteins in developing neocortical neurons (Pappas and Parnavelas, 1997), Purkinje cells (Larkfors *et al*, 1996), hippocampal neurons (Callazo, 1992) and embryonic dorsal root ganglia (Copray *et al.*, 1994). These data indicate that a stabilization of calcium homeostasis, through up-regulation of calcium-binding proteins, might be implicated in the neuroprotective mechanisms of BDNF.

In addition to the various alternative mechanisms that can regulate calcium homoeostasis during excitotoxicity, neuronal energy metabolism must be greatly potentiated to allow concurrent calcium extrusion to occur in response to increases in cytoplasmic calcium concentrations. In the ensuing section of this study, we have examined changes in neuronal energy levels under our experimental conditions, with and without glutamate, growth factors and taurine.

MITOCHONDRIAL ENERGY METABOLISM:

Energy metabolism is recognized as one of the fundamental processes that control the required balance between maintenance of neuronal structures and neuronal function during development and throughout adult life (Trenkner, 1990; Hoyer, 1993; Mattson *et al.*, 1993; Beal, 1995). The mitochondrial electro-chemical gradient (MtECG) is the main provider for cellular energy through a series of five enzyme complexes. The activation of these complexes, the maintenance of the MtECG and ultimately the amount of energy it produces is calcium-regulated (Hertz *et al.*, 1988; White and Reynolds, 1995; Budd and Nicholls, 1996). We, as

have others, recently demonstrated that depleted cellular energy-levels, caused by malfunction of the MtECG, increased the vulnerability towards excitotoxins and neurotoxins leading to neuronal cell death (Budd and Nicholls, 1995, 1996; Trenkner *et al.*, 1996; El Idrissi *et al.*, 1996). In the following experiments, using multiple factor inputs (taurine, bFGF, BDNF, glutamate and FCCP at various concentrations), we tried to define several neuronal energy states besides alive and dead, and investigated differences in neuronal vulnerability after varying the relative concentrations from these different signaling molecules. Here are the main findings.

Growth factors and taurine enhance mitochondrial function

Chen (1989) has developed an assay system that quantitatively evaluates the activity of the MtECG. The level of rhodamine 123 uptake has been shown to be an index of the activity of the MtECG. Using this fluorescent marker, we measured the activity of the MtECG under normal and excitotoxic conditions. We found that treatment of cerebellar granule cells with BDNF or bFGF in serum-free medium increased the activity of the MtECG when compared to untreated controls (Fig. 27). This increase was growth factor-specific since other growth factors, like epidermal growth factor (EGF), did not elicit such increases. Taurine (10 mM) alone or in combination with bFGF, BDNF or EGF significantly increased rhodamine uptake over untreated controls or in cultures treated with growth factors alone. Therefore, different mechanisms and combinations of those factors analyzed here, could lead to trophic support through modulating the activity of MtECG.

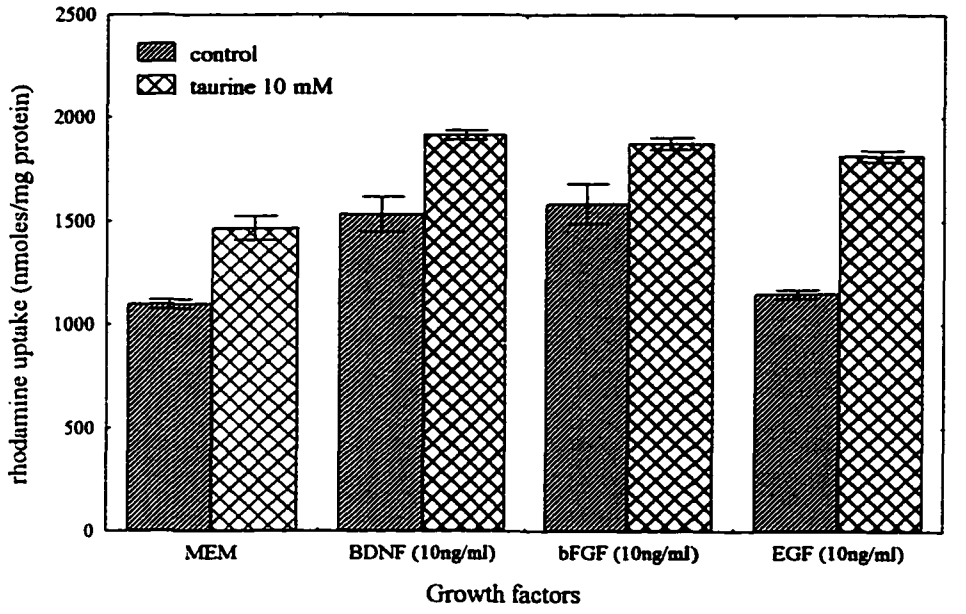


Figure 27. Growth factors and taurine enhance mitochondrial function. Mitochondrial electrochemical gradient as measure of energy levels was determined in neuron-enriched cultures using rhodamine 123 uptake. Cells were pre-incubated for 24 h with taurine or bFGF, BDNF or EGF (10 ng/ml) as indicated. Cells were plated in serum (10 % HS and 5% FCS), switched to serum-free medium after 24 h and grown for another 3 days. Rhodamine accumulation was determined as described in methods. Data represent mean ± SEM from 3 separate experiments. A two-way ANOVA involving the interactions between growth factors and taurine showed significance [$F(12,40) = 7.14$, $p < .001$]. The main effect of growth factors was significant [$F(3,40) = 9.133$, $p < .0001$], and the interaction involving taurine was significant [$F(1,16) = 118.30$, $p < .0001$]. Post hoc tests indicated that BDNF and bFGF caused significant increases in MtECG as compared to baseline ($p < .005$), whereas EGF did not. Taurine always caused a significant increase in the MtECG when compared to baseline or to any growth factor-treated cultures ($p < .05$).

Increased vulnerability to FCCP under serum-free culture conditions.

As shown earlier (Fig. 9 & 10), cultures grown in serum-containing medium proved less vulnerable to glutamate insults, whereas in serum-free conditions, neuronal cells were more sensitive to glutamate. Therefore, we first determined whether serum affected the MtECG and influenced the degree of vulnerability (sensitivity to glutamate), in cultured cerebellar granule cells with or without the addition of taurine and growth factors. As shown

in figure 28, removal of serum from the culture medium significantly decreased the MtECG as compared to cultures grown in serum-containing medium. Addition of carbonyl cyanide p-(trifluoromethoxy) phenyl hydrazone (FCCP), an uncoupler of MtECG at complex III, significantly reduced the MtECG activity. In control cultures, energy levels were significantly lower in cultures grown under serum-free condition. The decreased activity of the MtECG and subsequently the reduced energy levels, could explain the diminished trophic support for cerebellar granule cells grown in serum-free medium (Fig. 9 & 10).

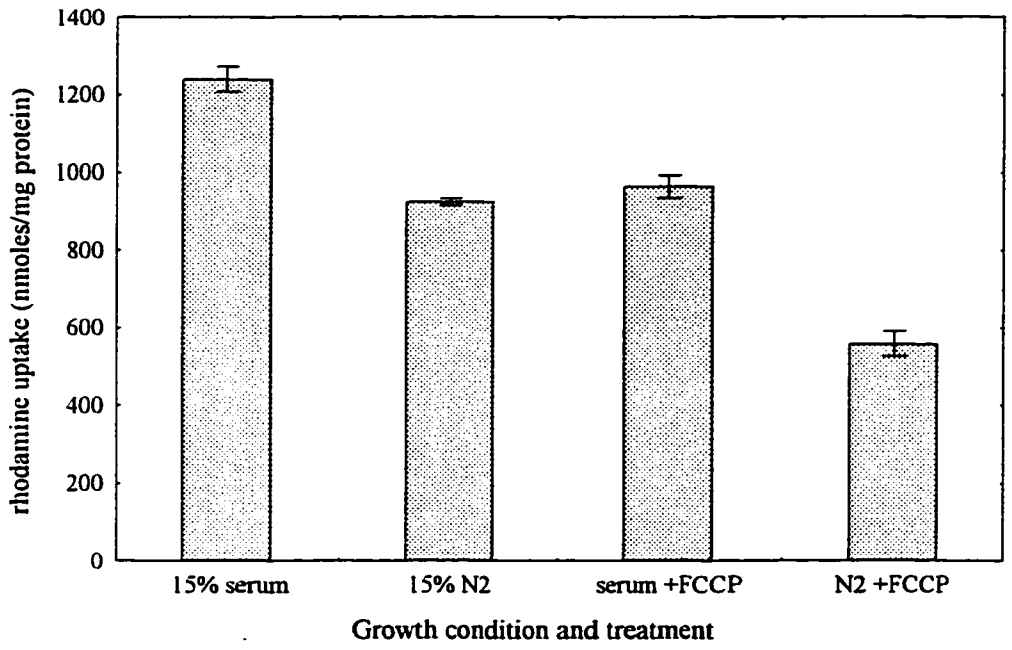


Figure 28. Increased vulnerability to FCCP under serum-free culture conditions. MtECG was compared between cultures grown in serum-containing (10 % HS+ 5 % FCS) or in serum-free medium (15 % N2 supplement) and/or treated with 1 μM FCCP. Removal of serum from the culture medium significantly decreased rhodamine uptake ($p < .001$), indicating a decrease in the MtECG. Treatment with 1 μM FCCP resulted in a significant decrease in rhodamine uptake as compared to controls in both serum ($p < .05$) and serum-free ($p < .01$) medium. The levels of rhodamine uptake in culture grown in serum and treated with FCCP were similar to those cultures grown in serum-free medium and not treated with FCCP. The decrease in MtECG activity under serum-free conditions, may be responsible for the increased vulnerability of cells to FCCP.

Reduction of energy by FCCP is dose-dependent

The differences in mitochondrial function, when cells were grown in serum compared to serum-free media, were significant, but uncoupling the gradient enhanced these differences even more. To characterize the mitochondrial activity in our system, we titrated FCCP concentrations to determine the range at which the uncoupler was effective. As shown in figure 29, rhodamine uptake into CGCs grown in serum-free medium was highly sensitive to FCCP. Concentrations as low as 1 μM caused a significant reduction in the MtECG, whereas 20 μM almost completely shutdown the mitochondrial function and led to neuronal cell death. Therefore, pharmacological inhibition of mitochondrial function was utilized here as a model for pathological conditions that target energy metabolism, to determine the importance of oxidative phosphorylation in regulating neuronal energy and neuronal survival.

Inhibition of the MtECG enhances glutamate excitotoxicity

The impairment of neuronal energy metabolism as a consequence of reduced mitochondrial function was shown to enhance glutamate excitotoxicity. Inhibition of mitochondrial function, here achieved through uncoupling of the MtECG, reduced ATP production, which in turn will lead to defective repolarization and prolonged or inappropriate opening of calcium-permeable channels, and consequently it will reduce the protective voltage-dependent Mg^{2+} block of NMDA receptors (Budd and Nicholls, 1995). Therefore calcium influx through NMDA receptors will be enhanced, creating an even greater demand for ATP. As shown in figure 30, both glutamate (1 mM) and FCCP (0.1 μM) inhibited the MtECG to the same extent. FCCP induced the inhibition in the MtECG through the direct

inhibition of complex III. On the other hand, glutamate induced an extensive calcium overload in these cells. As mitochondria are intracellular stores for calcium, overload of these organelles with Ca^{2+} resulted in the observed decrease in energy levels after excessive glutamate receptor stimulation. On the other hand, with reduced energy levels by FCCP treatment, glutamate caused a far more greater decrease in the MtECG. These data indicate that under pathological conditions that target the mitochondrial function, neurons become highly vulnerable to excitatory stimuli.

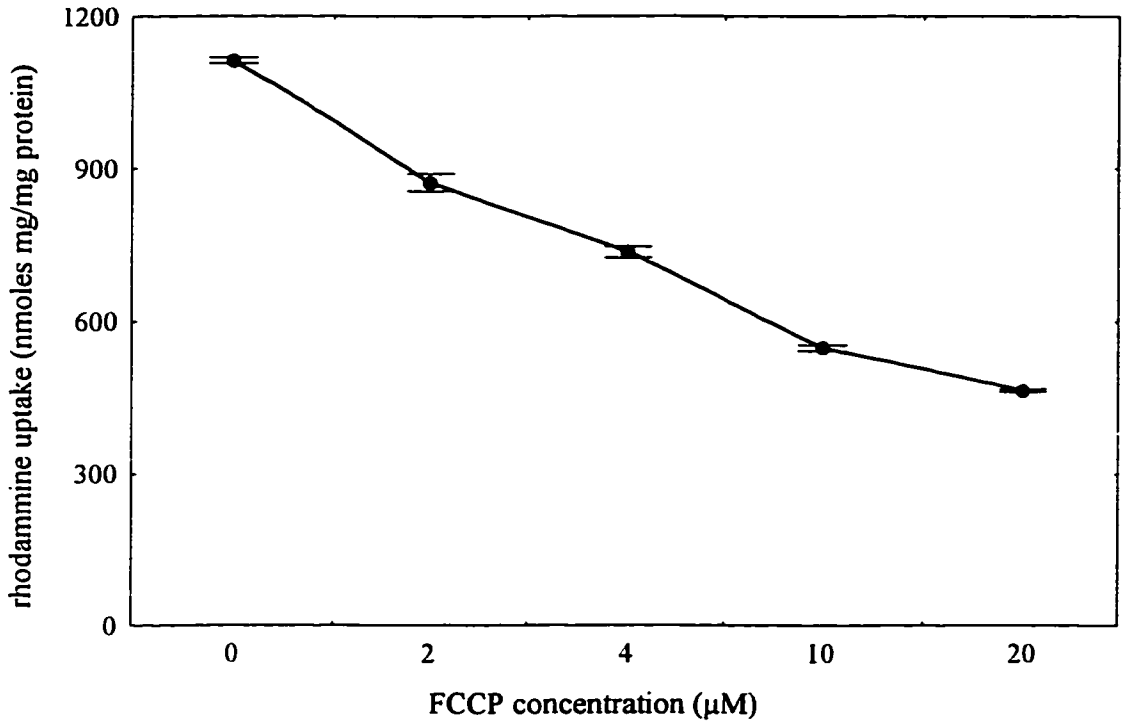


Figure 29. FCCP reduced the MtECG activity dose-dependently. To analyze the pharmacological inhibition of the MtECG by FCCP, we measured rhodamine uptake in the presence of increasing concentrations of FCCP. Cerebellar granule cells were maintained in serum-free medium for 5 DIV, and treated with FCCP for 30 min. There was significant inverse correlation between rhodamine uptake and FCCP concentrations ($r = -.97$, $p < .001$). Each tested concentration yielded a significantly different rhodamine uptake value than the concentration above or below ($p < .05$)

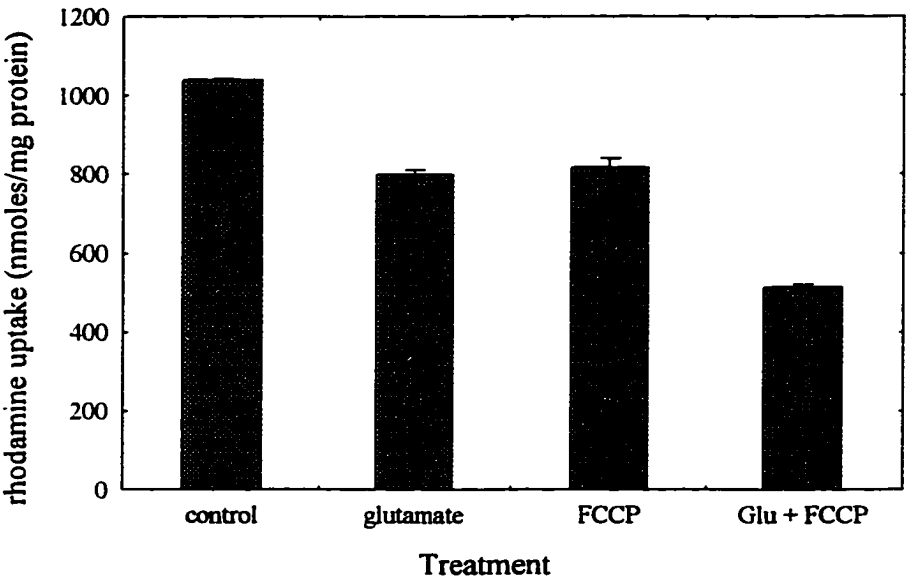


Figure 30. Inhibition of the MtECG enhances glutamate excitotoxicity. To test the idea that impairment in energy metabolism enhances glutamate excitotoxicity, we mildly inhibited complex III of the mitochondrial respiratory chain and treated the cells with excitotoxic concentrations of glutamate. Cerebellar granule cells were grown in serum-free for 5 DIV and treated with glutamate (1 mM), FCCP (1μM) or the combination of the two. Data represent mean ± SEM of three experiments. Glutamate or FCCP caused a significant reduction in rhodamine uptake as compared to untreated controls ($p < .05$). Combination of glutamate and FCCP caused a far more significant decrease in rhodamine uptake as compared to controls and to FCCP- or glutamate-treated cultures ($p < .005$ and $p < .05$ respectively). These data directly demonstrate that impaired energy metabolism exacerbates glutamate excitotoxicity

Serum, BDNF and taurine restored mitochondrial function

We have shown in figure 28 that neurons grown under serum-free conditions have accumulated rhodamine to a lesser extent than when grown in serum-containing medium. In order to determine whether addition of growth factors and taurine to serum-free medium would mimic the serum effects, we added BDNF or BDNF and taurine to cultures grown in serum-free medium. Figure 31 shows that adding BDNF (10 ng/ml) or BDNF and taurine (10 mM) to cultures grown in serum-free medium, increased MtECG-activity compared to

untreated cultures. However, rhodamine uptake did not reach that of cells grown in serum-containing medium, indicating that serum contains other growth factors that enhance mitochondrial function.

We also found that 1 mM glutamate significantly reduced MtECG activity with or without serum, however, the reduction was more pronounced in serum-free medium (Fig. 31). After uncoupling the MtECG with FCCP at concentrations that reduce only slightly the MtECG (0.1 μM) for 30 min, a significant decrease of rhodamine-accumulation was observed only under serum-free conditions, indicating that increased vulnerability must be related to reduced MtECG activity. This became clearer when a combination of subtoxic concentrations of FCCP (0.1μM) and glutamate (0.1 mM) significantly decreased rhodamine-uptake (loss of mitochondrial energy), to reach values similar to those obtained with 1 mM glutamate, 10 times without FCCP. Therefore we conclude that the degree of glutamate excitotoxicity depends on mitochondrial energy.

EAA agonist-induced decrease in the MtECG is not dose-dependent

Unlike inhibition of the MtECG-activity by FCCP (Fig. 29), the inhibition by glutamate receptor agonists was dose-independent (Fig. 32). Although these agonists activate different channels with selective permeability for different ions, their effects on the MtECG was similar and took place within 5 min post-depolarization. Furthermore, these decreases were specific, as pre-incubation with receptor-specific antagonists completely reversed the agonists effect (Fig. 33). Thus the initial energy loss could be in response to the activation of any of the glutamate receptor subtypes, since their activation, directly or indirectly, lead to

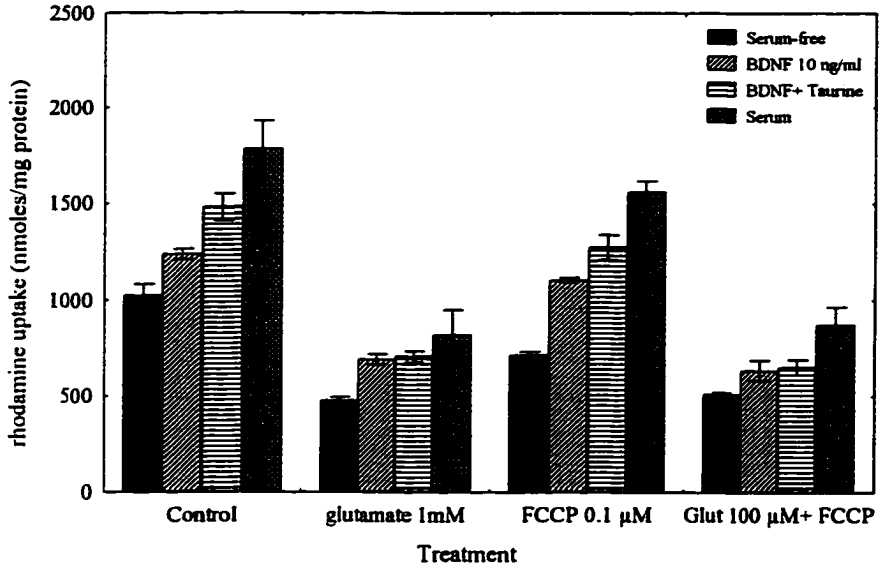


Figure 31. Serum, BDNF and taurine restored mitochondrial function. To determine whether taurine and BDNF would restore the MtECG induced by removal of serum, we cultured cells with or without serum and supplemented them with taurine and BDNF. After 5 DIV, cultures were treated for 30 min with a combination of glutamate (1 mM), FCCP (0.1 μM) or glutamate (100 μM) + FCCP (0.1μM). A two-way ANOVA involving the interactions between growth conditions (serum, serum-free, taurine and taurine + BDNF) and neurotoxic concentration of glutamate and FCCP was not significant [$F(3,32) = 1.56$, $p = .169$]. However, the main effect of growth conditions was highly significant [$F(3,32) = 25.36$, $p < .0001$]. The interaction of glutamate and FCCP was also significant [$F(2,32) = 55.35$, $p < .0001$]. Post hoc tests indicated that addition of serum to the medium significantly increased rhodamine uptake ($p < .005$). The levels of rhodamine uptake in cultures grown in serum were not significantly different from those in cultures grown in serum-free and treated with taurine and BDNF, indicating that these two factors substitute for the factors provided by serum. Treatment with FCCP (0.1 μM) did not decrease rhodamine uptake significantly under all conditions as compared to the corresponding controls. Treatment with (1mM) glutamate significantly decreased energy levels under all conditions as compared to the corresponding controls ($p < .05$). Glutamate (0.1μM) combined with FCCP (0.1 μM) resulted in the same levels of rhodamine reduction as glutamate (1 mM) alone.

cytoplasmic calcium overloads. Since the efficiency of the three glutamate-agonists used in this experiment was concentration-independent, the extent of calcium overload determines the degree of mitochondrial damage. Small increases in intracellular calcium concentrations

during normal neuro-transmission are brought back to resting levels with no subsequent mitochondrial damage, whereas calcium concentrations outside the physiological range led to irreversible mitochondrial damage and excitotoxic cell death. Therefore, the mechanism of prevention of excitotoxicity by taurine and growth factors is probably through lowering the intracellular calcium concentrations and prevention of mitochondrial damage.

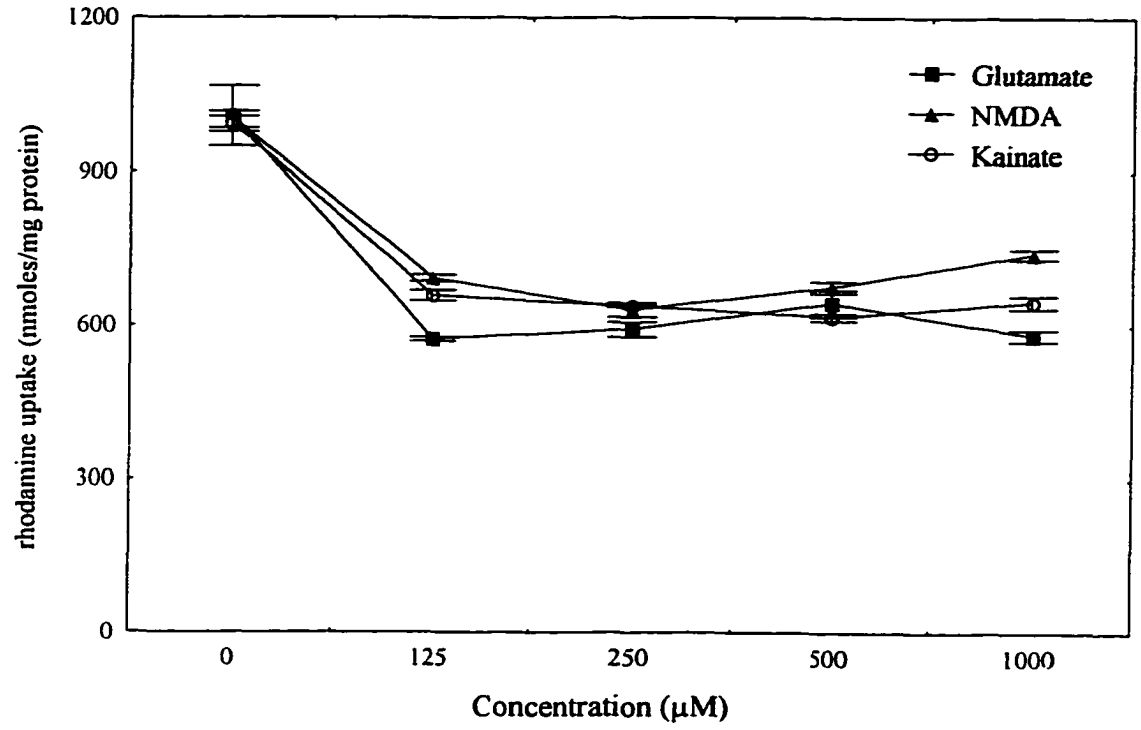


Figure 32. EAA agonist-induced decrease in the MtECG is not dose-dependent. Cerebellar granule cells were prepared in serum-free medium, cultured for 5 DIV and treated for 30 min with different concentrations of NMDA, kainate or glutamate, as indicated. Each data point represent the mean ± SEM of two sets of experiments. A two-way ANOVA showed a statistically significant main effect of EAA agonists treatment [$F(2,30) = 15.22$, $p < .0001$] and their concentrations [$F(4,30) = 203.25$, $p < .0001$]. The interaction between EAA agonists and concentration was also significant [$F(8,30) = 3.81$, $p < .01$]. Post hoc tests indicated that 125 μM of kainate, NMDA or glutamate caused a significant decrease in rhodamine uptake ($p < .001$). This EAA agonist-induced decrease in the MtECG was not potentiated by higher concentrations. Only at 125 and 1000 μM that the decrease in rhodamine uptake induced by glutamate was significantly different for that induced by NMDA, but it was not different from kainate.

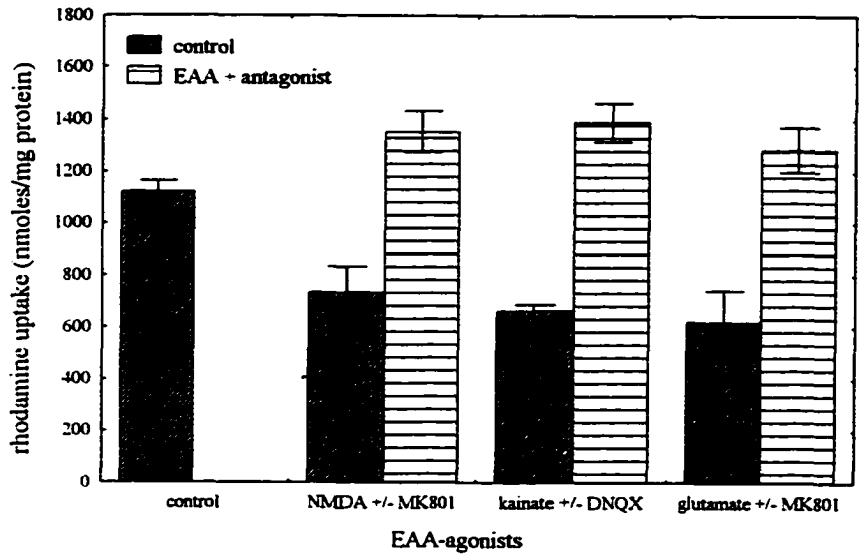


Figure 33. Specificity of EAA agonist-induced decrease in MtECG. To determine the specificity of EAA agonist-induced decrease in rhodamine uptake. we have used different glutamate receptor agonists and their corresponding antagonists. Neuronal cultures were prepared in serum-free medium, after 5DIV rhodamine uptake was measured after 30 min of depolarization with NMDA, kainate or glutamate (1 mM) in the presence or absence of MK801 or DNQX to block NMDA and kainate receptor function respectively. MK801 was used to block glutamate. Antagonists were applied 5 min before depolarization with different agonists. Data represent mean ± SEM of three experiments. A two-way ANOVA involving the interactions between glutamate receptor agonists and antagonists was significant [$F(9,32) = 3.54$, $p < .005$]. Also the main effect of agonists was significant [$F(3,32) = 19.56$, $p < .0001$]. The effect of antagonists was also significant [$F(3,32) = 78.72$, $p < .0001$]. Post hoc tests indicated that NMDA, kainate and glutamate caused a significant reduction in rhodamine uptake ($p < .005$) as compared to baseline. There was no significant difference between the reduction induced by NMDA, kainate or glutamate. In the presence of antagonists, the agonist-induced reduction in rhodamine uptake was eliminated and uptake levels where slightly, but not significantly, higher than controls.

Taurine and growth factors counteracted the glutamate-induced decrease in MtECG

After treating cerebellar granule cells with growth factors and taurine or the combination thereof, a significant increase in the function of the MtECG was observed, while glutamate (0.1 mM) significantly decreased the MtECG. However, pre-treatment with bFGF,

BDNF, taurine and their combinations 24 h before glutamate depolarization restored the MtECG-activity (Fig. 34). This increase in mitochondrial function with both taurine and bFGF under depolarizing conditions may well be the result of the calcium regulation.

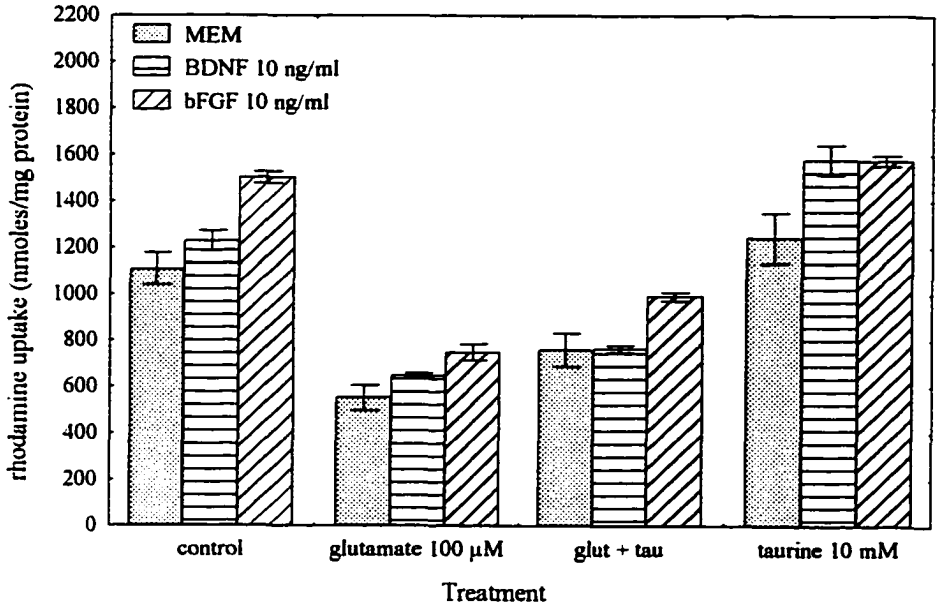


Figure 34. Taurine and growth factors prevented the glutamate-induced decrease in MtECG. In this experiment we tested the ability of growth factors alone or with taurine to stabilize mitochondrial function in the presence of glutamate. Cerebellar granule cells were prepared in serum-free medium as described. At 4 DIV they were treated with BDNF (10 ng/ml), bFGF (10 ng/ml) or taurine (10 mM) as indicated. 24 h later, cultures were depolarized with 100 μM glutamate for 30 min. Data represent mean ± SEM of three experiments. A two-way ANOVA involving the interaction between NAAs and growth factors was significant [$F(6,24) = 2.65$, $p < .05$]. The main effect of NNAs was significant [$F(3,24) = 155.9$, $p < .0001$]. The effect of growth factors was also significant [$F(2,24) = 30.35$, $p < .0001$]. Post hoc tests indicated that in growth factors-treated cultures (first group, controls), BDNF increased (not significant) rhodamine uptake, while bFGF caused a significant ($p < .005$) increase above baseline. In taurine- and growth factors-treated cultures (last group of bars), rhodamine uptake was increased (not significant). However, growth factors significantly enhanced taurine's function as compared to taurine alone ($p < .01$) or as compared to baseline ($p < .001$), suggesting a collaboration. Treatment with glutamate in the presence or absence of growth factors resulted in a significant ($p < .001$) decrease in rhodamine uptake. However, pretreatment with bFGF and taurine prior to glutamate depolarization (last bar in third group), almost completely counteracted the effects of glutamate, as the uptake levels was not statistically different from baseline but significantly ($p < .001$) higher than glutamate.

Similar results were obtained using the mitochondrial stain. JC-1 (Smiley *et al.* 1991). JC-1 is a fluorescent stain that forms aggregates within the mitochondria, due to the activity of the MtECG. Using JC-1 stain allowed for simultaneous analysis of the mitochondrial morphology and electrochemical gradient (Fig. 35 & 46), which can be microscopically visualized. The changes of the electrochemical gradient were reflected by changes in the fluorescent color, from high energy red through orange, to low energy green. Therefore, under the same experimental conditions as for rhodamine uptake, the results using JC-1 confirmed those obtained with rhodamine. There was a change in color from orange to green in the presence of glutamate (1 mM) indicating a decrease in the MtECG activity (Fig. 35).

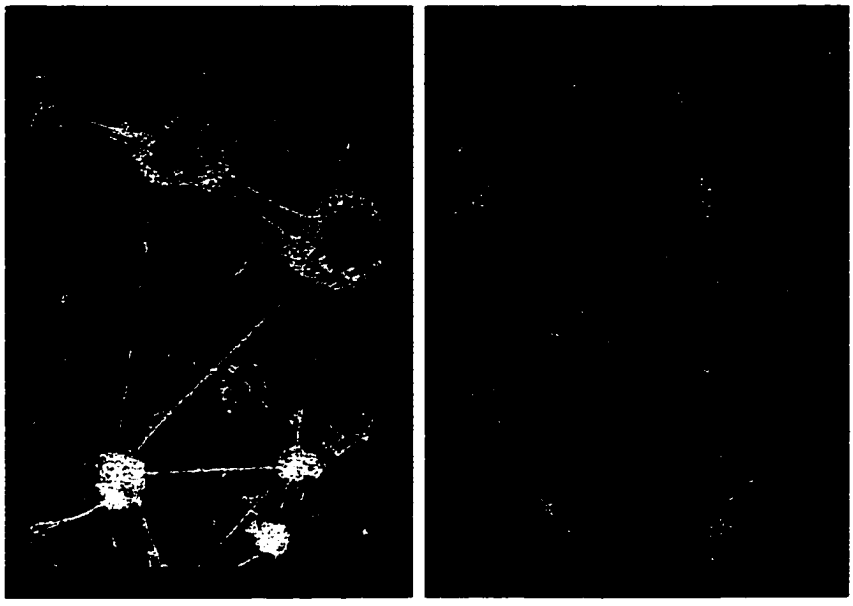


Figure 35. Mitochondrial function as revealed by JC-1. Mixed cerebellar granule cells were cultured as reaggregates. At 3 DIV, when extensive cables have been formed between reaggregates, cultures were treated with glutamate (100 μM) for 30 min. Control cultures are depicted on the left panel, glutamate-treated on the right. The predominant yellow color in control cultures is indicative of high MtEGC and energy, whereas the green color in glutamate-treated cultures (right) reflects low energy levels.

CONCLUSIONS

The findings described above support the idea that the functional and structural integrity of cerebellar granule neurons, and probably any other neurons, depends on an adequate formation and turnover of energy in the form of ATP (Hoyer, 1993). The availability of ATP in turn depends on the functional integrity of mitochondria. This was shown by directly uncoupling the MtECG with FCCP.

Treatment of cerebellar granule cells with low concentrations of FCCP (not toxic) increased significantly the vulnerability to glutamate, and a pronounced reduction in the MtECG was observed. This enhanced vulnerability to glutamate, we assume, is the cause of neuronal cell death; it has been suggested as a mechanism for several neurodegenerative diseases (Hoyer *et al.*, 1991, Hoyer, 1993; Beal *et al.*, 1993). These studies have demonstrated that neuronal energy metabolism and excitotoxicity-associated disturbances in neuronal function are each capable of inducing neuronal death; both excitotoxicity and energy failure are closely associated, the co-occurrence of the two will rapidly culminate in cell death at much lower thresholds.

Calcium uptake studies revealed that intracellular calcium concentrations correlated with extracellular glutamate concentrations and the time of its presence. Furthermore, calcium accumulation, observed after short exposure to glutamate, has been shown to cause mitochondrial damage (Wang *et al.*, 1994; White and Reynolds, 1996). Glutamate, NMDA or kainate treatments resulted in a significant decrease in the MtECG. This decrease however, was not dose-dependent as shown in figure 32. If calcium influx reduces the mitochondrial functions, then small concentrations of excitatory amino acids could reduce the energy only

transiently, whereas high concentrations as shown produce permanent mitochondrial damage followed by cell death.

Taurine, bFGF and BDNF enhanced the mitochondrial function (Fig. 27), suggesting a mechanism for their support of cell survival. Furthermore, these factors reduced the glutamate-induced decrease in the MtECG. It is possible that these effects are mediated through stabilization of intracellular calcium homeostasis during glutamate depolarization. This is supported by the finding that blockade of the glutamate receptors by specific antagonists completely inhibited glutamate-induced cell death (Fig. 8), calcium accumulation (Fig. 12, 14) and decrease in the mitochondrial function (Fig. 33).

To test if the ability of these factors to regulate calcium homeostasis would extend to the regulation of Ca^{2+}-dependent enzymes, we measured the activity of two kinases: protein kinase C (PKC) and protein kinase A (PKA). PKC is Ca^{2+}-dependent, whereas PKA is Ca^{2+}-independent, cAMP-dependent. As shown by others (Favaron, *et al.*, 1990; Mattson, 1991), abnormal activity of protein kinases results in abnormal protein phosphorylation. Since protein phosphorylation plays a pivotal role in cellular signal transduction, and abnormal protein phosphorylation is associated with several neurodegenerative diseases, we sought to analyze the activity of protein kinases and the resulting protein phosphorylation pattern.

PROTEIN KINASES AND PROTEIN PHOSPHORYLATION:

Protein phosphorylation is widely recognized as an important event in transmembrane signal transduction. It is therefore not surprising that a large number of protein

phosphorylating enzymes - protein kinases- exist. In general, they are divided into serine/threonine kinases and tyrosine kinases on the basis of the phosphate-accepting residue in the substrate protein.

PKC is a serine/threonine kinase that was first characterized on the basis of its activation *in vitro* by Ca^{2+}, phospholipids and diacylglycerol (Nishizuka, 1992; Asaoka *et al.*, 1992). It has been established that PKC is not a single entity and that many closely related PKC isotypes exist, perhaps providing an explanation for the range of processes in which PKC has been implicated.

As suggested by Mattson, (1991) pathologically increased activity of protein kinases are involved in neuronal degeneration and death in cultured neurons, to mediate neurotoxic actions of glutamate (Favaron, *et al.*, 1990), and participate in the development of brain pathology triggered by ischemia (Damanska-Janik and Zalewska, 1992). Both *in vitro* and *in vivo*, neuroprotection was achieved by administering the PKC inhibitors H7 or staurosporine (Mattson, 1991; Candeo *et al.*, 1992), supporting the view that hyper-phosphorylation is involved in neuronal death.

In the ensuing set of experiments we have examined the activity of PKC and PKA in the presence of growth factors and NAAs. Since taurine and growth factors modulated Ca^{2+} uptake, they are expected to regulate calcium-dependent PKC- activity. This regulation could serve as a further example for taurine's role as calcium homeostasis-modulator. Furthermore, regulation of protein kinases should be revealed by changes in protein phosphorylation.

Taurine, BDNF and bFGF down-regulate glutamate-induced PKC activity

PKC has been shown as one of the calcium-activated enzymes linking glutamate receptor activation to excitotoxicity (Choi, 1990). We investigated the effects of taurine and growth factors on the activity of this enzyme. As has been reported earlier (Coffey *et al.*, 1993; Eboli *et al.*, 1994), glutamate (1 mM) induced a significant increase in PKC activity (Fig. 36). Twenty-four h pretreatment of the cultures with taurine (10mM), prevented this increase and led to lower activity levels than untreated control cultures (Fig. 36). Similarly, addition of BDNF or bFGF (10 ng/ml) prevented the increase in PKC activity in response to glutamate (Fig. 36). When cells were pretreated with both taurine and bFGF significant decrease in PKC activity was observed. Taurine and BDNF on the other hand did not change PKC-activity. These data suggest that taurine, BDNF and bFGF modulate PKC activity, probably through regulation of intracellular calcium, as these factors were found to affect intracellular calcium concentrations.

Interaction of growth factors and taurine during the regulation of PKA activity

PKA is an essential regulator of various forms of synaptic plasticity and the activity of different ion channels, including NMDA and non-NMDA receptors (Leonard and Hell, 1997). Here, we analysed PKA activity in the presence of growth factors and NAAs. The advantage of the assay used is that it allows us to determine both the amount of activity induced by different treatment - active PKA - and the activity induced by exogenously adding cAMP to the lysate -total PKA -, which is an indication of the total amount of the enzyme available.

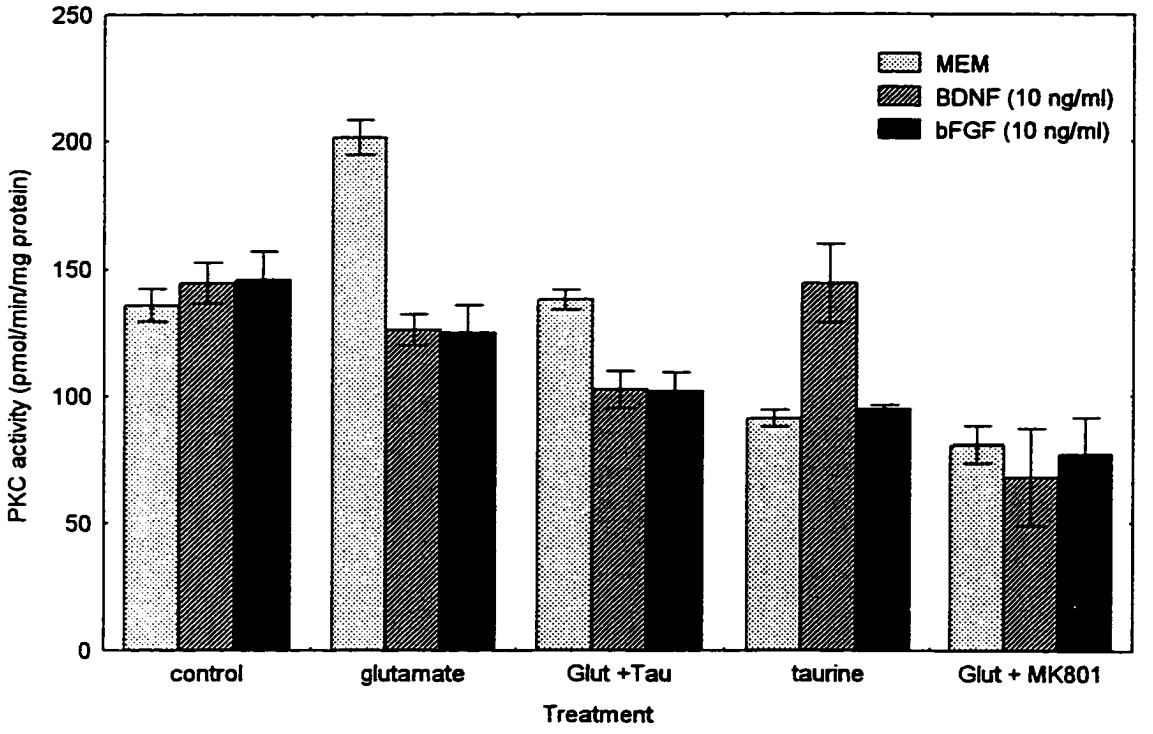


Figure 36. Regulation of PKC by taurine, BDNF and bFGF. Cerebellar granule cells were cultured in serum-free medium for 4 DIV, and treated with BDNF, bFGF (10 ng/ml) or taurine (10 mM) 24 h before the assay. Cells were depolarized with glutamate with or without these factors for 30 min. subsequently culture medium was removed and cells were lysed in ice-cold homogenization buffer as described. Data represent the mean ±SEM of three separate experiments. A two way ANOVA involving the interactions between NAAs (glutamate and taurine) and growth factors (BDNF and bFGF) were significant [$F(8,30) = 5.94$, $p < .001$]. The main effect of NAAs was highly significant [$F(4,30) = 27.69$, $p < .0001$]. The effect of growth factors was also significant [$F(2,30) = 10.74$, $p < .001$]. Post hoc tests indicated that glutamate significantly ($p < .005$) increased PKC activity as compared to controls. Pre-treatment with BDNF, bFGF and/or taurine inhibited the glutamate-induced PKC activity, and the resulting activity levels were not statistically different from controls. Addition of MK801 (10 μM), 2min before glutamate depolarization, blocked activity levels significantly ($p < .05$) even lower than controls.

Unlike other protein kinases, PKA consists of two different types of subunits that dissociate when the enzyme is activated; a regulatory (R) subunit, which is the receptor for cAMP, and a catalytic (C) subunit which phosphorylates the substrate. In the absence of cAMP, the enzyme exists as an inactive tetramer, R_2C_2. When intracellular cAMP levels are elevated, the cyclic nucleotide binds with a high affinity to the R subunit. This decreases the affinity of the R subunit to the C subunit by approximately four orders of magnitude and leads to dissociation of the holoenzyme into an $R_2.(cAMP)_4$ dimer and two free C subunits that are catalytically active (Taylor *et al.*, 1988).

By inducing changes in intracellular cAMP levels with NAAs and growth factors, we will gain a certain activity level of PKA -called active PKA-. These cAMP-levels may not be high enough to activate all the available enzyme. Therefore, by adding exogenous cAMP to aliquots of cell lysates, all the available enzyme -total PKA- should be activated. This measure would indicate the availability of the enzyme. As shown in figure 37, treatment of cerebellar granule cells, grown under serum-free conditions, with glutamate, taurine, bFGF, BDNF or the combination thereof, had no measurable effect on the active PKA levels.

In the cell-free system, increasing cAMP revealed different modulatory effects of NAAs and GFs. Taurine and glutamate, alone or in combination, had no effects on PKA activity, whereas, BDNF and bFGF induced a significant change. Mainly, BDNF in the presence of taurine reduced significantly the activity, bFGF alone or in combination with taurine increased the activity significantly. BDNF-induced decreases and bFGF-induced increases in PKA-activity were neutralized when these two factors were combined (Fig. 37).

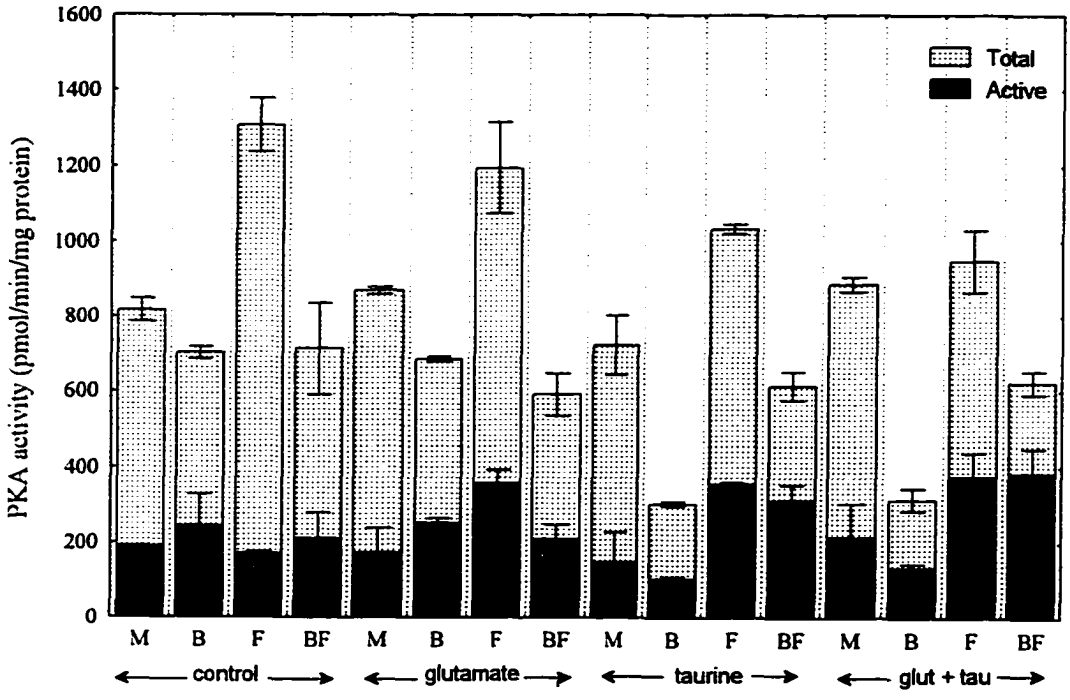


Figure 37. Growth factors differentially regulate PKA. Cerebellar granule cells were cultured in serum-free medium for 5 DIV, and treated with BDNF, bFGF (10 ng/ml) or taurine (10 mM), or the combination thereof, 24 h before the assay. Cells were depolarized with glutamate in the presence of absence of these factors for 30 min. Culture medium was removed and cells lysed in ice-cold homogenization buffer as described. PKA activity was determined in the absence of exogenously added cAMP "active PKA" or in the presence of added cAMP "total PKA". Data represent the mean ±SEM of three separate experiments. Abbreviations: M: MEM+15%N2, B: BDNF, F: bFGF, B+F: BDNF+bFGF. For active PKA, a two way ANOVA involving the interactions between NAAs (glutamate and taurine) and growth factors (BDNF and bFGF) was not significant [$F(9,16) = 1.16$, $p=.38$]. The main effect of NAAs was not significant [$F(3,16) = .96$, $p=.43$]. The effect of growth factors was also not significant [$F(3,16)= 3.1$, $p = .056$]. For total PKA, a two way ANOVA involving the interactions between NAAs (glutamate and taurine) and growth factors (BDNF and bFGF) was significant [$F(9,16) = 2.86$, $p< .05$]. The main effect of NAAs was significant [$F(3,16)= 10.57$, $p < .001$]. The effect of growth factors was also significant [$F(3,16)= 95.46$, $p < .0001$]. Post hoc tests indicated that "total PKA" was mainly regulated by growth factors and taurine, but not with glutamate. bFGF alone or in the presence of glutamate, taurine or both, caused a significant ($p< .05$) increase as compared to baseline. BDNF on the other hand, caused a significant ($p< .005$) decrease, only when taurine was present (last two panels). The combination of BDNF and bFGF resulted in levels of activity not significantly different from controls.

Thus we conclude that growth factors influence the intracellular availability of PKA, and suggest that the regulation of PKA by growth factors may be mediated through the expression of this enzyme. Furthermore, the PKA-modulation by growth factors was enhanced by the presence of taurine, suggesting another level of interaction between these factors.

Regulation of protein phosphorylation by growth factors and neuroactive amino acids

BDNF and bFGF exert their biological activity by binding to specific receptors, FGFR1 and TrkB, respectively, that possess an intrinsic tyrosine kinase activity, whereas glutamate activates cell surface receptors that are linked to ion channels permeable mostly to Na^+ and Ca^{2+}. Taurine, on the other hand, does not bind to cell surface receptors but rather to a high affinity uptake transporter. We found that regulation of intracellular calcium homeostasis was common to these signaling molecules. Thus we examined the role of calcium regulation on protein phosphorylation.

Western blot analysis of phosphorylated proteins indicated that BDNF, bFGF and glutamate all induced a time-dependent increase in protein phosphorylation on both serine/threonine and tyrosine residues (Fig. 38 A-D). As expected, glutamate induced a strong serine/threonine compared to tyrosine phosphorylation (Fig. 38 A&B) due to the second messenger systems triggered by glutamate, mainly calcium. Thus, indicating the dependency of serine/threonine kinases on calcium, as compared to glutamate-stimulated tyrosine kinases. Taurine on the other hand, reduced glutamate-induced phosphorylation (Fig. 38 A&B), probably through regulation of intracellular calcium homeostasis, demonstrating once again taurine's balancing effects under glutamate-depolarization.

Treatment with the growth factors BDNF and bFGF also induced protein phosphorylation on both serine/threonine and tyrosine residues (Fig. 38 C&D). BDNF- and bFGF-induced protein phosphorylation was more rapid (5 min) as compared to glutamate induction, which took place 10 min after glutamate addition. While growth factors initiate signaling mechanisms by directly binding to cell surface receptors and mediating auto-phosphorylation of these receptors and subsequently other regulatory proteins, glutamate induces influx of extracellular ions, mainly calcium, that activate protein kinases. Thus this may explain the rapid induction of protein phosphorylation by growth factors when compared to glutamate.

Examination of the serine/threonine and tyrosine phosphorylation patterns induced by BDNF, bFGF and glutamate indicate that some of the phosphorylated proteins, based on molecular weight, may be commonly induced by these factors. Although speculative at present, these findings might suggest a convergence in the signaling mechanisms of these molecules.

Figure 38. Regulation of protein phosphorylation by growth factors and NAAs. Protein phosphorylation was analyzed in cerebellar granule cells grown in serum-free-medium and treated for the indicated times with BDNF, bFGF, glutamate and taurine. Phosphorylated proteins were detected with monoclonal antibodies against phospho-tyrosine, serine- and threonine. Anti-phospho-serine and threonine antibodies were simultaneously used because substrate proteins are usually phosphorylated on both residues. Lines represent phosphorylation profiles which were obtained by quantifying all individual bands under the same length of treatment.

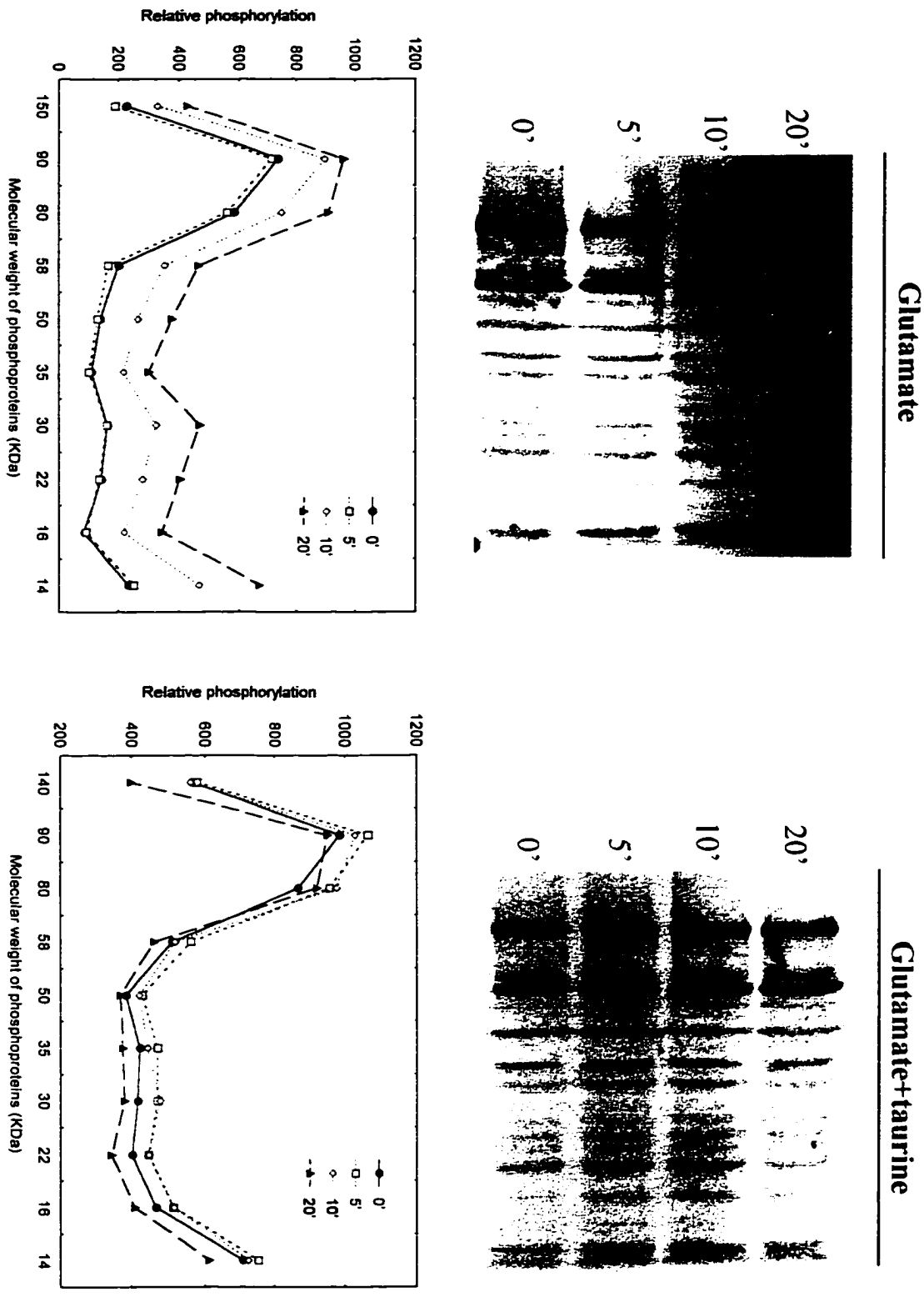


Figure 38 A. Glutamate-induced serine/threonine phosphorylation: regulation by taurine.

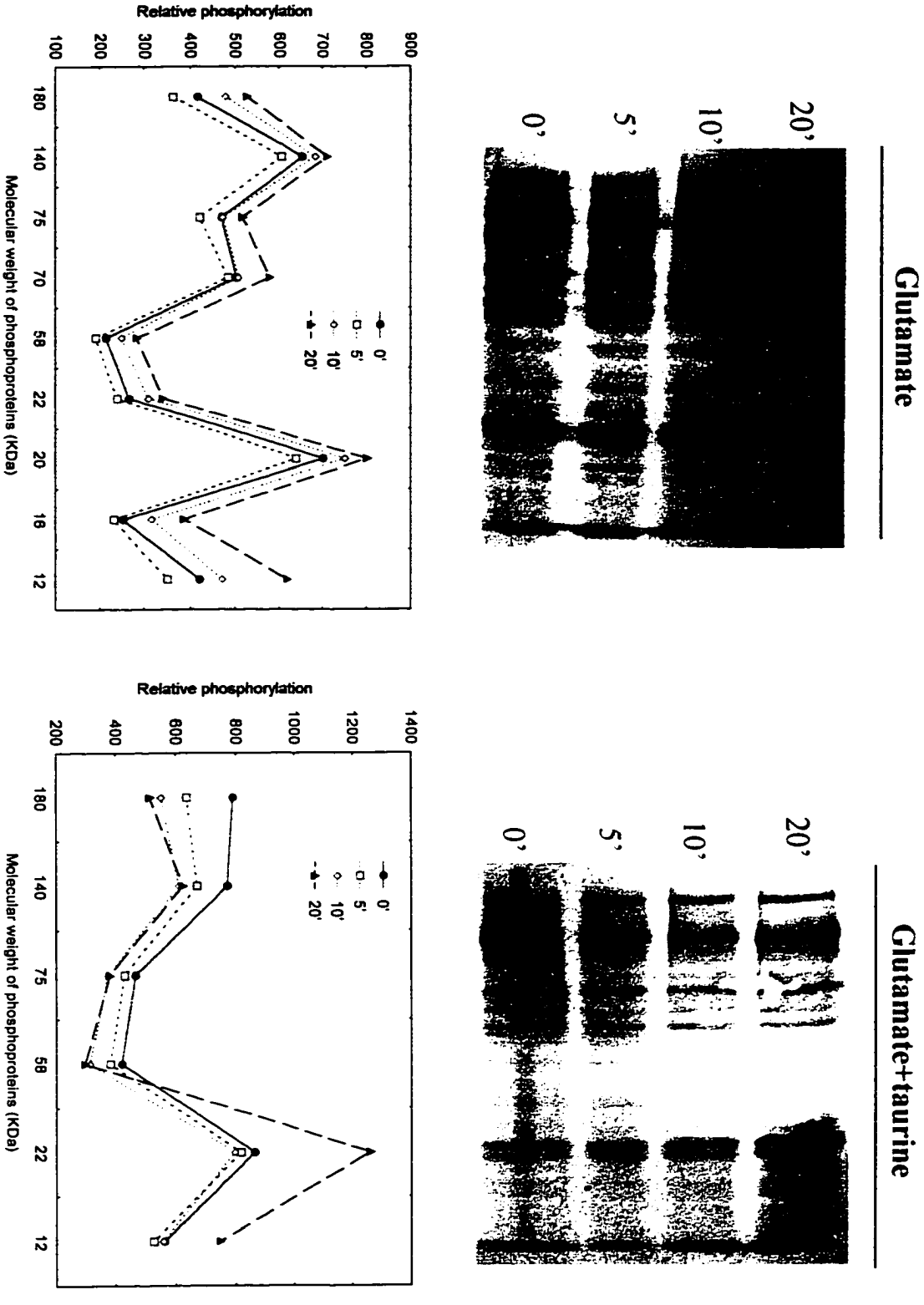


Figure 38 B. Glutamate-induced tyrosine phosphorylation: regulation by taurine

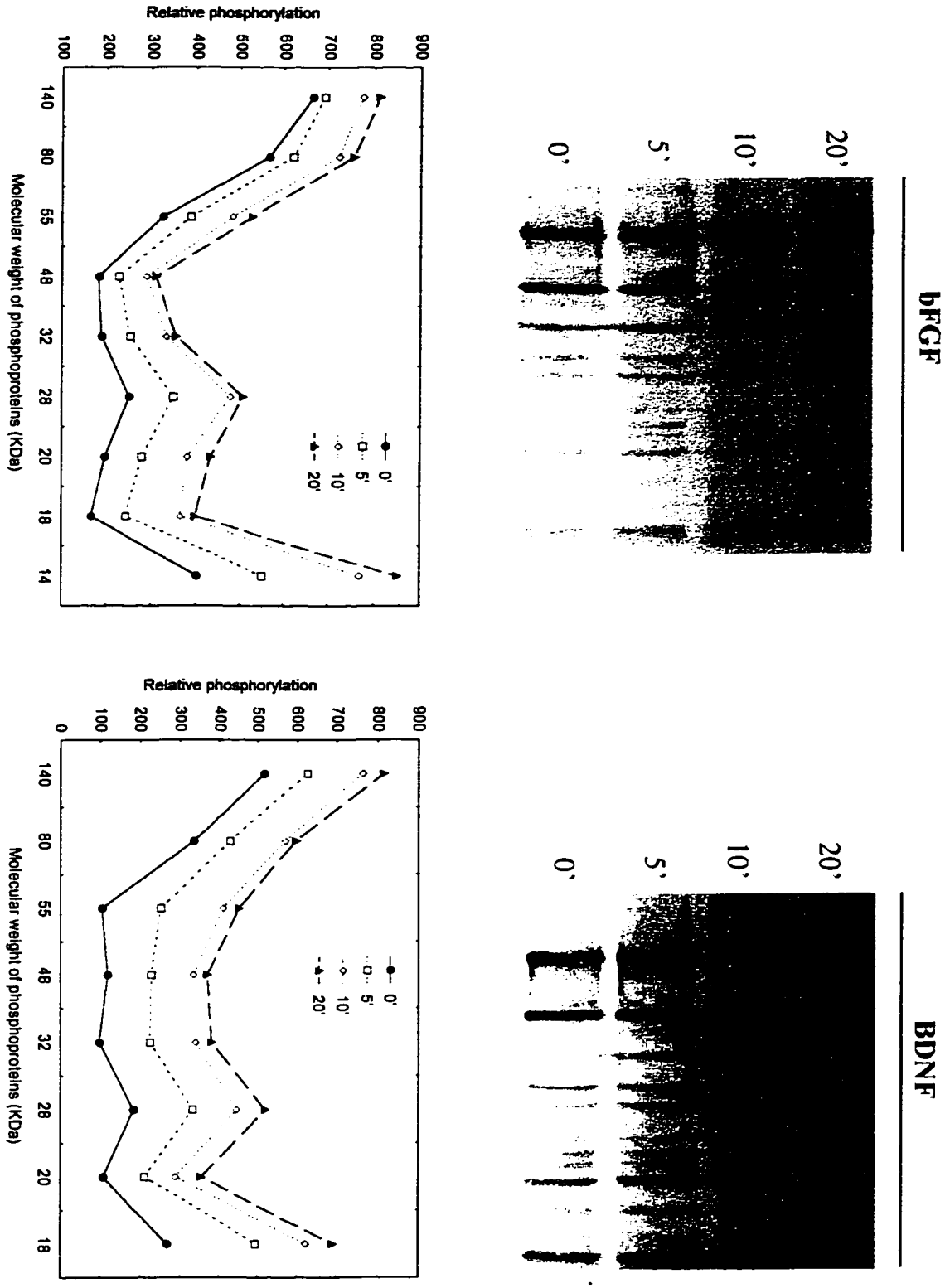


Figure 38 C. BDNF- and bFGF-induced serine/threonine phosphorylation.

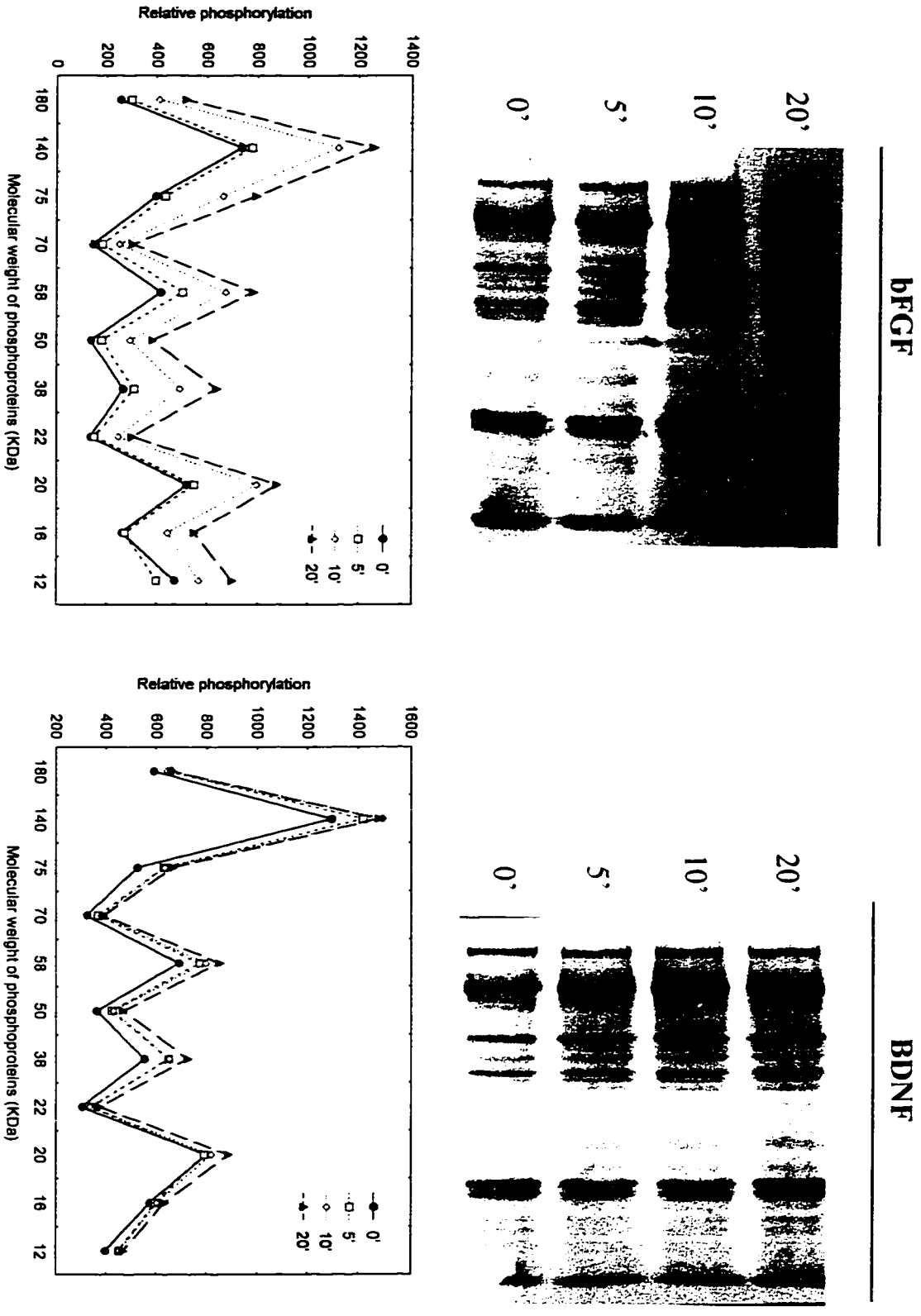


Figure 38 D. BDNF- and bFGF-induced tyrosine phosphorylation.

Taurine down-regulates glutamate- but not BDNF-induced phosphorylation

In order to look for possible pathways involved in GFs and NAA-regulated granule cells survival, as a first step, we have investigated the effects of BDNF and glutamate on protein phosphorylation in these cells using 2-D gel analysis. The polypeptides (95, 55, 20KDa) were reproducibly found to show changes in phosphorylation.

Figure 39 shows a typical western blot of two-dimensional gel electrophoresis analysis of phosphoproteins (PP) identified with anti-phospho-tyrosine monoclonal antibodies. BDNF (10 ng/ml) induced phosphorylation of two sets of polypeptides of apparent molecular weight of 95 (PP95) and 55 Kda (PP55). Each set of the same molecular weight PP consisted of at least three labeled spots with different apparent isolelectric points. Addition of 1 mM glutamate (15 min) enhanced the phosphorylation of the PP55 but not the PP95. Pre-treatment of CGCs with 10 mM taurine for 24h, significantly inhibited the phosphorylation of the PP55 as shown by quantitative analysis of the phosphorylated proteins (Fig.40).

Quantitative analysis of the phosphorylated proteins revealed that the phosphorylation levels of the PP95 did not show significant changes after treatment with glutamate and taurine. Thus stimulation of CGCs with 1 mM glutamate for 15 min or 10 ng/ml BDNF for 24 h stimulated phosphorylation by tyrosine kinase activity. While BDNF-induced tyrosine phosphorylation is an inherent property of TrkB receptors and may be calcium-independent, the glutamate-induced tyrosine phosphorylation could be mediated through a calcium-dependent mechanism. This is supported by the finding that taurine down-regulates only the glutamate-stimulated tyrosine phosphorylation, presumably through calcium regulation.

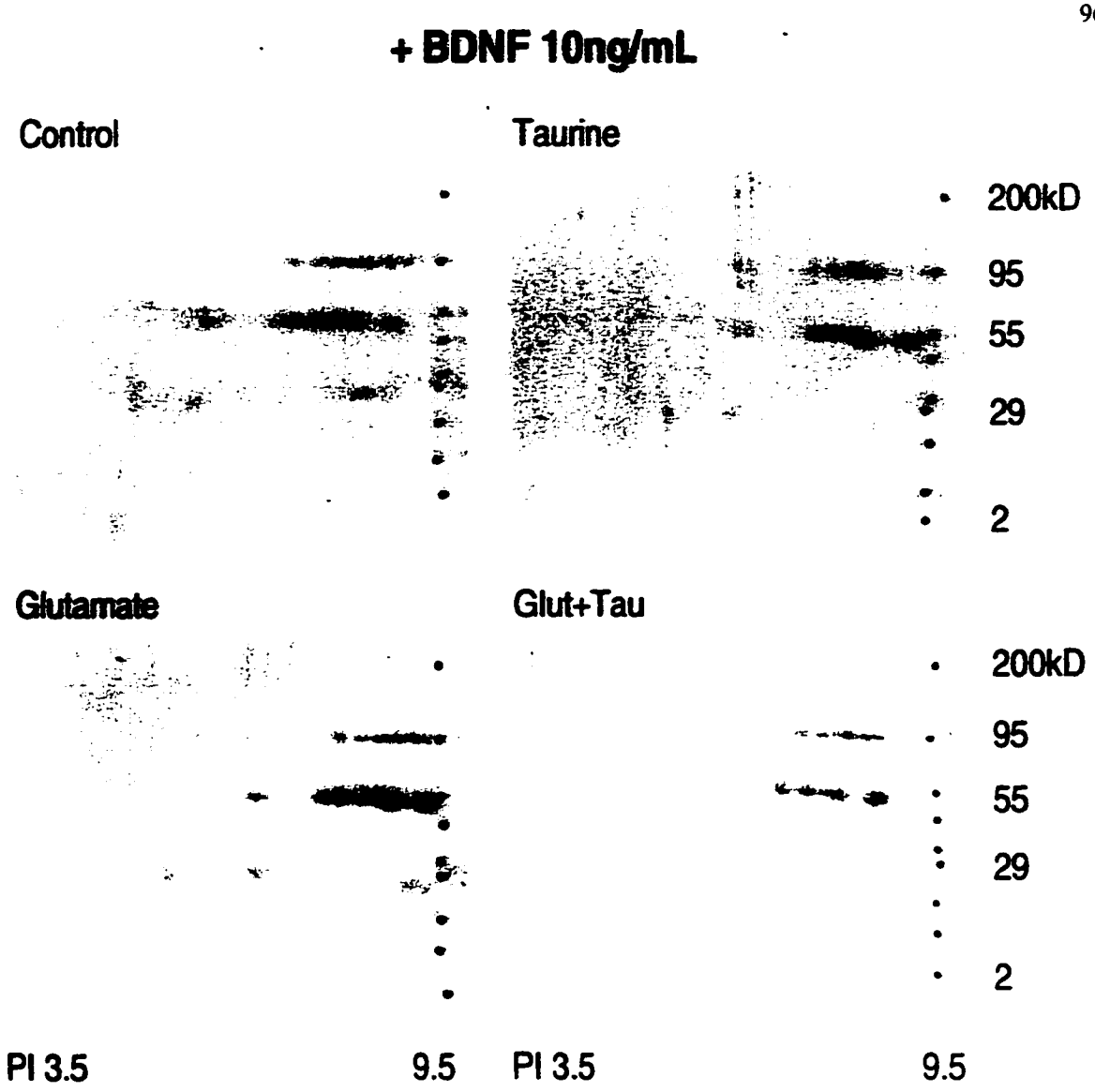


Figure 39. Western blot of 2D gel electrophoresis of PP in the presence of BDNF.

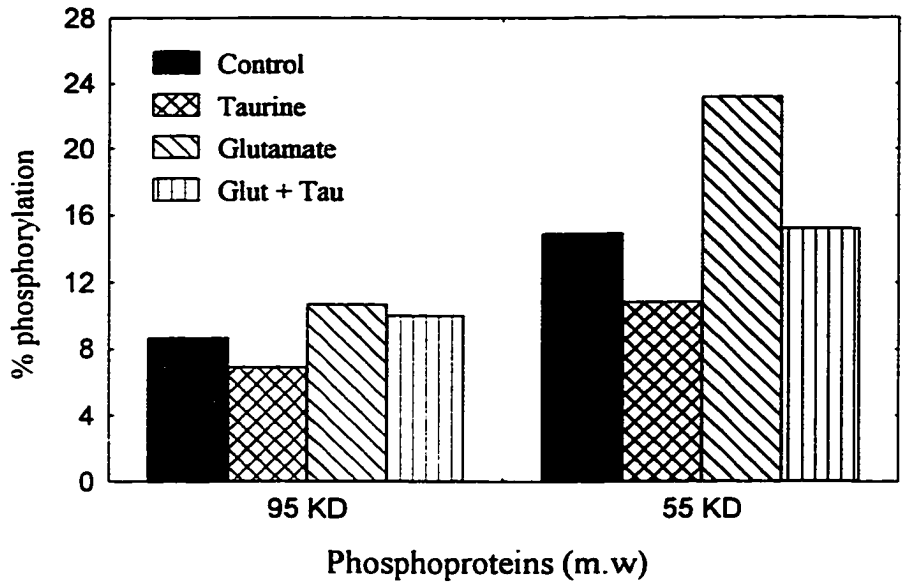


Figure 40. Taurine differentially down-regulated protein phosphorylation. When cultures were treated with BDNF (10 ng/ml), different proteins were tyrosine-phosphorylated, mainly a set of 95KDa and 55 KDa PP. Taurine and glutamate modulated only the phosphorylation of 55 KDa.

Preliminary results to identify the tyrosine phosphorylated proteins through N-terminal sequencing of the 55 Kda polypeptides revealed alpha and beta tubulin as a substrate for tyrosine phosphorylation in response to BDNF and glutamate. It is not clear at present which tubulin isoform is tyrosine-phosphorylated, by virtue of their association as a heterodimer. However, both alpha and beta tubulin have been shown to be tyrosine- phosphorylated (Nishida *et al.*, 1987; Cox and Maness, 1993; Ley *et al.*, 1994; Teng and Greene, 1994; Aletta, 1996). Moreover, posttranslational modification of tubulin through phosphorylation has been shown to alter microtubule-dynamics during neurite formation and to modify the function of cytoskeletal proteins (Maness and Matten, 1990; Cox and Maness, 1993; Teng

and Greene, 1994; Aletta, 1996; Blagosklonny *et al.*, 1997; Gradin *et al.*, 1998). It is conceivable that BDNF-induced tubulin-phosphorylation plays a role in the stability of microtubules, while, glutamate-induced hyper-phosphorylation could promote tubulin disassembly and neurites to collapse concomitantly with excitotoxicity. Taurine inhibited glutamate- but not BDNF-induced tubulin-phosphorylation consistent with its neuroprotective effects.

CONCLUSIONS

The main finding of these experiments demonstrate that growth factors and NAAs regulate differentially the activity of PKC and PKA. Furthermore, the activation of these enzymes, and probably others that we have not examined, phosphorylated a specific set of proteins, which was regulated by taurine.

Exposure to glutamate resulted in a significant increase in PKC activity, suggesting that prolonged activation of PKC may be related to glutamate neurotoxicity. Consistent with this, down- regulation of PKC achieved almost complete neuroprotection from glutamate toxicity (Favaron *et al.*, 1990; Manev *et al.*, 1990). We found that taurine, bFGF and BDNF in the presence of glutamate all down-regulated PKC activity, suggesting the involvement of PKC in the neuroprotective mechanisms of these agents.

The regulation of PKC by taurine, glutamate, BDNF and bFGF was compatible with their active calcium-modulation, whereas the calcium-independent PKA was differentially regulated. As expected, PKA- activity was not significantly affected by these factors, due to the lack of cAMP-generation by these factors. Growth factors modulated PKA expression

levels, whereas BDNF and taurine down-regulated the expression, bFGF alone independent of taurine, up-regulated PKA expression.

Since both PKA and PKC play important roles as mediators of trans-membrane signaling events, alteration of their activity and/or expression levels will undoubtedly affect protein phosphorylation. As a first step, we have examined tyrosine-phosphorylation of substrate proteins and begun to identify them. We found that the phosphorylation of tubulin, a major cytoskeletal protein, is regulated by BDNF, glutamate and taurine, which adds to the list of cellular functions fulfilled by taurine. More work however is needed to identify other substrate proteins that are target for pathological phosphorylation.

We thank Dr. Jorge Ghiso for the his assistance in sequencing the alpha and beta tubulin.

Identification of genes differentially expressed by BDNF, taurine and glutamate

We have begun to analyze the effects of GFs and NAAs on gene expression using differential display polymerase chain reaction (DDRT-PCR). These results are preliminary. This is potentially a powerful method for identifying genes that are over- or under-expressed in response to external signals. In this technique, specific subpopulations of mRNA are amplified using reverse transcription and PCR. mRNAs are reverse-transcribed in subsets using specific anchored oligo (dT) primers that recognize different fractions of the total mRNA population. The resulting cDNA is then amplified with the same anchored oligo (dT) primer and a short arbitrary primer (table 1). Labeled dCTP is included in the reactions and

the labeled products are separated on a DNA sequencing gel and visualized by autoradiography.

In the first experiment, we treated cerebellar granule cells with glutamate, BDNF or taurine. We have used 3 anchored 3' primers (dT1, dT2 and dT3) and 2 arbitrary 5' primers (A1 and A5), which gave us a total of six reactions. Figure 43 shows the result of such an amplification. Most, but not all, of the amplified messages are the same in each of the reactions, suggesting that most of the messages are not differentially expressed by these treatments. These commonly expressed messages could be "house keeping" genes. There are some distinct differences, however, between the amplified products generated from the different treatments (Fig. 41), suggesting factor-specific gene induction.

Undoubtedly altered gene expression lies at the heart of the regulatory mechanisms that control the cell biology. Comparisons of gene expression in response to different external stimuli provide the underlying information we need to analyze the biological processes that control cell survival, differentiation, maturation, and death. These results demonstrate the potential of this technique to identify differentially expressed mRNAs and to clone their genes.

We are grateful to Dr. Robert B. Denman for giving us the opportunity to do these experiments in his laboratory, and for providing us with all the necessary reagents and advice.

Table 1. Differential Display Primers.

3' Primers	**Sequence**
dT1	5' GCG CAA GCT_{12} GC3'
dT2	5' GCG CAA GCT_{12} GG3'
dT3	5' GCG CAA GCT_{12} GA3'
5' Primers	**Sequence**
A1	5' CGG ATC GAC TCC AAG3'
A5	5' CGG GTC TGC TAG GTA3'

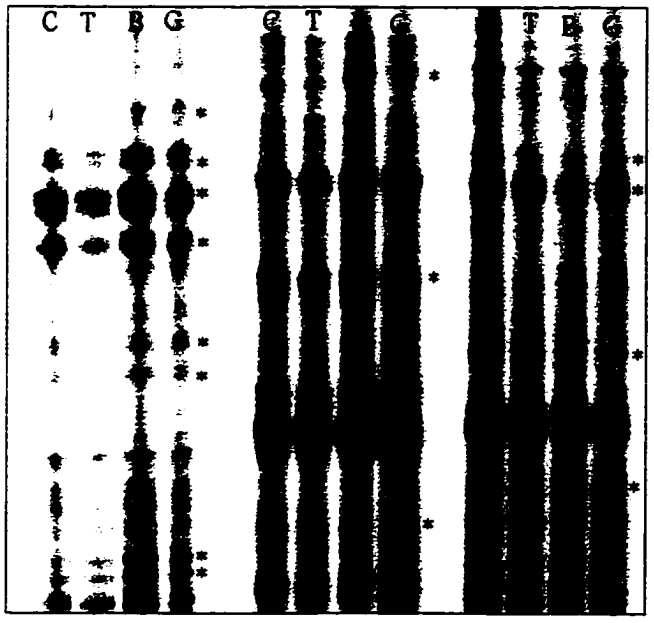


Figure 41. Differentially displayed genes. Representative region of the autoradio-gram showing differentially displayed bands, as detected by the 3' primers dT1, dT2 and dT3, respectively (left to right) and by the 5' primer A1. (C) control, (T) taurine 10 mM, (B) BDNF 10 ng/ml, (G) glutamate 100 μM. Asterisks indicate bands affected by different treatments

DISCUSSION

The findings of this study can be summarized as follow:

Culture conditions had significant effects on the response of cerebellar granule cells, neuronal viability was reduced under serum-free conditions. Neurons co-cultured with astrocytes, or in the presence of serum survived better.

BDNF, bFGF and taurine had trophic effects on the survival of cerebellar granule neurons, as all these factors improved neuronal viability.

Excessive stimulation of any of the glutamate receptor subtypes elicited excitotoxicity. The extent of glutamate-induced cell death was greater under serum-free conditions.

Growth factors and taurine had excitoprotective effects, and attenuated glutamate-induced excitotoxicity.

Glutamate depolarization resulted in an intracellular calcium accumulation, which was dependent on the extracellular concentration of glutamate and the time of its presence.

NMDA receptors were the main route of calcium entry into cerebellar cells. Non-NMDA receptors and VSCCs were also contributors to the glutamate-induced calcium influx.

The function of the NMDA subtype of glutamate receptors was highly regulated. In addition to the glycine modulation and the magnesium block, also serum in the culture medium significantly reduced NMDA-induced calcium accumulation. Such inhibition of NMDA receptor function was mimicked by bFGF and taurine.

Taurine and bFGF synergistically reduced the glutamate-induced calcium uptake, however through different mechanisms.

Taurine is the most abundant free amino acid in cerebellar granule cells *in vitro*, and its uptake is mediated via a high affinity uptake transporter.

Taurine maintains intracellular calcium homeostasis under depolarizing conditions, and may play an important role as an organic osmoregulator.

Mitochondrial energy metabolism is an important determinant of cell vulnerability. Impairment of energy metabolism, through inhibition of the MtECG, significantly enhanced excitotoxicity.

Taurine and growth factors enhanced mitochondrial function and attenuated glutamate-induced reduction in the MtECG.

The survival-promoting effects of taurine, bFGF, and BDNF were mediated through stabilization of intracellular calcium homeostasis and mitochondrial energy metabolism.

Taurine, BDNF and bFGF down-regulated glutamate-induced PKC-activation, probably through calcium modulation.

BDNF in combination with taurine down-regulated PKA-expression, whereas bFGF up-regulated PKA-expression.

The cytoskeletal protein tubulin was identified as a substrate for tyrosine phosphorylation in the presence of BDNF and glutamate. Taurine down-regulated only glutamate- but not BDNF-induced tubulin phosphorylation.

Taurine, BDNF and glutamate controlled specific gene expression.

This study was conducted to determine whether and how neuroactive amino acids and growth factors interact during crucial steps in early postnatal cerebellar development and during excitotoxic conditions. We found that the development, maintenance and survival of cerebellar granule cells were dependent on a balanced interaction between these factors. This most likely reflects the *in vivo* situation, as excess or decline in the availability of any signaling molecule has been associated with some form of neurological malfunction.

One of the essential mechanisms during neuronal development is the regulation and maintenance of calcium homeostasis (Mattson *et al.*, 1993; Mattson and Cheng, 1993; Kater *et al.*, 1994; Kater and Lipton, 1995). Calcium ions are ubiquitous intracellular second messengers, acting as key regulators of numerous cellular functions. Because of this, neurons must tightly regulate the concentration of free cytosolic calcium ions in order to survive and function. Furthermore, loss of intracellular calcium homeostasis has been shown to be associated with models of brain pathologies, such as glutamate excitotoxicity (Dykens *et al.*, 1987; Choi, 1987, 1988; Mattson *et al.*, 1993; Mattson and Cheng, 1993; Eimerl and Schramm, 1995). In this study, we have chosen representatives from distinct groups of signaling molecules and their combinations that are known to regulate cerebellar development: taurine and glutamate as representatives of neuroactive amino acids; bFGF and BDNF as examples of two distinct growth factor families. Since all of these molecules are present at a given time in the intracellular and the extracellular environment of the cerebellum and regulate early development and neuronal function, we have tried to characterize their interactions and ask question whether or not these molecules induce the same cellular regulatory systems and could substitute for each other, whether the regulation of calcium influx is synergistic or additive and whether these mechanisms are complementary depending

on concentration and availability.

We as well as others (Brewer *et al.*, 1993; Yuzaki *et al.*, 1993; Shambaugh *et al.*, 1994; Trenkner *et al.*, 1996; Wood, 1997; Wood *et al.* 1997; Ye and Sontheimer, 1998), have established that the viability of neurons is greatly affected by their extracellular environment; more neurons survived in the presence of serum and astroglial cells (Fig. 9A). Additions of factors with known neurotrophic support on cerebellar granule cells (bFGF, BDNF and taurine), did not further improve cell survival beyond untreated controls, suggesting that optimal conditions were provided by serum and astroglia. When glial cells were removed from these cultures, purified cerebellar granule neurons showed a significant decrease in cell viability, when compared to mixed cultures. Furthermore, in serum-free medium, cell viability was significantly compromised, providing evidence that serum has trophic effects. Therefore, this tissue culture system which does not contain serum or glial cells was utilized to determine whether these various factors affected the survival and function of neuronal cells.

Figures 9 & 10 demonstrate clearly the protective effects of the particular media under excitotoxic stress. Serum containing cultures with astrocytes provided the most protection, possibly because trophic factors in serum and those released from glial cells protected to a maximum. This idea is supported by the finding that in serum-free medium, taurine, bFGF (Fig. 9) and BDNF (Fig. 10) did protect a significant number of cerebellar neurons from glutamate-induced excitotoxicity. The fact that the actual number of surviving cells was less than that of cells maintained in serum- and glia-containing media, might indicate that other mechanisms than those suggested also play a major role, such as removal of glutamate by high-affinity uptake and removal of excitotoxic concentrations of glutamate through conversion of glutamate into glutamine (Fonnum, 1984; Rosenberg and Aizenman, 1989;

Rosenberg, 1991), or as Casper and Blum (1995) have shown that in mixed cultures of dopaminergic neurons and astrocytes, glutamate uptake was significantly increased by bFGF through its mitogenic effect on astrocytes, reducing therefore the extracellular concentration of excitotoxins.

Our results suggest that neuroprotection is controlled by other mechanisms than those involving transport and elimination of glutamate from the medium. These include regulation of intracellular calcium homeostasis and control of neuronal energy metabolism. This is suggested by the neuroprotective effects of bFGF and taurine on purified neuronal cultures without astroglial cells (Fig. 9 B-D). Support for these alternative mechanisms comes from experiments in which glutamate-induced calcium uptake was assessed in the presence of growth factors (Mattson *et al.*, 1993; Cheng *et al.*, 1995), showing quantitative correlations between excitotoxicity and total NMDA receptor-mediated calcium accumulation in neurons (Choi, 1988; Mattson, 1988; Hartley *et al.*, 1993; Eimerl and Schramm, 1994). We have demonstrated a correlation between the concentration of extracellular glutamate and calcium accumulation in neurons over time (Fig. 11), and we have shown that calcium accumulation was directly related to the time glutamate was present in the medium (Fig. 21 & 23). Thus, one common denominator of bFGF and taurine appears to be, among others, their ability to regulate intra-neuronal calcium homeostasis. In the serum-free medium used here, which is comparable to the composition of cerebrospinal fluid (CSF), glutamate-induced calcium uptake and excitotoxic neuronal death was significantly higher than in the presence of serum, suggesting that serum contains factor (s) that affect the glutamate-induced calcium accumulation that can lead to excitotoxicity. When incubated with bFGF and/or taurine for 24 h prior to glutamate-depolarization, intracellular calcium accumulation was significantly

reduced (Fig. 21 & 23), as it was by increasing the serum concentration in the medium (Fig.15).

The excitoprotective mechanisms of bFGF have been shown to be mediated through the regulation of the expression of specific glutamate-receptor subunits. bFGF selectively increased levels of the GluR1 subunit of the AMPA receptor (Cheng *et al.*, 1995) and suppressed the expression of a 71KDa NMDA receptor protein in hippocampal neurons (Mattson *et al.*, 1993), resulting in the reduction of neuronal vulnerability to glutamate toxicity (Mattson *et al.*, 1993). This mechanism is not limited to hippocampal neurons. In cerebellar granule cells, Brandoli *et al.* (1998) showed that both BDNF and bFGF reduced the expression of NR2A and NR2C subunits of the NMDA receptors , which may explain why BDNF and bFGF protected neuronal cells from glutamate excitotoxicity.

Numerous studies have demonstrated the presence of taurine in neurons and glia in multiple areas of the brain (Palkovits *et al.*, 1986; Huxtable, 1992; Sturman, 1993). In this study we showed that the intracellular concentration of taurine in CGCs was relatively high as compared to other amino acids. Earlier, we have suggested that taurine and glutamate interact in a balanced fashion, particularly under excitotoxic conditions (Trenkner, 1990; Trenkner *et al.*, 1996). The release of taurine from neurons and glial cells has also been associated with this neuroprotective effect (Trenkner, 1990; Trenkner *et al.*, 1996; Bianchi *et al.*, 1996; Segovia *et al.*, 1997; Saransaari and Oja, 1997; Katoh *et al.*, 1997). On the other hand, increasing the concentration of extracellular glutamate, by infusion of selective inhibitors of the high-affinity glutamate uptake, correlated significantly with increasing concentrations of taurine. The increase of extracellular taurine was blocked by NMDA and AMPA/kainate receptor antagonists (Segovia *et al.*, 1997). Taking these results together, the

large amount of taurine released simultaneously with the activation of glutamate receptors is apparently neuroprotective. Yet an other mechanism has been proposed involving taurine as inhibitor of depolarizing effects thought increasing membrane chloride conductance (Oja *et al.*, 1990; Quinn and Harris, 1995). Should this mechanism be involved, direct measurements of chloride conductance should give the answer.

The evidence presented here concerning calcium uptake regulation by taurine can be interpreted in two ways. First, taurine acted only after glutamate depolarization (Fig. 15 & 22). However, we could not detect a direct interaction with glutamate-specific subtypes like NMDA or kainate receptors, suggesting that the modulatory role of taurine has to be based on different mechanisms. As demonstrated in figure 22, prevention of calcium accumulation by taurine required approximately 20 min. in our cultures (serum-free medium) when taurine was added together with glutamate. However, cultures which were pre-loaded with taurine began to regulate calcium accumulation after less than two minutes (fig. 22). Such kinetics do not agree with receptor-mediated reactions, since depolarization usually occurs significantly faster (in seconds rather than minutes). In fact such kinetics have been described for osmoregulation and ion-exchange. Taurine as well as β-alanine, glycine and other organic osmolites have been described as volume- and osmo-regulators (Hoffman and Simonson, 1989). When applied to calcium homeostasis, ion exchange and volume regulation, including calcium exchange, taurine might very well balance extracellular and intracellular calcium concentrations through water release and uptake or through high affinity transport systems as shown for taurine, β-alanine and glycine. Our experiments support this idea, since calcium uptake occurred over 30 min. to reach its maximum. But our results also show that the calcium modulation has to be taurine specific because β-alanine (Fig. 25) and glycine (Fig.

19) did not modulate or inhibit calcium uptake like taurine although they are viewed as organic osmolites.

GABA is the inhibitor of the glutamate system and GABA's inhibitory function is receptor-mediated and activated immediately in response to GABA-agonists. Although taurine has been shown to bind to GABA receptor sites, taurine does not inhibit calcium accumulation through this site. At the same time, bFGF acting as calcium-uptake modulator, activates second messenger cascades that regulate the degree of depolarization as shown in figure 23. Therefore, we would like to conclude that although the mechanisms are significantly different, taurine as well as bFGF modulate calcium uptake to the same degree. Due to taurine's property to regulate calcium homeostasis as well as osmolarity, taurine could be instrumental in bFGF's function during calcium uptake modulation.

In contrast to taurine and bFGF, BDNF did not directly affect the glutamate-induced calcium accumulation (Fig. 22), though protected cerebellar cells against glutamate excitotoxicity (Fig. 10). The regulation of intracellular calcium homeostasis offers a plausible explanation for the neuroprotective role of taurine and bFGF. However, the results of this study inferred a different mechanism for BDNF.

Energy metabolism is recognized as one of the fundamental factors that control the maintenance of neuronal structures and their function during development and throughout adult life (Hoyer *et al.*, 1991; Hoyer, 1993; Mattson *et al.*, 1993; Beal, 1995). Also neuronal development and maintenance of function depends on cellular energy (Budd and Nicholls, 1995, 1996; Trenkner *et al.*, 1996; Budd *et al.*, 1997). We and others, have recently demonstrated that depletion of cellular energy, caused by malfunction of the MtECG, increased the vulnerability towards excitotoxins and neurotoxins leading to neuronal cell death

(Trenkner, *et al.*, 1996; El Idrissi *et al.*, 1996; Budd and Nicholls, 1995, 1996). In this study we showed *in vitro* that taurine (10 mM) alone or in combination with bFGF or BDNF facilitated an increase in mitochondrial activity. This increase was taurine-, bFGF- and BDNF-specific since EGF had no effect (Fig. 27). However, EGF significantly reduced the glutamate-induced calcium uptake similar to bFGF (not shown), indicating that calcium homeostasis is controlled by several mechanisms. Although the exact mechanisms of taurine's action are not fully understood, taurine plays a significant role in the regulation of glutamate-induced signaling events. In particular, taurine has opposing effects to those of glutamate. Furthermore, the net cellular effects of glutamate are modulated by the interaction between growth factors and taurine. Thus, the cascade of events culminating in a decreased Ca^{2+} response and an increased neuronal energy metabolism after depolarization, could represent a general mechanism by which taurine and growth factors can prevent glutamate-mediated neuronal death after glutamate receptors were stimulated. Although only speculative at the present time, the possibility exists that, by reducing the intracellular responses to glutamate, factors with neuroprotective properties, such as taurine, BDNF and bFGF, might prevent the formation of cytotoxic metabolites such as nitric oxide, peroxidases, and free-radicals (Dykens *et al.*, 1987; Maiese *et al.*, 1993; Dykens, 1994; Mattson *et al.*, 1995; Kume *et al.*, 1997) that are thought to be responsible for the final toxicity of glutamate. Consistent with this, Lam *et al.*, (1998) have shown that NGF suppressed glutamate-stimulated nitric oxide synthase (NOS) activity and protected hippocampal neurons from NO toxicity.

The ability of growth factors and taurine to regulate calcium homeostasis was extended to the calcium-dependent enzyme PKC. PKC is involved in glutamate excitotoxicity (Favaron *et al.*, 1990; Mattson, 1991; Damanska-Janik and Zalewska, 1992; Candeo *et al.*,

1992). Taurine, BDNF and bFGF down-regulated the glutamate-induced increase in PKC activity, probably as a consequence of calcium modulation under glutamate depolarization.

Because glutamate neurotoxicity is related to the destabilized intracellular Ca^{2+} homeostasis, it has been suggested that the proteins involved might be the ones regulating the rate of Ca^{2+} influx or efflux (Manev *et al.*, 1990). Recently, it has been shown that PKC reduces the Mg^{2+} block of the Ca^{2+}-permeable NMDA receptor channel (Chen and Huang, 1992), suggesting that the phosphorylation of these receptors, which have the principal role in glutamate toxicity, may enhance the NMDA-mediated responses. In addition, NMDA receptor function is modulated by PKA (Xie and Lewis, 1997), and that all three NMDA receptor subunits, NR1, NR2A and NR2B are substrates for PKA as well as PKC phosphorylation (Leonard and Hell, 1997). Regulation of non-NMDA receptors by PKA and PKC has also been shown (Traynelis and Wahl, 1997; Maiese *et al.*, 1996). The GluR1 subunit of the AMPA receptor is phosphorylated by PKA on ser-845 residue, which potentiated the function of these receptors (Roche *et al.*, 1996). Furthermore, desensitization of metabotropic glutamate receptors is mediated by phosphorylation of the mGluR5 subunit of these receptors by PKC (Nakahara *et al.*, 1997; Gereau and Heinemann, 1998). These studies have provided evidence that glutamate receptors are directly phosphorylated and functionally modulated by protein kinases.

Glutamate receptors are only few substrates which are phosphorylated by PKA and PKC. Interestingly, in the context of this study, cystein sulfonic acid decarboxylase (CSAD), the rate-limiting enzyme in taurine biosynthesis, was found to be activated when phosphorylated by PKC (Wu *et al.*, 1998; Tang *et al.*, 1998), which indicated that taurine biosynthesis could be up-regulated under excitotoxic conditions. In cerebellar granule cells,

BDNF induced the expression of maturation-specific proteins (synaptophysin and tau), and the growth associated-protein (GAP-43). These inductions were mimicked by a PKC activator, phorbol ester (Coffey *et al.*, 1997), indicating that PKC is either a component of, or feeds into the signaling mechanisms of BDNF during differentiation and maturation of cerebellar granule cells.

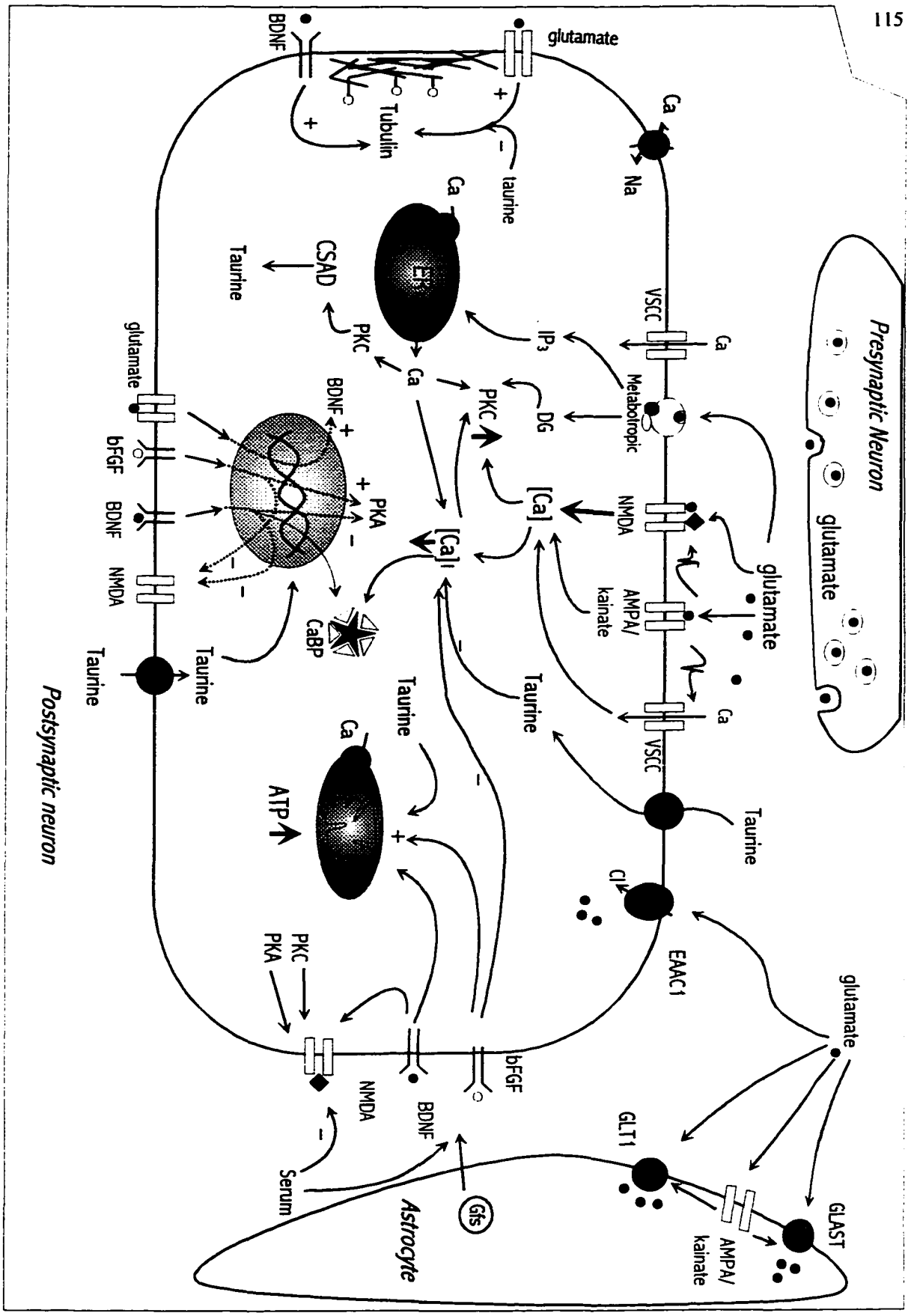
These examples clearly demonstrate the importance of PKC and PKA as mediators of trans-membrane signaling events. In light of findings that H7 treatment protected neurons in culture from toxicity by an overstimulated PKC activity (Mattson, 1991) and reduced the consequences of brain ischemia in rats (Joó *et al.*, 1989).

An other important finding of this study is the regulation of protein phosphorylation by GFs and NAAs. Protein phosphorylation has been shown as key regulator of cell survival, morphological differentiation, neurite outgrowth, and synaptogenesis (Aletta *et al.*, 1989; Girault *et al.*, 1992; Teng and Greene, 1994; Nakao *et al.*, 1995; Nakao *et al.*, 1996). On the other hand, hyper-phosphorylation of cytoskeletal proteins has been involved in several neurodegenerative diseases (Tanaka *et al.*, 1998). We have shown that glutamate significantly increased the phosphorylation levels of 55 KDa proteins, that were identified by N-terminal sequencing as alpha and beta tubulin. It is not clear at present if both the α and β isoforms were tyrosine-phosphorylated, by virtue of their close association to form a heterodimer. However, tyrosine phosphorylation of tubulin has been shown to inhibit the ability of this subunit to polymerize into microtubules (Ley *et al.*, 1994). Here, we suggest that glutamate causes a hyper-phosphorylation of tubulin, which in turn destabilizes cytoskeletal mircotubules, an event concomitant with excitotoxicity, whereas BDNF-induced tubulin phosphorylation may enhance microtubules stability. Taurine on the other hand, significantly

reduced the glutamate- but not BDNF-induced tubulin phosphorylation, suggesting an additional mechanism for taurine's neuroprotection.

In conclusion, growth factors and NAAs interact through modulation of calcium homeostasis after glutamate depolarization. Some of these factors induce the same regulatory mechanisms (e.g. taurine and bFGF reduced calcium uptake; taurine and BDNF increased rhodamine uptake; glutamate and BDNF induced tubulin phosphorylation; taurine, bFGF and BDNF all decreased cell death and neuronal cell vulnerability) and appear to support each other (Fig. 42). These findings further suggest that neuronal cells utilize alternative pathways to regulate and maintain their function. These pathways are accessible to cells depending on their developmental stages as well as the availability of these factors. We conclude that under conditions where more than one factor is present, which is likely the situation *in vivo*, these factors regulate each other's function to promote optimal environmental condition for cell survival and function.

Figure 42. Sequence of events triggered by growth factors and neuroactive amino acids. Glutamate released from the presynaptic neuron binds to its receptors on the postsynaptic neurons and on astroglial cells. Calcium influx occurs through receptor-gated ion channels such as the NMDA receptor or voltage-sensitive calcium channels (VSCC) that are activated by excitatory transmitter receptors such as the AMPA/kainate receptor. Calcium release from endoplasmic reticulum (ER) stores is stimulated by inositol triphosphate (IP_3), which is liberated upon activation of metabotropic receptors coupled to inositol phospholipid hydrolysis. ATP-dependent Ca^{2+} pumps in the plasma memdrane, mitochondria and ER remove calcium from the cytoplasm. Calcium-binding proteins (CaBP) provide an other mechanism of reducing $[Ca^{2+}]_I$. As a consequence of increased $[Ca^{2+}]_I$, several Ca^{2+}-dependent enzymes can be activated in a non-controlled manner and lead to apoptotic or necrotic cell death. Taurine and bFGF reduced $[Ca^{2+}]_I$ accumulation and the reactions associated with it in the presence of glutamate. Under conditions which favor the activation of PKC, such as presence of glutamate in the absence of taurine, bFGF or BDNF, CSAD is activated through its phosphorylation by PKC, which leads increased taurine biosynthesis. Activation of PKC also regulates the function of NMDA receptor through subunits-phosphorylation. The presence of astrocytes in the cultures, serum in the medium, or exogenously added GFs induced an increase in the MtECG and protected neurons against excitotoxicity. Both BDNF and glutamate induced tyrosine phosphorylation of the cytoskeletal protein tubulin. Taurine down-regulated only the glutamate-induction. BDNF, bFGF, glutamate and taurine differentially regulate gene expression. Glutamate transporters play important role in the termination of glutamatergic synaptic transmission. In addition to being co-transporters, these proteins also function as Cl^- channels. Depicted here are the GLT1 and GLAST restricted to astrocytes, and the EAAC1 found in neurons (Trotti *et al.*, 1998). Influx of Cl^- associated to postsynaptic transport could shorten postsynaptic excitation.



MATERIALS AND METHODS:

Cell preparation:

a. *Mixed cultures*: Cerebellar granule cells were prepared from 7-day-old mice as previously described (Trenkner and Sidman, 1977; Trenkner, 1991). Briefly, the entire cerebellum was removed and single cell suspensions were prepared by trypsinization and trituration (1 % trypsin in Ca^{2+} / Mg^{2+}-free isotonic phosphate buffer). Cells were washed and resuspended in culture medium (basal medium Eagle supplemented with 0.25% glucose, 2mM glutamine, 10% horse serum (HS), 5 % fetal calf serum (FCS) and 25 U/ml both penicillin and streptomycin). Cells were seeded into a poly-lysine coated or un-coated dishes depending on the questions to be answered and incubated at 37°C in a moist chamber under 5% CO_2 (for more detail, see Trenkner, 1991)

b. *Enriched neuronal cultures*: Cultures were prepared as for mixed cultures, but after 24 h *in vitro* the medium was replaced by serum-free medium. The cultures were grown in a humidified CO_2 / air (5% / 95%) atmosphere at 37°C in a slightly modified Dulbeccos's medium (MEM) with elevated glucose (30 mM) as the only source of energy, and reduced glutamine (0.8 mM) concentrations. MEM was supplemented with 15 % N-2 derivative containing 100 μg/ml transferrin, 20 μg/ml putrescine, 12.8 ng/ml progesterone, 10.4 ng/ml selenium, 25 ng/ml insulin, 0.8 ng/ml thyroxine. Cytosine arabinoside (Ara C) was added at a final concentration of 2 μM to curtail the number of astrocytes that proliferate in the cultures.

c. *Purified neurons in serum-containing medium*. In order to prepare cultures of purified granule neurons, cells were obtained from 3 or 4 day old mice and single cell suspensions prepared as described above. Early postnatal stages had to be used because cells were less

attached and adhesion molecules were less expressed. Granule neurons were purified in a Percoll gradient (35-65%) based on cellular size and selective adhesiveness of neurons and glial cells to plastic (for more detail, see Trenkner, 1991).

d. *Purified neurons in serum-free medium*. Cultures were prepared as above, seeded in serum-containing medium for 24h and medium was replaced by serum-free medium consisting of MEM supplemented with 15% N-2 supplement. Because of the high neuronal yield (98 %) it was not necessary to add Ara C to the medium.

Exposure to excitatory amino acid

Neuronal survival was determined after exposing early postnatal cerebellar cells to glutamate, NMDA, Kainate (Sigma Chemical Co.), or AMPA (Tocris Neuramin), at room temperature in various culture media, after three days *in vitro*, a time when extensive synaptic interconnections have developed (Trenkner and Sidman, 1977; Trenkner, 1990). Cultures were washed once with the growth medium to remove the bulk of extracellular excitatory amino acids, and cultures were returned to the incubator. Neuronal injury was assessed 24 h after the initial treatment. For $^{45}Ca^{2+}$uptake, cells were exposed to excitatory amino acids in Locke's solution at room temperature for the indicated time. For all other experiments, exposure to glutamate or other glutamate receptor agonists was carried out for the indicated times in culture medium.

Assessment of neuronal cell survival:

Morphological assessment: When growing as monolayer, the integrity of presumptive neurons, glia and fibroblasts is readily appraised with phase-contrast microscopy. Neurons

were identified and distinguished from glial cells by morphological parameters, by shape, size and processes (Trenkner and Sidman, 1977; Trenkner, 1990; Trenkner, 1991). Neurons were scored alive when the cell surface of their neurites were smooth and multiple, and the surface of cell bodies were smooth and round to oval in shape (Fig. 43). In degenerating neurons, neurites were fragmented and beaded, and the soma was rough, swollen, vacuolated, and irregular in shape. The percentages of dead neurons represent counts of all presumptive granule neurons within two randomly chosen fields encompassing almost 60% of total well area.

Figure 43. Morphological determination of cell viability. Photograph of enriched neuronal culture as seen under phase contrast microscopy. Cells with smooth cell bodies and neurites were scored as alive, whereas cells with rough cell bodies and fragmented neurites were counted as dead. The bright spots in the photograph are remaining nuclei of dead cells. (x200).

Intravital staining of the culture: Monolayers were washed with Locke's solution and stained with the mixture of two dyes, fluorescein diacetate (FDA) and propidium iodide (PI) (Jones and Senf, 1985; Faravon *et al.*, 1988). Living cells were labeled with FDA (15 μg / ml), a nonpolar ester that crosses the cell membrane and is hydrolyzed by intracellular esterases to produce a green-yellow fluorescence in the cytoplasm. Dead cell nuclei were identified with PI (4.5 μg/ml), which interacts with DNA to yield a bright red fluorescent complex. Cells

were immediately observed with a standard epi-illumination fluorescent microscope (450 exci.. 520 barrier). Fluorescein-labeled cells were observed with the interference filters usually used for fluorescein detection, whereas PI-labeled cells were observed with the filters used for rhodamine (Van-Vliet *et al.* 1989). A field containing about 200 cells was examined, each cell observed was labelled with either FDA or PI.

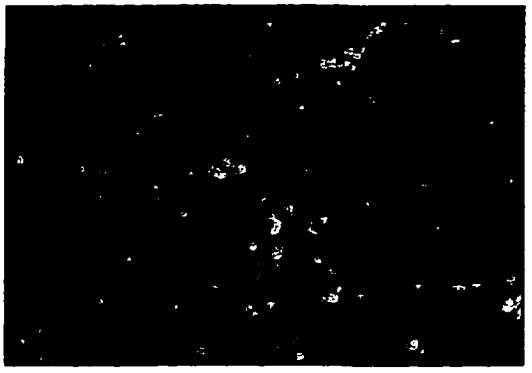


Figure 44. Intravital staining of cultures. Cell viability was determined by simultaneously staining the cells with FDA and PI. Cells labeled green are alive whereas red spots are PI-labeled nuclei of dead cells. The specificity of these cell-viability markers is apparent as no cells is double labeled with both dyes. (x 200)

Determination of mitochondrial electrochemical gradient:

JC-1 staining: As described by Smiley *et al.* (1991), a stock solution of JC-1 was prepared by disolving 1 mg in 1 ml dimethyl sulfoxide (DMSO). Fresh staining solution (10 μg / ml, final concentration) was prepared by diluting the stock solution in warm (37^{0}C) culture medium. 50 μl were immediately applied to each well, which was then kept in the incubator for 10 min. Cells were rinsed in warm dye-free culture medium and covered with a cover slip. A Zeiss axiophot microscope with epifluorescence optics was used to visualize the mitochondria. For visualizing the green fluorescence of JC-1 monomer, any filter combinations (such as Zeiss's barrier LP 515-565 nm, dichoic mirror FT 510 nm, and exciter 450-490 nm) used for fluorescein dye are adequate. Likewise, any rhodamine filter sets (such as Zeiss's barrier LP 590 nm, dichroic mirror FT 580 nm, and exciter BP 546/12 nm) are

suitable for detecting the red fluorescence of J-aggregates. However, to visualize green and red simultaneously, a long-pass filter system (such as Zeiss's barrier LP 520 nm, dichoic mirror FT 510 nm, and exciter 450-590 nm) was used. Fluorescent images were recorded on Kodak professional Ektomatic p800/1600 color films at exposure index (E.I) 1600 for green and yellow fluorescence as shown in figure 46.

Figure 45. Visualization of mitochondria by JC-1. Cerebellar granule cells were stained with JC-1 to reveal the MtECG. Mitochondria with high energy were seen yellow while the green color represent low MtECG. The soma of cerebellar granule cells contain a small cytoplasmic ring around the nucleus where mitochondria are found. Mitochondria are also abundant in the cellular processes. X 400.

Quantitative determination of rhodamine 123 uptake: As described by Chen *et al.* (1989), a stock solution (1mg/ml) of rhodamine 123 was disolved in DMSO and stored at 4^0C. After neurons were exposued excitatory amino acids, the medium was removed, and rhodamine 123 was added to a final concentration of 10 μg / ml in fresh medium. After 30 min at 37^0C, cultures were washed 3 times with pre-warmed growth medium. To allow the release of accumulated rhodamine, 2 ml of butanol were added to each culture dish, and incubated at room temperature for one hour. Total cellular accumulation was determined using a spectrofluorometer with the excitation wavelength set at 508 nm and emission wavelength set at 536 nm.

^{45}Ca Uptake:

Cells were washed twice with Locke's solution [154 mM NaCl, 5.6 KCl, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 2.3 mM, 5.6 mM glucose, and 5 mM HEPES (pH 7.4)]. $^{45}CaCl_2$ was added to reach a final volume of 0.25 ml including 2 x 10^5 cpm of $^{45}CaCl_2$, ~10 s before the addition of the agonist. After 20 min at room temperature, the cells were rapidly washed three times with 0.5 ml of ice cold Locke's solution containing 2 μM MK-801, a relatively high concentration of MK-801 to ensure fast $^{45}Ca^{2+}$ channel block. finally the cells were dissolved in 0.5 ml of 0.1 M NaOH and counted. All ^{45}Ca assays were run in triplicates.

Protein Kinase C assay:

PKC activity was measured by incorporation of ^{32}P from [γ-^{32}P]ATP into a synthetic peptide from myelin basic protein (MBP), that contains the consensus site for PKC phosphorylation, as a specific substrate. Cells are rinsed with PBS, and collected in an extraction buffer (20mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5 % Triton X-100, 25 μg/ml each aprotinin and leupeptin (protease inhibitors). Cells were then homogenized on ice on a pre-cooled dounce homogenizer and centrifuged for 2 min in a microcentrifuge. 25 μl of the supernatant was added to 5μl lipid preparation (100 μM phorbol 12-myristate 13-acetate [PMA], 2.8 mg/ml phosphatidyl serine, Triton X-100 mixed micelles) to activate PKC and water (10 μl) then incubated at room temperature for 20 min. subsequently, 10 μl of [γ-^{32}P]ATP/substrate (250 μM Ac-MBP(4-14), 100 μM ATP, 5 mM $CaCl_2$, 100 mM $MgCl_2$, 20mM Tris, pH 7.5) were added to each sample, immediately placed at 30°C, and incubated for 5 min. Since timing was extremely critical, samples were scattered at 15 sec intervals. The reaction was stopped by removing and spotting 25 μl from each tube onto a phosphocellulose disc to

immobilize ^{32}P-labeled peptide. Unincorporated [γ-^{32}P]ATP was removed by washing with 1% phosphoric acid and water. PKC-specific activity was expressed as pmol/min and was normalized to total protein.

cAMP-dependent protein kinase (PKA):

Granule cells from 7 day old mice were grown for 5 days in 35 mm dishes Assays were performed according to the protocol of the supplier's description (GIBCO BRL, Grand Island, NY) by adding 10 μl of cell homogenate to 20 μl of assay mixture containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP, 0.25 mg/ml BSA, 50 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), and immediately placed at 30°C, and incubated for 5 min. The reaction was stopped by removing and spotting 25 μl from each tube onto a phosphocellulose disc to immobilize ^{32}P-labeled peptide. Unincorporated [γ-^{32}P]ATP was removed by washing with 1% phosphoric acid and water. Values obtained in the absence of exogenously added cAMP represent the "active PKA", whereas those in the presence of exogenous cAMP represent "total PKA". PKA-specific activity was expressed as pmol/min and was normalized to total protein.

Gel Electrophoresis and Western Blotting:

Granule cells were grown for 5 days in 35 mm dishes, the culture medium was removed and cells were lysed. For 2-dimensional gel electrophoresis the lysis buffer consisted of 9 M urea solubilization buffer (9.0 M urea, 4 % IGEPAL CA-630, 2 % ampholytes, 1 % dithiothreitol). For 1-dimension gels, the lysis buffer (ice cold) consisted of 20 mM p-nitrophenyl phosphate, 20 mM Tris-HCl, pH 7.5, 50 mM NaF, 50 mM sodium orthovanadate, 80 mM β-

glycerophosphate, pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$. Cell lysates were collected and centrifuged for 30 min at 10.000 g. The proteins were separated on sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) at 7.5 % polyacrilamide and transferred to nitrocellulose membranes. Blots were blocked with 0.2 % non-fat dry milk (NFDM) in PBS and incubated overnight at +4^{0}C with a mouse monoclonal anti-phosphotyrosine antibody at 0.4 μg/ml (Santa Cruz). After two washes in blocking buffer, the filters were incubated for 1h at room temperature with a rabbit anti-mouse immunoglobulin (Santa Cruz)(1:2000) diluted in blocking buffer.

Differential Display

Total RNA was isolated from approximately 3.5 x 10^6 cells with TRI reagent™ according to the manufacturer's instuction (Molecular Research Center, Cincinnati, OH, USA). The final RNA pellet was solubilized in 125 μl of diethyl pyrocarbonate (DEPC)-treated water and stored at - 70 ^{0}C until it was used. RNA recoveries was monitored by UV-vis spectroscopy at 260 and 280 nm.

Differential display was performed as described by Linskens *et al.* (1995) and Sung and Denman , (1997). First- strand cDNA was synthesized by mixing one μg of total RNA with 2.5 μl of 20 μM of one of the 3' PCR primers listed below and 9.5 μL of DEPC-treated water. Following a 10 min heat denaturation at 75 ^{0}C, the sample was snap-cooled on ice. Subsequently a master mixture consisting of 5 μL of 5 x Moloney murine leukemia virus reverse transcriptase (MMLV-RT) buffer, 1 μL (40 U) of RNasin®, 2.5 μL of 0.1 mM dithiothreitol (DTT), 2.5 μL of 0.25 μM dNTPs and 1μL (200 U) of MMLV-RT was added; the sample was then incubated for 70 min at 37^{0}C in a water bath. One microliter of this

reaction mixture was amplified in the presence of [α-^{32}P] dCTP (25 cycles consisting of denaturation at 94^{0}C for 1.5 min; annealing at 42^{0}C for 30 s and 57^{0}C for 1 min; and a final extension at 72^{0}C for 1.5 min) and run out on a 6% non-denaturing polyacrilamide gels to produce the differential display. The gels were transferred to 3MM chromatography paper (Whatman, Clifton, NJ, USA) and dried; differentially displayed bands were detected on Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) after 2-5 h exposure.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), using Bio-Rad reagent and bovine serum albumin as a standard.

Statistical Analysis

Multifactorial analyses of variance and covariance were used to identify overall condition effects. Significant changes were determined by post hoc comparisons of means using the Tukey HSD test. Significance was set at a confidence level of 95 %. Data are presented as mean ± SEM

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IMAGE EVALUATION
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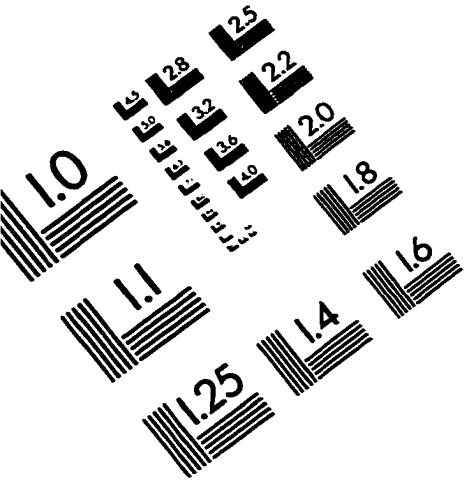

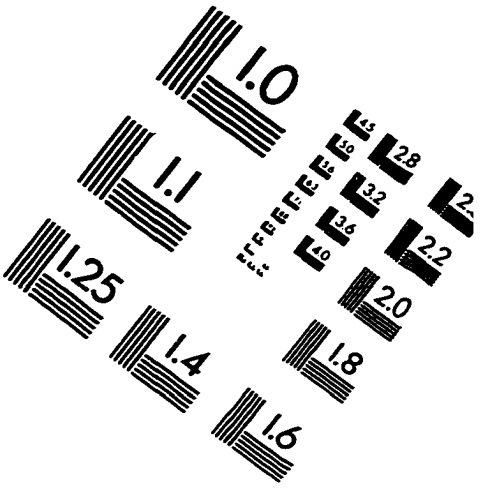

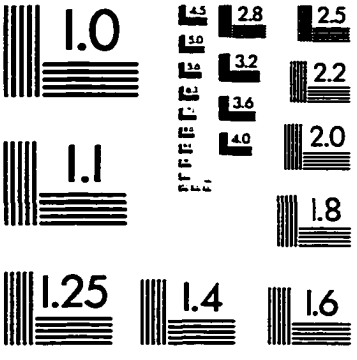

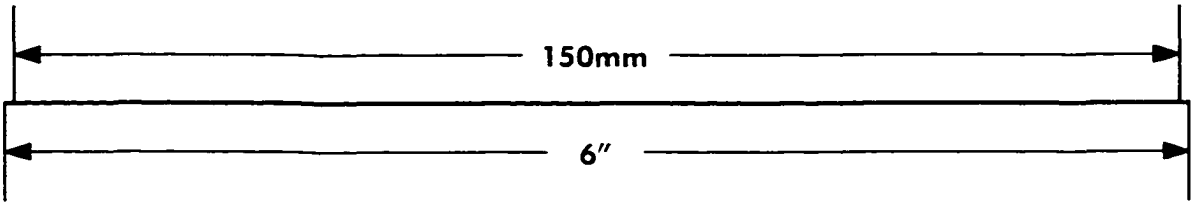

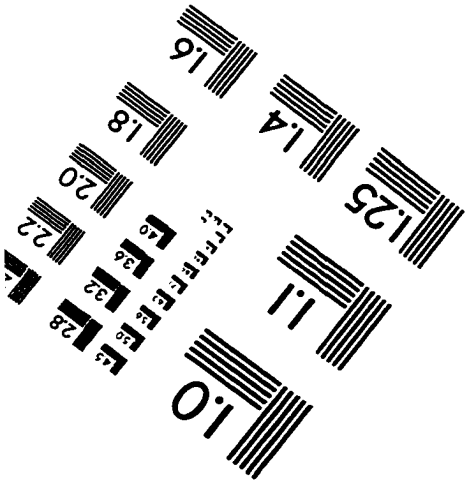

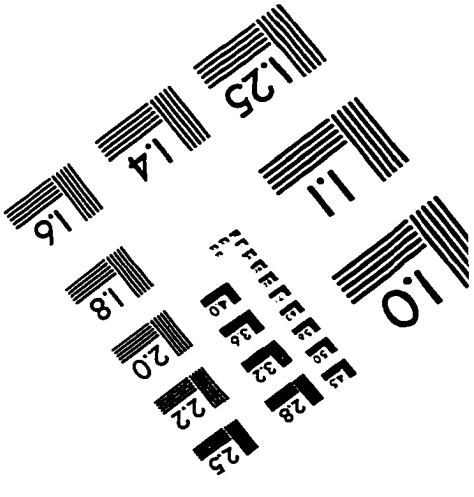

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