EVALUATION OF CHICK EMBRYO SPINAL MOTONEURON CULTURES FOR THE STUDY OF NEUROTOXICITY

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CHAPTER I.

INTRODUCTION

The study of neurotoxicity involves the determination of whether the chemical under investigation causes any deviation from the normal structure and function of the nervous system. Neurotoxicity can be brought about by a large variety of physiological, pharmacological, and biochemical mechanisms. Toxins can selectively interfere with basic metabolic processes such as energy metabolism, lipid metabolism, RNA and protein synthesis, biochemical reactions responsible for the maintenance of myelin, axonal transport, nerve membrane permeability, and conduction of action potential or synaptic transmission.

Because of the functional and structural complexity of the nervous system, the detection of toxic effects poses greater difficulty than with other target organs. Hence, multidisciplinary in vivo (behavioral, neurochemical, and neuropathological) and in vitro preparation (brain slices, synaptosomes and cell cultures) studies are essential for determining the nature and mechanism of toxicities. Since there are a multiplicity of mechanisms, there is no single in vitro biochemical or electrophysiological screening test capable of detecting all types of neurotoxic phenomenon. Valid in vitro methods for detection of toxicity require either a detailed knowledge of the mechanism (biochemical, structural and electrophysiologic) of the toxic effects under consideration or a good structural model of the target system incorporating all the factors that could influence toxicity.

A variety of in vitro models widely used in neurobiology are currently used in neurotoxicology research. These include: whole embryo, whole organ, organotypic

explants and slices, reaggregate cultures, primary cell cultures and neural cell lines. These culture systems are easily reproduced and provide economical means for the study of chemical neurotoxicity, as reasonable additions to the more conventional in vivo bioassays.

Included in the many advantages of in vitro culture systems are the ability to control experimental exposure conditions and the minimal amounts of test chemical required for analysis. As with other approaches, the cell culture system is not without limitations. These include: the reduced cellular complexity which may not represent the more complex nervous system, the lack of a Blood Brain Barrier and the lack of hepatic and extrahepatic metabolic systems that are normally present in the intact animal. Regardless of these limitations, in vitro models provide useful approaches for the screening of chemicals of neurotoxicity potential, as well as to obtain data about the mechanism of action of the chemical under investigation. In the present study chick embryo spinal motoneuron cultures are used:

1. To assess the response of cultured motoneurons to exogenous application of naturally occurring neurotransmitters (glycine, gamma-aminobutyric acid [GABA], and N-methyl D-aspartate [NMDA]) and chemical neurotoxins (strychnine, picrotoxin) using an electrochromic dye, Di-4-Anneps, which measures the change in the membrane potential of cells.
2. To study the cytotoxic effects of glycine, GABA, NMDA, strychnine, picrotoxin and bulbocapnine using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide] (MTT) assay which is based on the reduction of the soluble yellow tetrasolium MTT to a blue insoluble formazan product by the mitochondria of the cells.

CHAPTER II.

LITERATURE REVIEW

Embryonic Development

Postmitotic motoneurons appear in the spinal cord of the chick embryo between embryonic age 2.5 and 5 days ($E_{2.5}$ and E_5) (Hollyday and Hamburger, 1977). After all postmitotic motoneurons have established contact with their target muscle, the phase of developmental motoneuron death begins and leads to the loss of approximately half of those initially generated (Oppenheim et al., 1989).

Survival of embryonic motoneurons is regulated by neurotrophic factors derived from the target muscle. A study of the survival requirement of early and late developed motoneurons from E_5 chick spinal cord showed that large motoneurons developed 1.5 days earlier than the small motoneurons (Mettling et al., 1995). Large motoneurons were found to be more mature functionally and biochemically and had an acute requirement for trophic support from muscle derived factors. Small motoneurons can develop into large motoneurons suggesting that they represent a general transitional stage in motoneuron development (Mettling et al., 1995). The results suggest that there is a defined period which elapses between the initial appearance of a motoneuron and its acquisition of trophic dependence. This delay may be due to the time required for target innervation.

Different survival properties have been shown for embryonic wing motoneurons and embryonic leg motoneurons. Although spinal motoneurons in chick embryos are born in a rostrocaudal gradient, the timing of their naturally occurring cell death varies.

Brachial spinal motoneurons die later than lumbar spinal motoneurons. This delayed opoptosis in the brachial motoneuron population is intrinsically programmed, at least partially through a higher sensitivity to trophic factors (Mettling et al., 1993). Target derived neurotrophic factors have been found to reduce naturally occurring cell death in the lateral motor column of the spinal cord of chick embryos (Oppenheim et al., 1992).

Survival of motoneurons in culture

Because of their trophic dependence for growth and differentiation, motoneuron cultures established from E_6 chick spinal cord survived for less than 2 days in chemically defined medium (Juurlink et al., 1991). The addition of serum and muscle extract to the medium resulted in the survival of about 20% of the motoneurons for 8 days. It has been suggested that trophic factors derived from muscle are dependent upon the presence of another factor found in the serum (Juurlink et al., 1991). Purified factors such as ciliary neurotrophic factors (CNTF) and brain derived neurotrophic factors (BDNF) have been shown to facilitate motoneuron recovery in vitro (Megal et al., 1991; Arakawa et al., 1990; Oppenheim et al., 1992). Highly enriched embryonic chick motoneurons in culture were supported at survival rates higher than 60% by CNTF. In contrast to CNTF, the members of the neurotrophene gene family (nerve growth factor [NGF], BDNF, neurotrophene-3 [NT-3]) did not support the survival of chick embryo motoneurons in culture (Sendtner et al., 1991; Hughes et al., 1993a).

Co-culture of motoneurons with astrocytes resulted in a twofold increase in neuronal survival. The addition of insulin and insulin-like growth factor I to such cultures further increased motoneuron survival. It is suggested that the effect of insulin and insulin-like growth factor I is possibly mediated by astrocytes (Ang et al., 1992).

Recent studies reported that fibroblast growth factor 5 is a major muscle-derived survival factor which supported the survival of highly enriched cultures of embryonic chick motoneurons (Hughes et al., 1993b).

Membrane properties

Membrane potential is a complex entity attributed to the passive concentration difference which results from the presence of charged but impermeable anions within the cell, metabolically maintained concentration differences coupled with differences in passive permeability, particularly between potassium and sodium ions, and by an electrogenic action of the sodium pump (Paul, 1975).

At rest the interior of the axon membrane is -70 mV negative to the exterior. The resting potential is essentially a diffusion potential based chiefly on the 3 to 5 fold higher concentration of K^+ ion in the axoplasm as compared with the extracellular fluid and the relatively high permeability of the resting membrane to K^+ ions. Na^+ and Cl^- ions are present in higher concentration in the extracellular fluid than in the axoplasm but their concentration gradients across the membrane are somewhat lower than that of K^+. These ionic gradients are maintained by an energy dependent active transport or pump mechanism which involves ATPase activated by Na^+ at the inner and K^+ at the outer surface of membrane.

The first successful introduction of a glass microelectrode to a mammalian motoneuron was done on the spinal cord of an anesthetized cat (Brock et al., 1952). According to the results obtained from the above study, the resting potential, action potential and reversal potential are -70 mV, -95 mV, and -25 mV, respectively. The motoneuron action potential is mediated by fast Na^+ and K^+ conductances (Hille, 1977).

During nerve impulse or action potential, the internal resting potential changes from negative to positive. This reversal of potential is due to sudden, selective increases in the permeability of the membrane to Na^+ ions which flow rapidly inward in the direction of their concentration gradient. Rapid replacement of this charge by increased permeability of K^+ causes repolarization of the membrane (Cole, 1968; Naharashi, 1975). Following the action potential in spinal motoneurons there is a brief after-hyperpolarization (AHP). The ionic basis for the AHP appears to be an increase in K^+ conductance induced by an increase in intracellular Ca^{2+} concentration (Coombs et al., 1955; Gustafson, 1974). AHP is usually preceded by a small after-depolarization (ADP) due to the increase in Na^+ permeability which follows excitation (Harada and Takahashi, 1983). Results indicate that AHP and ADP are dependent upon extracellular Ca^{2+}. Ca^{2+} ions entering during the ADP activate K^+ conductance and cause AHP (Krnjevic and Liseiwicz, 1972). In support of this evidence intracellular injection of EDTA markedly suppresses AHP (Krnjevic et al., 1975). Extracellular application of Ca^{2+} antagonist divalent cations (CO, Mn) inhibits Ca^{2+} influx and reduces AHP.

Receptors and Channels

A channel is regarded as an excitable molecule that responds to stimuli such as membrane potential change, neurotransmitter or other chemical or mechanical deformation. Ionic channels are found in the membranes of all cells. Their functions include: establishing resting membrane potentials, shaping electrical signals, gating the flow of messenger Ca^+ ions, controlling cell volume, and regulating the net flow of ions across like membrane.

Voltage-gated ion channels primarily respond to membrane potential changes. Ligand-gated channels of fast chemical synapses respond to specific chemical neurotransmitters such as ACh, glutamate, glycine, and GABA.

Inhibitory aminoacid receptors

Fast inhibitory synapses involve anion permeable channels. These pores are permeable to (Cl^-, SCN^-, I^-, Br^-, No_3^-) and the physiological reversal potential is near the equilibrium potential for Cl^- ions. GABA and glycine are the predominant inhibitory neurotransmitters which act on inhibitory receptors in the vertebrate nervous system. Glycine is a major inhibitory transmitter in the spinal cord and brain stem, where it acts by activating a chloride conductance. The post-synaptic glycine receptor has been purified and contains two transmembrane subunits and a peripheral membrane protein.

DNA sequencing of the α and β subunits has revealed a common structural organization and strong homology between nicotinic acetylcholine receptors and GABA receptor proteins (Bechade et al., 1994). Walstrom and Hess (1994) found that the formation of the transmembrane channel is consistent with the prior binding of at least 3 glycine molecules to receptor form A_α and two glycine molecules to form A_β. At saturating glycine concentrations an average of 70% of the whole-cell-current amplitude was associated with form A_α and 30% with A_β. Studies on the effect of the divalent cation Zn^{2+} on inhibitory glycine receptor currents on rat embryonic spinal cord neurons revealed the existence of distinct high and low affinity Zn^{2+} binding sites on the ligand binding α subunit of glycine receptor. These sites may be implicated in the regulation of synaptic efficacy within the glycinergic pathway. Nanomolar concentrations of Zn^{2+}

enhanced the chloride ion current produced in response to the brief application of glycine ion in cultured rat spinal cord neurons (Bloomenthal et al., 1994).

Most spinal cord neurons respond to both GABA and glycine suggesting co-expression of GABA and strychnine sensitive glycine receptors in individual cells. Much less is known about the cellular localization of GABA receptors in spinal cord neuron. Immunofluorescent staining study showed that most GABA receptor positive cells exhibited prominent glycine receptor immunoreactivity (Bohlhalter et al., 1994). The co-existence of GABA and glycine receptors suggests a possible synergism in the action of GABA and glycine in spinal cord neurons. Fatima and Barry (1992) reported on the similarity between GABA and glycine activated currents and their response to a combination of saturating concentrations of both GABA and glycine which implied that the two agonists activated a comparable number of anatomically distinct channels with very similar permeation properties. Early expression of GABA and glycine receptors has been reported in rat neocortical neurons, chick lumbo-sacral spinal motoneurons and embryonic rat dorsal-horn neurons (Siebler et al., 1993; Philippe et al., 1993; Wang et al., 1994).

Excitatory aminoacid receptors

Excitatory aminoacids open cation permeable channels. The predominant fast excitatory neurotransmitter of the vertebrate CNS seems to be L-glutamate. The pharmacology of glutamate response clearly indicates that more than one type of glutamate receptor is present; including NMDA, kainate, and quisqualate (Watkins and Olverman, 1987).

Non-NMDA receptors of neurons mediate the rapid excitatory signalling of the CNS. They account entirely for the fast excitatory postsynaptic potentials of motoneurons. When glutamate or quisqualate is abruptly applied, postsynaptic channels open for only a millisecond or two and then close despite the continuous presence of agonists (Tang et al., 1989; Trussell and Fischback, 1989).

The NMDA receptors seem too specialized for transduction by elevating intracellular free Ca^{2+}. The NMDA receptor channel is 5 to 10 times more permeable to Ca^{+} ions than to Na^{+} and K^{+} (Mayer and Westbrook, 1987; Asher and Nowak, 1988). The NMDA receptor channels have high glutamate sensitivity and voltage dependence. Glutamate, aspartate and taurine immuno-reactivities were clearly detected in the ventral horn of cat spinal cord (Shupliakov et al., 1993). Another study reported the existence of androgen receptors in the motoneurons of rat spinal cord (Matsuura et al., 1993). Research findings indicated that initial synaptic transmission in embryonic motoneuron was mediated solely by NMDA receptors (Ziskind, 1990). Dose response curves of motoneurons to L-glutamate, NMDA and kainate demonstrated that motoneurons are sensitive to these agonists prior to the formation of synapses between afferents and motoneurons. Another study on neonate rat motoneurons indicated that stimulation of dorsal roots elicited a monosynaptic excitatory postsynaptic potential mediated by glutamate/aspartate acting predominantly on the quisqualate/kainate subtypes of glutamate receptors (Jiang et al., 1990). Dopamine enhances glutamate activated currents by about 200% in cultured embryonic chick motoneurons. It is suggested that dopamine is acting most probably on avian homology of the dopamine receptor family. Dopamine causes cAMP to increase which results in increased activation of kainate-gated channels during glutamate-mediated transmission (Smith et al., 1995).

CHAPTER III.

EVALUATION OF CHICK EMBRYO SPINAL MOTONEURON CULTURES FOR THE STUDY OF NEUROTOXICITY

INTRODUCTION

Cell culture systems represent an important animal test alternative to traditional in vivo testing that helps to minimize the number of animals used and to provide economical and reproducible system with well controlled experimental exposure conditions for the study of chemical neurotoxicity. Furthermore, results from initial cell culture studies may lead to more efficient experimental design for animal experiments that could save time and animal numbers, as well as expenses.

Cell cultures enriched in spinal somatic motoneurons are increasingly used as test systems for the detection of factors that are trophic or toxic for motoneurons. Cell cultures comprised solely of neurons are particularly well suited to toxicology analysis since the properties of any function under study are directly attributable to neurons without interference from other cell types. These cultures are used not only to demonstrate non-specific tissue injury and cell death, but also for the detection and quantitative assessment of specific functional, biochemical, and structural defects that may not inevitably lead to cell death.

Functional and biochemical events at the membrane interface, most importantly, the active transport of ions (pump), selective membrane permeabilities, and the associated receptors, are extremely important sites of toxicant action. Disruption of these generic cell functions will alter the membrane potential of the cell. This change in the membrane

potential of cell is considered as a bioindicator signalling the effect of the chemical under study (Stringer and Blankemeyer, 1993).

An electrochromic styryl dye, Di-4-Anneps, has frequently been used as an indicator of fast changes in membrane potential (Pvzner et al., 1993). The fast response of this styryl dye is caused by a direct potential-sensitive change in the electronic distribution within the dye. The voltage sensitivity of the fluorescence of Di-4-Anneps is characterized by a decrease of the intensity of fluorescence during depolarization (Fromherz and Cambacher, 1991). The fluorescence of Di-4-Anneps has been found to decrease approximately 1% for every 100 mV depolarization (Chien and Pine, 1991). Previous studies using Di-4-Anneps to determine the effect of toxicants on the membrane potential (Blankemeyer et al., 1992) have shown that this fluorescence assay is useful to evaluate molecular events and mechanisms associated with toxicities. Although a simple assay does not give accurate assessment of the modulation of cell function, information obtained from the fluorescence assay could be used as a guideline to further implement other studies that can validate the result. In the present study, the response of chick embryo motoneurons to various concentrations of strychnine, picrotoxin, bulbocapnine, L-glutamate and NMDA was studied using a vital dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide] (MTT). This in vitro cytotoxicity assay is based on the reduction by mitochondria of the soluble yellow tetrasolium MTT to a blue insoluble formazan product. The formazan product can be measured spectrophotometrically and is directly proportional to the number of metabolically active cells. This technique allows quantitative evaluation of toxic cell damage and death for the predictive screening of toxic agents.

Selective cellular responses other than cell death were studied using naturally occurring neurotransmitters, glycine, gamma aminobutyric acid (GABA), and N-methyl D-aspartate (NMDA) in order to determine the expression of the respective neurotransmitter receptors by motoneurons (E_7). The main objective of this study was to assess the usefulness of chick embryo spinal motoneuron cultures for neurotoxicity study. We chose these cells because they provide a homogenous population of cells approximately 95% as determined by calcitonin gene related peptide (Juurlink et al., 1990) and they generate an action potential at embryonic age 4 (E_4) which makes them suitable for membrane potential study.

MATERIALS AND METHODS

Isolation of Motoneurons

Specific pathogen-free fertile chicken eggs obtained from SPAFAS, Inc. (Illinois) were incubated for six days (E^6). Motoneurons were isolated from the E_6 chick embryo according to the procedure outlined by Juurlink (1992). After freeing the embryo from the egg, the spinal cord was isolated and freed of meninges and dorsal root ganglia. Spinal cords were incubated at 37°C for 30 minutes in 0.025% trypsin in Ca^{2+} and Mg^{2+} free puck's solution. The trypsin solution was then replaced by Dulbecco's modified eagle's medium nutrient mixture (DMEM)/F_{12} containing 0.02% DNase and the spinal cords were dissociated in a stomacher laboratory blender for one minute. After the cells were dissociated, larger fragments were allowed to settle and the supernatant was centrifuged at 180 g for ten minutes. The cell pellet was then resuspended in 5 ml DMEM/F_{12} - 0.02% DNase and layered on top of 3.5% bovine serum albumen and

centrifuged for 15 minutes at 100 q. to remove cell debris. The cell pellet was then resuspended in 5 ml DMEM/F_{12} - 0.02% DNase and layered on a discontinuous metrizamide gradient consisting of 5 ml of 6.4% metrizamide solution and centrifuged at 500 g for 30 minutes. The motoneuron fraction situated at 0% - 6.4% interface was isolated and diluted in DMEM/F_{12} - 0.02% DNase and centrifuged at 180 g for ten minutes and resuspended in chemically defined medium consisting of DMEM/F_{12} (1:1), $3x10^{-8}$ μl sodium selenite, $2x10^{-8}$ μl progesterone, 5 μg/ml transferin, 5 μg/ml insulin and 10 mM KCl. After cell counts were done the cells were diluted to $4x10^{4}$ cells/ml in growth medium containing 5% heat inactivated horse serum.

Cell Culture

The culture substratum used was poly-D-lysine coated on 96 well culture plates (Falcon). Each well contained 200 μl of growth media containing approximately 8,000 cells. After the cells were seeded, the culture plates were incubated at 37°C in a humidified 5% CO_2-in-air atmosphere. After 12 hours of incubation the cells were examined under the microscope (40x) to confirm the development of neurite outgrowth.

Preparation of Test Chemicals and Solutions

Glycine, GABA, NMDA, bulbocapnine, strychnine, and picrotoxin were obtained from Sigma Chemical Company, St. Louis, Missouri. Serial dilution of the test chemicals was done using distilled water. Different types of physiological Ringer's solutions were prepared fresh using triple distilled water on the day of the experiment.

Cytotoxicity Study

Cytotoxicity study was carried out using a vital dye, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide] (MTT). This colorimetric assay is based on the reduction of the soluble yellow tetrasolium dye to a blue insoluble formazan product by the mitochondria of viable cells. The formazan product can be measured spectrophotometrically and is directly proportional to the number of metabolically active cells in the culture. Different concentrations of strychnine (0.625 - 5 μmol), picrotoxin (0.625 - 5 μmol), bulbocapnine (0.625 - 5 μmol), NMDA (25 - 200 μmol), and L-glutamate (25 - 200 μmol) were added in E_7 motoneuron cultures. After 24 hours of incubation with the test chemical, 0.01 ml of MTT was added to each well and incubated for four hours at 37°C for cleavage of MTT. After four hours the MTT formazan product in wells containing live cells will appear as black, fuzzy crystals on the bottom of the well. Isopropanol/HCl solution (0.1 ml) was added to each well. The HCl converts the phenol red in tissue culture media to a yellow color which does not interfere with MTT formazan measurement. Isopropanol dissolves the formazan to give a homogenous blue solution suitable for absorbance measurement. After one hour, the absorbance was measured on an ELISA plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Concentration response cytotoxicity curves were generated for each chemical tested. The relative cytotoxicity of test chemicals was compared by computing the concentration needed to reduce the absorbance of MTT by 50% of control cells. Such midpoint cytotoxicity determinations were designated as MTT_{50} values.

Measurement of Membrane Potential

Membrane potential of E_7 motoneuron cultures was measured using the electrochromic dye, Di-4-Anneps. This is a dialkylaminostyrel pyridium sulfonate dye with positive and negative charges interspersed in the membrane leaflet. Changes in fluorescence of the dye occur directly in response to changes in membrane potential. The change in fluorescence was used to determine the response of the cells to each chemical agent tested. Cells were washed thrice with Ringer's solution to remove any growth media. Di-4-Anneps was added to each well with a final concentration of 2 μm. After thirty minutes, cells were washed thrice again to remove unbound dye.

Each test chemical (glycine, GABA, strychnine, picrotoxin, and NMDA) was tested in three different cell groups. Fluorescent data was collected immediately after the test chemical was added. The culture plate was put under the microscope and cells were excited with 480 nm light from a PTI Delta Scan (Photon Technologies, Inc., Princeton, NJ) monochromator based excitation source. The excitation light passed through a dichromic mirror and microscope objective and the reflected excitation light and fluorescence from the cell were returned through the objective. The excitation light was removed by a high pass optical filter. Fluorescence was measured by a photomultiplier tube operating in photon counting mode. The concentration of each test chemical was determined from results of previous experiments.

Data for each experimental group was divided by the mean value of the negative control groups and recorded as percent of negative control fluorescence. Change in fluorescence was computed as a difference from negative control group. Cells without

any chemical added and maintained in Ringer's solution were considered negative control groups and 1% KCl (14μmol) was used as a positive control.

Data Analysis

Data analysis of the fluorescence was performed with a graph pad (San Diego, CA). Data for each experimental group was divided by the mean value of the negative control groups and recorded as percent of negative control fluorescence. Curves were fitted by non-linear regression using the INPLOT program. EC_{50} were determined using the 50% point of maximum effects of test agents on fluorescence.

Individual cytotoxicity data points for each concentration of toxicant computed as a mean of three replicate groups were used to construct concentration response cytotoxicity curves. Midpoint cytotoxicity (MTT_{50}) was determined by linear regression.

RESULTS

The response of E_7 motoneurons to the neurotoxic effects of picrotoxin, strychnine, and bulbocapnine was studied using the MTT assay. Concentration response cytotoxicity curves generated with the MTT assay (Figure 1a) show the response of E_7 motoneurons to different concentrations of picrotoxin (0.625 - 5 μmol), strychnine (0.625 - 5 μmol) and bulbocapnine (0.625 - 5 μmol) after an exposure period of 24 hours. E_7 motoneurons respond in a concentration dependent manner for all the toxins applied. The relative cytotoxicities of test agents were compared by computing the concentration needed to reduce the absorbance of MTT by 50% of the control cells. According to the midpoint (MTT_{50}), cytotoxicity values determined from the concentration response

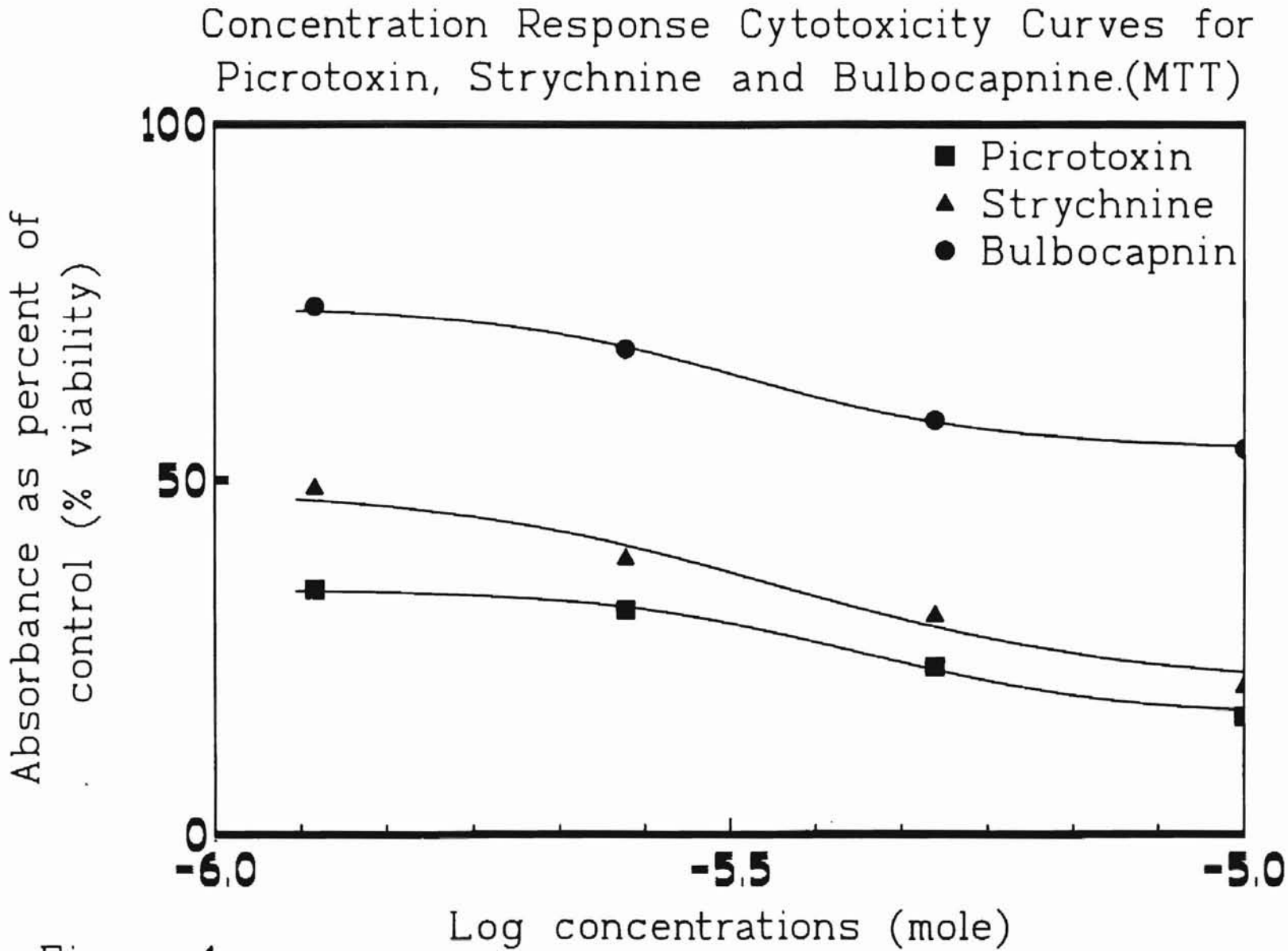
Concentration Response Cytotoxicity Curves for
Picrotoxin, Strychnine and Bulbocapnine.(MTT)
100
50
0
Absorbance as percent of
control (% viability)
Picrotoxin
Strychnine
Bulbocapnin
-6.0
-5.5
-5.0
Log concentrations (mole)

Figure 1a

cytotoxicity curve (Table I), picrotoxin was ranked first, followed by strychnine and bulbocapnine.

In order to investigate the response of motoneurons to the cytotoxic effects of the excitatory aminoacids, motoneurons were incubated with varying concentrations of L-glutamate (25 - 200 μmol) and NMDA (25 - 200 μmol) for 24 hours. The MTT test results indicated that E_7 motoneurons respond in a concentration dependent manner (Figure 1b) for both NMDA and L-glutamate. Midpoint cytotoxicity values (MTT_{50}) determined from concentration response cytotoxicity curve indicate that L-glutamate is more cytotoxic than NMDA.

Specific cellular responses other than cell death were studied by spectrofluorometric method using the styryl dye, Di-4-Anneps. The effect of the naturally occurring neurotransmitter GABA on the membrane potential of E^7 motoneuron cultures was measured using Di-4-Anneps (Table II). Bath application of 100μmol GABA caused 30% change in fluorescence (70% fluorescence as percent of negative control). KCl (14μmol) used as a positive control caused a 28% change in fluorescence (72% fluorescence as percent of negative control).

Bath application of strychnine (0.625 μmol) caused 14% change in fluorescence. When 100 μmol glycine was added on the cells pretreated with strychnine, the change in the fluorescence was only 1%, while glycine alone caused 35% change in fluorescence. This result indicates that the effect of glycine was completely blocked by strychnine which is glycine antagonist. Similarly, picrotoxin (0.625 μmol) caused 98% fluorescence (2% change in fluorescence). When 100 μmol GABA was added on cells pretreated with picrotoxin, it changed the fluorescence by only 2% while GABA alone

TABLE I

Concentrations of test agents needed to cause 50% change in fluorescence of Di-4-Anneps (EC_{50}) and concentrations needed to reduce the absorbance of MTT by 50% of control cells (MTT_{50})

Toxin	EC_{50} (μmol)	MTT_{50} (μmol)
Picrotoxin	1	3.83
Strychnine	2.5	4.54
Bulbocapnine	--	9.9
NMDA	75.1	158
L-glutamate	70.5	118

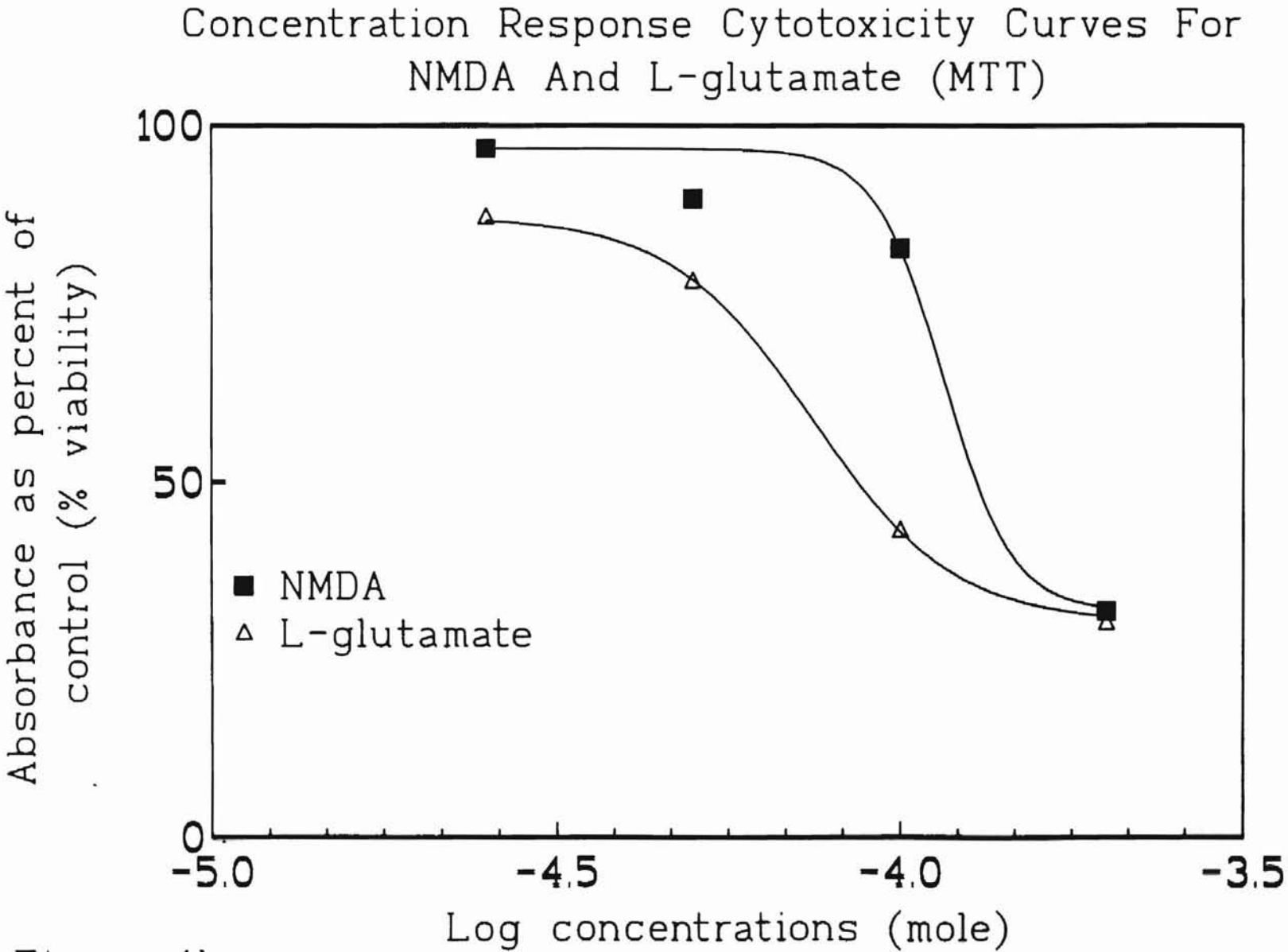
Concentration Response Cytotoxicity Curves For
NMDA And L-glutamate (MTT)
Absorbance as percent of control (% viability)
100
50
0
NMDA
L-glutamate
-5.0
-4.5
-4.0
-3.5
Log concentrations (mole)

Figure 1b

TABLE II

The response of E_7 motoneurons to bath application of KCl (positive control), neurotransmitter agonists, GABA and glycine, and antagonists, picrotoxin and strychnine

Test Agent	Concentration (μmol)	% Fluorescence of Negative Control	% Change in Fluorescence
KCl	14	72	28
Glycine	100	65	35
Strychnine	0.625	86	14
Strychnine and Glycine		85	15
GABA	100	61	39
Picrotoxin	0.625	98	2
Picrotoxin and GABA		96	4

caused 39% change in fluorescence. This result indicates that the GABA antagonist, picrotoxin, completely blocked the effect of GABA on the cells.

Bath application of 200μmol NMDA caused a 61% change in fluorescence (39% fluorescence as percent of control), while addition of glycine on the same cells treated with NMDA caused 59% change in fluorescence (41% fluorescence as percent of control) (Table III). The same experiment was repeated on different groups of cells maintained in a solution containing 0.1% Mg^{2+}. NMDA (200μmol) caused 43% change in fluorescence (57% fluorescence as percent of control). Addition of glycine on NMDA treated cells caused 56% change in fluorescence. This result indicates that the effect of NMDA on membrane potential is depressed in Mg^{2+} containing solution and glycine plays a role in potentiating the effect of NMDA. The response of E_7 motoneurons for varying concentrations of NMDA (200-25μmol), L-glutamate (200-25μmol), picrotoxin (5-0.62μmol), and strychnine (20-2.5μmol) was studied using Di-4-Anneps. E_7 motoneurons responded in a concentration dependent manner for all the chemicals tested (Figure 2a,b). The maximum change in fluorescence for NMDA, L-glutamate, picrotoxin, and strychnine was 60%, 60%, 52%, and 51% change in fluorescence, respectively. The EC_{50} which was determined using the midpoint (50%) of the maximum effect of NMDA, L-glutamate, picrotoxin, and strychnine is shown in Table I. According to the EC_{50} values obtained from the concentration response curve, picrotoxin was found highly effective in changing the membrane potential.

DISCUSSION

Ion channels of excitable membranes are major target sites for a variety of neurotoxicants (Narahashi, 1975). Certain natural toxins and environmental agents have

TABLE III

The response of E_7 motoneurons to bath application of NMDA and glycine in Mg^{2+} free Ringer's and 0.1% Mg^{2+} supplemented Ringer's solution

Solution	Test Agent	Concentration (μmol)	% Fluorescence of Negative Control	% Change in Fluorescence
Mg^{2+} free Ringer's	NMDA	100	39	61
Mg^{2+} free Ringer's	NMDA and glycine	100	31	69
Ringer's with 0.1% mg^{2+}	NMDA	100	57	43
Ringer's with 0.1% Mg^{2+}	NMDA and glycine	100	44	56

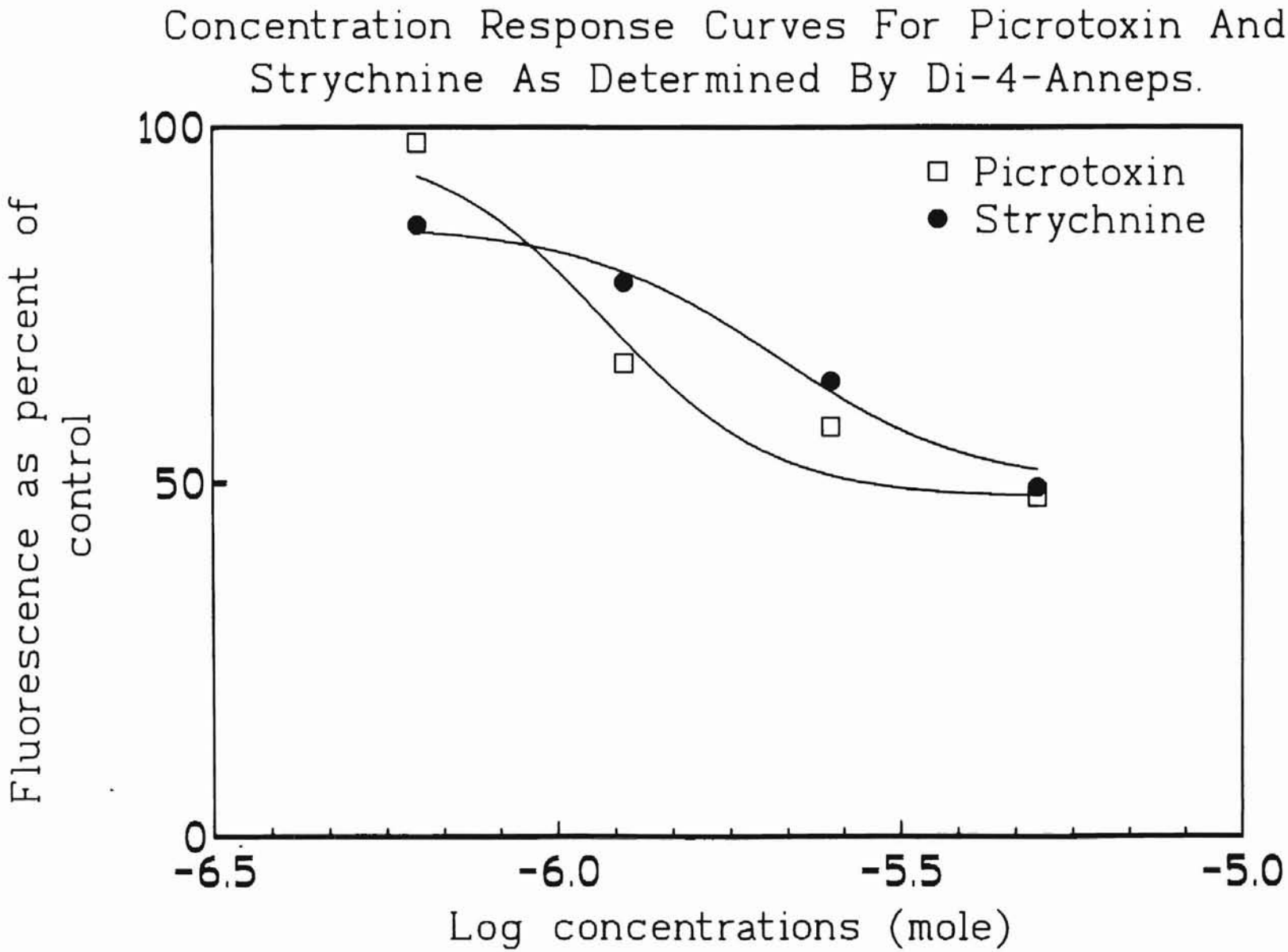
Concentration Response Curves For Picrotoxin And Strychnine As Determined By Di-4-Anneps.
100
50
0
Fluorescence as percent of control
Picrotoxin
Strychnine
-6.5
-6.0
-5.5
-5.0
Log concentrations (mole)

Figure 2a

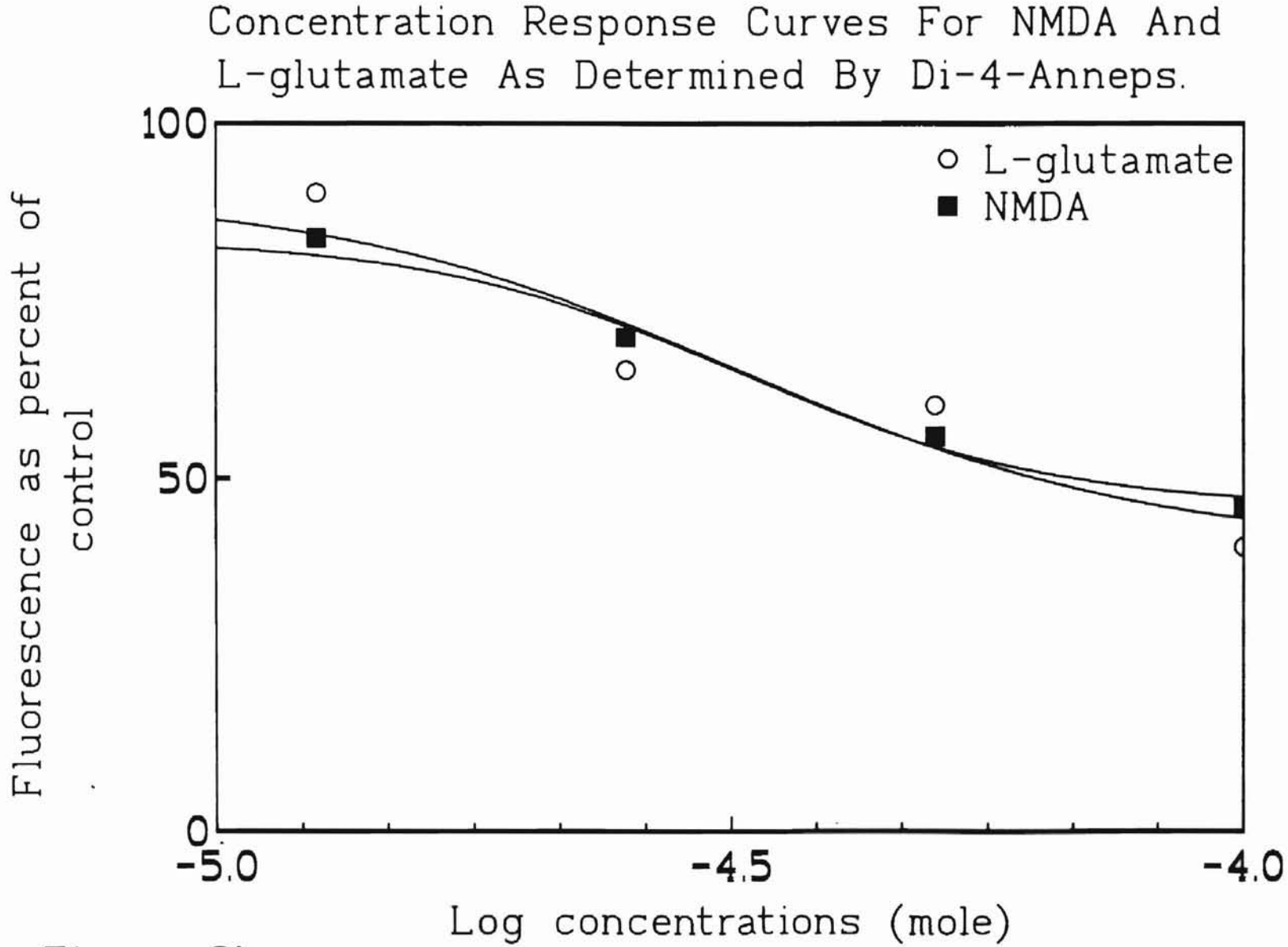
Concentration Response Curves For NMDA And L-glutamate As Determined By Di-4-Anneps.
100
50
0
Fluorescence as percent of control
L-glutamate
NMDA
-5.0
-4.5
-4.0
Log concentrations (mole)

Figure 2b

been found to act on nerve membrane ion channels in a highly specific manner. Loss of the activity of the ion channels results in a loss of function of the nerve cell. Because the generation and integration of electrogenic potential is dependent upon active ion transport across the membrane, any disruption of this activity causes a change in membrane potential. In this study we examined the effects of naturally occurring neurotransmitters and their antagonists (toxicants) on embryonic (E_7) chick spinal motoneuron cultures by measuring toxic cell damage and death using a viability assay (MTT) and the change in membrane potential using an electrochromic dye, Di-4-Anneps.

The results demonstrate that chick embryo motoneurons in culture express receptors for GABA, glycine, NMDA, and dopamine. The response of chick embryo motoneurons to strychnine in a dose dependent manner in both studies (spectrofluorometry and cytotoxicity study) and the ability of strychnine to block the effect of glycine indicates that motoneurons in culture express glycine receptors. Strychnine acts as a competitive antagonist of the inhibitory neurotransmitter, glycine, at a post-synaptic inhibitory site. Receptor binding studies indicate that both strychnine and glycine interact with the same receptor complex although possibly at different sites (Curtis and Johnston, 1974). The response of chick embryo motoneurons to picrotoxin in a dose dependent manner and the ability of picrotoxin to block the effect of GABA indicates the existence of GABA receptors. Picrotoxin directly blocks the Cl^- channels of GABA and decreases the frequency of channel opening. According to the values for EC_{50} (Di-4-Anneps) and MTT_{50}, chick embryo motoneurons were found highly susceptible to picrotoxin followed by strychnine.

The response of chick embryo motoneurons to GABA and glycine was membrane depolarization as opposed to the hyperpolarizing effect typical of adult motoneurons. In

differentiating motoneurons, glycine and GABA induce chloride dependent membrane depolarization and a large decrease in membrane resistance (Wu et al., 1992). In support of this finding (Wang et al., 1994) reported developmental loss of GABA and glycine induced depolarization in embryonic rat dorsal horn neurons in culture. It is well established that both glycinergic and GABAergic mechanisms play a role in recurrent inhibition of motoneurons in the mammalian spinal cord; much less is known about their role in avian species. A postembedding immunocytochemistry study revealed that Ca^{2+} binding protein, parvalbumen and GABA localize in the majority of chick lumbosacral spinal motoneurons (Philippe et al., 1993). Another study found that glycine receptors and ion channels were expressed in amphibian oocyst after injection of chicken retina mRNA (Zhu et al., 1994). Electrophysiological and biochemical investigations have also shown that some motoneurons located in the spinal cord of the young chicken display GABA-like immunoreactivity (Philippe et al., 1990). A high frequency of co-localization of GABA and glycine receptors has been reported by Taal and Holstege (1994). Both types of receptors have very similar distribution patterns in the cell membrane and are frequently localized suggesting a possible opportunity for synergism in the action of GABA and glycine in spinal motoneurons (Schneider and Fyfe, 1992).

NMDA receptors represent a distinct class of glutamate-gated channels permeant to monovalent cations and Ca^{2+}. Studies on rat embryos indicated that NMDA receptors mediate poly- and monosynaptic potentials (Ziskind, 1990). At least five distinct sites are recognized in NMDA receptors, namely NMDA, glycine, phenylclidin, Mg^{2+}, and Zn^{2+} sites. The result of our experiments show that NMDA causes less effect in changing the membrane potential in the cells maintained with Mg^{+} supplemented solution. One of the features of NMDA receptor channels is that they are blocked by

low concentrations of Mg^{2+} in a voltage dependent manner. Mg^{2+} ion induced block occurs only at the negative potential range and increases with hyperpolarization. Mg^{2+} ions enter the channel when it opens and bind to multiple sites within the channel (Nowak et al., 1984).

Application of glycine in NMDA treated cells causes a drastic change in membrane potential (13%). Glycine has been found to play an important role in the modulation of NMDA receptors. In support of our finding, Johnson and Asher (1987) have shown that glycine potentiates the NMDA responses in cultured mouse brain neurons. It appears that glycine binds to an NMDA receptor glycine site as a co-agonist while NMDA binds to another site.

Subsequent exposure of chick motoneurons to different concentrations of NMDA elicited a dose dependent change in membrane potential. As indicated in our result, the blocking effect of Mg^{2+}, the potentiating effect of glycine and the response of motoneurons in a dose dependent manner are used to confirm the expression of NMDA receptors by motoneurons. The susceptibility of motoneurons to the naturally occurring neurotransmitter glutamate was very high as compared to NMDA. NMDA activated channels are permeable to Ca^{+} as well as Na^{+} and K^{+}. Thus prolonged exposure to agonists such as L-glutamate will result in a massive influx of Ca^{2+} and cause cell death.

Chick embryo motoneurons responded to bulbocapnine in a dose dependent manner. Bulbocapnine, which is derived from Corydalis cava roots, has blocking activity on dopamine receptors. Dopamine receptors have been identified in cultured embryonic chick motoneurons (Smith et al., 1995).

CHAPTER IV.

SUMMARY AND CONCLUSIONS

The performance of chick embryo motoneuron cultures (E_7) as investigated by two different techniques (Di-4-Anneps and MTT) has proven useful and informative in neurotoxicological studies. The results of the spectrofluorometric study (Di-4-Anneps) and the cytotoxicity study (MTT) indicate that E_7 motoneurons express receptors for neurotransmitter, GABA, glycine, NMDA, and dopamine.

Motoneuron cultures (E_7) are appropriate to study the mode of action of neurotoxic compounds using receptor binding interactions. Using the spectrofluorometric assay (Di-4-Anneps) we were able to study the response of E_7 and to determine the expression of the respective receptors by E_7 motoneurons in culture.

In addition to their mechanistic and investigative potential, E_7 motoneuron cultures can be used as a pre-development screen for agents with interesting pharmacological profiles as well as neurotoxic potential. Using the cytotoxicity assay (MTT) we were able to further confirm the expression of receptors as well as to determine the relative toxicities of test agents.

The presence of receptors and the ability of motoneurons to generate action potentials at an early embryonic state (E_4) makes this culture system a reliable model to conduct mechanistic studies using spectrofluorometry as well as predictive screening tests for agents of pharmacological and toxicological potential using cytotoxicity studies. The problems with motoneuron culture include the time consuming dissection and the relatively small amount of material obtained for toxicological analysis. Furthermore, motoneuron cultures are considerably fastidious and it is difficult to maintain them over

long periods of time. To alleviate this problem, the rapid development and availability of trophic factors that support the growth of motoneurons in culture for extended periods of time is crucial.

CHAPTER V

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VITA

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