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City University of New York, 1994

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ASCORBIC ACID IN MESENCEPHALIC CULTURES:

EFFECTS ON DOPAMINERGIC NEURON DEVELOPMENT

by

Henry H. Kalir

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

1994

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Abstract

ASCORBIC ACID IN MESENCEPHALIC CULTURES:
EFFECTS ON DOPAMINERGIC NEURON DEVELOPMENT
by
Henry H. Kalir

Advisor: Professor Catherine Mytilineou

Ascorbic acid (AA) exists in high intracellular concentrations in fetal rat brain. In mesencephalic (MB) cultures prepared from E14 rat embryos, AA drops sharply to undetectable levels when no ascorbic acid is added to the medium, thus creating a model of scorbutic neuronal tissue and affording the study of AA's effects on its development and function. Cultures were treated both chronically and acutely with 0.2 mM AA, and compared to controls (scorbutic cultures) by using morphological and biochemical indices.

In the chronically-treated AA cultures the following were noted on 7 and 14 days in vitro (DIV):

1. A marked increase in glial proliferation demonstrated by glial fibrillary acidic protein staining.
2. Increased neurite growth and number of dopaminergic neurons on tyrosine hydroxylase (TOH) staining.
3. A significantly higher dopamine (DA) uptake ([^{3}H]DA uptake).
4. Significantly higher levels of DA and 3,4-dihydroxyphenylacetic acid (DOPAC).

The capacity to accumulate and retain the intracellular AA developed gradually as the cultures matured. Ascorbic acid reached

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the embryonal levels by DIV 14. These data show that although neuronal cultures can survive and grow in the absence of detectable levels of ascorbic acid, its chronic presence exerts a broad effect on dopamine neuron morphology and biochemical functioning either directly or through increased glial proliferation, or possibly both.

In an attempt to clarify the mechanism by which AA affects these changes, the acute effects of AA on the levels of DA in MB cultures were investigated. These DA levels were then correlated with the results of TOH immunocytochemistry, [^{3}H]DA uptake and AA levels over a period of 48 hrs following the addition or omission of 0.2 mM AA in DIV10 mesencephalic cultures. The results showed an increase in the number of TOH stained neurons and the extent of staining after 24 hrs exposure to AA, with an increase in the DA content only after 48 hrs. The AA levels reached a maximum value at 24 hrs and declined in a manner similar to the chronically AA-treated cultures. The [^{3}H]DA uptake remained unchanged. These data indicate that the cultures not previously treated with AA are capable of AA accumulation and retention in a manner similar to the chronically AA-treated cultures, and that the acute effects on the DA content are not due to an increase in neuronal survival and could be the result of regulation of TOH activity.

Finally, in order to clarify the mechanism by which AA affects these acute DA changes, the effects of AA on TOH activity were investigated by measuring the levels of L-dihydroxyphenylalanine (L-DOPA) in naive, L-AA and D isoascorbic acid (D-AA - the biologically inactive epimer) treated young cultures from DIV 0 through 2. During this period, the cultures are relatively immature,

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but contain TOH (as evidenced by TOH immunocytochemical staining and a baseline L-DOPA level), while the other synthetic and catabolic enzymes of DA are relatively inactive, as indicated by the very low levels of DA and DOPAC. Thus these young MB cultures provide a good model for exploring factors affecting the in vivo TOH activity. These experiments showed a marked increase in L-DOPA levels at 24 hrs only in the L-AA treated group but not in the D-AA or control groups, and indicate a possible regulatory effect on the TOH activity.

The data presented in this thesis indicate that AA probably plays a significant role in regulating DA metabolism through its effects on TOH activity and possibly other enzymes in the DA synthetic and catabolic chain.

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Acknowledgements

This is my second graduate degree, separated from my MD by a period of nearly 2 decades. My experience during this time has served to reinforce my strong belief that people cannot thrive in a vacuum. I therefore would like to express my deep gratitude to the following people who so generously have filled my world with their wisdom, knowledge, friendship, love and encouragement.

To my mentor - Dr. Catherine Mytilineou for her boundless enthusiasm, support and encouragement. May all future graduate students be blessed with such a mentor. To the members of my advisory committee: Dr. M. Van Woert - for his advice and generosity in allowing me the use of his HPLC equipment, and to Dr. Tim Brannan for his advice and friendship. To Dr. Bernard Cohen - for all his generous help and steadfast encouragement. To Ms. Julie Shen, who sadly passed away in 1993, for all her technical assistance and ever positive outlook of life. To all my colleagues and friends in the Departments of Neurology and Neurobiology at the Mt. Sinai School of Medicine- for the pleasant and productive time. To all my colleagues and friends in the Department of Psychiatry at UMDNJ-Newark for their encouragement in the final phases of the dissertation-writing process. Last, but not least, to my parents: My mother - Anat Kalir, MD who showed me that even Auschwitz cannot bring down those who persevere, and my father - Asher Kalir, PhD, Professor of Medicinal Chemistry and organic chemist non pareil, for being such a great role model throughout my life. May all be blessed with such parents. And finally, to my children - David and Eva for making it all worthwhile.

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TABLE OF CONTENTS

Approval page........ ii
Abstract........ iii
Acknowledgements........ vi
Table of Contents........ vii
List of Figures........ ix
List of Tables........ x
Chapter 1: Overview and background........ p. 1
1.1 Overview........ p. 1
1.2 Ascorbic Acid........ p. 6
1.2.1 Ascorbic Acid synthesis........ p. 8
1.2.2 Ascorbic Acid catabolism........ p. 10
1.2.3 Ascorbic Acid as an antioxidant........ p. 12
1.3 Dopamine........ p. 15
1.3.1 Dopamine synthesis........ p. 16
1.3.2 Dopamine catabolism........ p. 18
1.3.3 Dopamine storage and release........ p. 20
Chapter 2: Materials and methods........ p. 22
2.1 Source of chemicals, drugs and antibodies........ p. 22
2.2 Cell cultures........ p. 22
2.2.1: Cell culture preparation........ p. 22
2.2.2: Cell culture maintenance........ p. 23
2.2.3: Ascorbic Acid Treatment........ p. 23
2.3 Ascorbic Acid assay........ p. 24
2.4 Catecholamine analysis........ p. 25
2.5 [^{3}H] Dopamine uptake assay........ p. 26
2.6 Immunocytochemistry assays........ p. 26
2.6.1 Tyrosine Hydroxylase staining........ p. 27
2.6.2 GFAP staining........ p. 27
2.7 GSH assay........ p. 27
2.8 Protein determination........ p. 28
2.9 Statistical analysis........ p. 28

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Chapter 3: Experiments: Results and discussion.............. p. 31
3.1 Results.. p. 31
3.1.1 Chronic L-AA exposure...................................... p. 31
3.1.1a Effects on culture L-AA content............. p. 31
3.1.1b Effects on culture DA & DOPAC content... p. 33
3.1.1c Effects on [^{3}H] Dopamine uptake............. p. 36
3.1.1d Effects on TOH + neurons............................ p. 38
3.1.1e Effects on culture glial cells........................ p. 40
3.1.2 Acute L-AA exposure... p. 42
3.1.2a Effects on culture L-AA content.............. p. 42
3.1.2b Effects on culture DA content.................... p. 45
3.1.2c Effects on [^{3}H] Dopamine uptake.............. p. 48
3.1.2d Effects on TOH + neurons............................ p. 50
3.1.2e Effects on GSH content................................. p. 53
3.1.3 AA effects on TOH activity.................................. p. 56
3.1.3a Cellular accumulation of AA stereoisomers.. p. 56
3.1.3b Effects of AA stereoisomers exposure.... p. 61
3.2. Discussion.. p. 63
Chapter 4: Summary and conclusions....................................... p. 70
References.. p. 73

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List of Tables

Table 1 Advantages of neuronal cultures in AA studies............. p. 4
Table 2 AA's currently known functions and effects.................... p. 5

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List of Figures

Fig. 1 Ascorbic Acid structure.......... p. 7
Fig. 2 Ascorbic Acid biosynthesis.......... p. 9
Fig. 3 Ascorbic Acid catabolism.......... p. 11
Fig. 4 Dopamine biosynthesis.......... p. 17
Fig. 5 Dopamine catabolism.......... p. 19
Fig. 6 Dopamine reuptake and storage.......... p. 21
Fig. 7 Typical AA Chromatogram.......... p. 29
Fig. 8 Typical CA separation chromatogram.......... p. 30
Fig. 9 AA content - chronic treatment.......... p. 32
Fig. 10a DA content - chronic AA treatment.......... p. 34
Fig. 10b DOPAC content - chronic AA treatment.......... p. 35
Fig. 11 [^{3}H]DA accumulation - chronic AA treatment.......... p. 37
Fig. 12 Tyrosine hydroxylase staining - chronic AA tx.......... p. 39
Fig. 13 GFAP staining - chronic AA treatment.......... p. 41
Fig. 14a AA content - acute AA tx - naive cultures.......... p. 43
Fig. 14b AA content - acute AA tx- chronic AA tx cultures.... p. 44
Fig. 15a DA content - acute AA tx - naive cultures.......... p. 46
Fig. 15b DA content - acute AA tx - chronic AA tx cultures... p. 47
Fig. 16 [^{3}H]DA accumulation - acute AA treatment.......... p. 49
Fig. 17 TOH+ neuronal count - acute AA treatment.......... p. 51
Fig. 18 Tyrosine hydroxylase staining - acute AA tx.......... p. 52
Fig. 19a GSH content - acute AA treatment.......... p. 54
Fig. 19b GSSG/GSH ratio - acute AA treatment.......... p. 55
Fig. 20 L-AA content - young MB cultures.......... p. 58
Fig. 21 L-AA and D-AA content in young D-AA tx cultures. p. 59
Fig. 22 Total AA content - young MB cultures.......... p. 60
Fig. 23 L-DOPA content - young MB cultures.......... p. 62

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Chapter 1: Overview and Background

1.1 Overview

Ascorbic acid (AA) is an essential nutrient present in all tissues (Hammarstrom 1966). Humans, primates and other mammals such as the guinea pig have lost the ability to synthesize AA from glucose. They lack the liver microsomal enzyme L-gulonolactone oxidase and therefore cannot convert L-gulonolactone to 2-keto-L-gulonolactone and to L- ascorbic acid (Chatterjee 1970; Levine 1986). Mammals which are capable of synthesizing AA can do so only in the liver, so that tissues in other organs still need to have the capacity to import AA. In the absence of an external AA intake, scurvy develops with prominent connective tissue disturbances such as gum swelling and hematomas and mucosal bleeding (Levine 1986). This condition has been recognized clinically by Lind - a Royal Navy physician who first carried out a controlled clinical trial in 1747 and demonstrated that oranges and lemons are capable of reversing the scorbutic symptoms. AA itself was isolated only in 1928 and its structure determined in 1932.

In 1907 the guinea pig was identified as lacking AA synthetic capabilities, thus affording an animal model of scurvy. In subsequent studies with this model it soon became apparent that while most tissues could be made to lose all of their AA content when the animals were fed a scorbutogenic diet for 2-3 weeks, the brain presented an exception by holding on to ~25% its AA content (Hughes 1956; Saner et al. 1975). When the brain AA content was measured in various animals, its concentration was found to be high relative to

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other tissues (Hammarstrom 1966; Hughes et al. 1971). In fetal rat brains its concentration was found to be higher than the adult rat's by a factor of ~1.5 (Kratzing et al. 1985), but it continued to remain high (~200 μg/gm) throughout the life of the animal (Milby et al. 1982). To maintain these levels a complex system has evolved which enables the mammalian brain in all animals regardless of their AA synthetic capabilities, to import, store and conserve AA. The intracellular AA stores also served as a source for the homeostatic control of the extracellular fluid AA concentration (Schenk et al. 1982; Wilson 1990). In the intact mammalian brain a large percentage of the AA content is washable immediately, and thus presumably is extracellular (Kratzing et al. 1982). Recently, a high affinity, stereo-selective uptake mechanism for L-AA has been found to exist in astrocytes (Wilson 1989). Similar uptake mechanisms also have been demonstrated to exist in other cells such as neutrophils (Washko et al. 1989; Washko et al. 1990), adrenomedullary chromaffin cells (Diliberto et al. 1983) and osteoblasts (Wilson and Dixon 1989). Therefore, to understand the intact brain's ability to hold on to its AA when all the other tissues have lost theirs, other mechanisms have to be considered. The blood brain barrier and the choroid plexus apparently endow the brain with this ability: The blood brain barrier prevents AA from crossing it, thus isolating the AA content of the intact brain from the rest of the body's AA pool, while the choroid plexus has been found to have a highly specific AA transport system, which allows AA to be actively imported (Spector 1989). The precise array of cellular and subcellular storage sites has not been mapped out to date.

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Given the brain's high AA content, especially in the fetal brain, the question of its role in development and function arises. Here studies have been hindered by the lack of a truly scorbutic brain model, as discussed above, so that no current data are available as to its effects on brain development. The advantages of neuronal cultures in studying the role of AA in the nervous system are listed in Table 1. The reported actions include a general function as an antioxidant- both intracellularly and extracellularly (Frei et al. 1989; Halliwell and Gutteridge 1990; Hugh et al. 1987; Vatassery et al. 1989; Wayner et al. 1986), stimulating the release of acetylcholine (ACh) and norepinephrine (NE) from synaptic vesicles (Kuo et al. 1979), decreasing the binding of various ligands to their receptors (Leslie et al. 1980; Wiener et al. 1989), increasing the number of ACh receptors in myogenic cells (Knaack and Podelski 1985) and slowing the rate of NE clearance from the body (Saner et al. 1975). Specific biochemical actions have been demonstrated only in a few reactions involving oxidation, such as the β-hydroxylation of dopamine to NE (Diliberto and Allen 1981) and the hydroxylation of proline to hydroxyproline in connective tissues (Levine 1986). Current knowledge concerning AA's effects on tissue development consists of mostly generalized observations in non-neural tissues. It has been demonstrated to inhibit malignant T-cell proliferation (Helgestad et al. 1990) and shows antimetabolic activity in human and animal tumors (Pavelic et al. 1989). In cell cultures it induces calcium deposition in chick chondrocytes (Leboy et al. 1989; Wu et al. 1989), increases radioactive proline incorporation into collagen in rat bone cultures (Spindler et al. 1989) and markedly induces alkaline

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phosphatase activity in both. In cultured trabecular meshwork cells it promotes fibronectin and laminin production (Yue et al. 1990). See Table 2 for a summary of the above. These studies notwithstanding, no studies on AA's effects on neural tissue development have been reported to date in the literature.

Table 1: Advantages of neuronal cultures in the study of AA's role in CNS

1. **Cultures afford a scorbutic model of neuronal tissue - no blood brain barrier & choroid plexus involvement.**

2. **Cultures afford the study of AA's effects on development and functioning of neural tissue, without any other organ involvement.**

3. **Cultures afford the study of AA uptake and storage sites on whole tissue from a specific area and also its separate components.**

4. **Cultures afford assessing AA's antioxidant or prooxidant capacity.**

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Table 2: AA's currently known functions & effects

1. ***In defined biochemical reactions:***

   a. **Cofactor in the hydroxylation of proline residues in collagen.**

   b. **Cofactor in dopamine β-hydroxylation to norepinephrine.**

2. ***In various tissue systems.***

   a. **Induction of mineral deposition (Ca^{2+}) in cultured chondrocytes.**

   b. **Modulation of fibronectin and laminin production in trabecular-meshwork cell cultures.**

   c. **Reduction of ovarian aromatase activity in rats.**

   d. **Stimulation of alkaline phosphatase activity in bone cell cultures.**

   e. **Increases the ACh receptor density on muscle cells.**

   f. **Mediates the a-amidation of a-MSH in intermediate pituitary lobe cell culture.**

   g. **Modulation of various ligand-receptor bindings.**

   h. **Inhibition of tumor growth (T cell, mammary & melanoma).**

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1.2 Ascorbic acid

AA is a relatively simple compound (Fig. 1). It is a vitamin for humans, primates and mammals such as the guinea pig, which have lost the ability to synthesize it from glucose. They lack the liver microsomal enzyme L-gulonolactone oxidase and therefore cannot convert L-gulonolactone to 2-keto-L-gulonolactone and to L- ascorbic acid.

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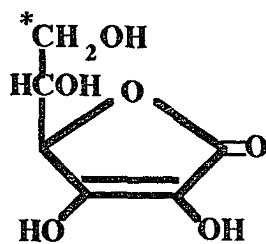

Fig. 1 **Ascorbic Acid**

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1.2.1 Ascorbic acid synthesis

Mammals which are capable of synthesizing AA from glucose can do so only in the liver, so that tissues in other organs still need to have the capacity to import AA (Chatterjee 1970; Levine 1986). The synthetic pathway of AA from glucose is outlined in Fig. 2 below.

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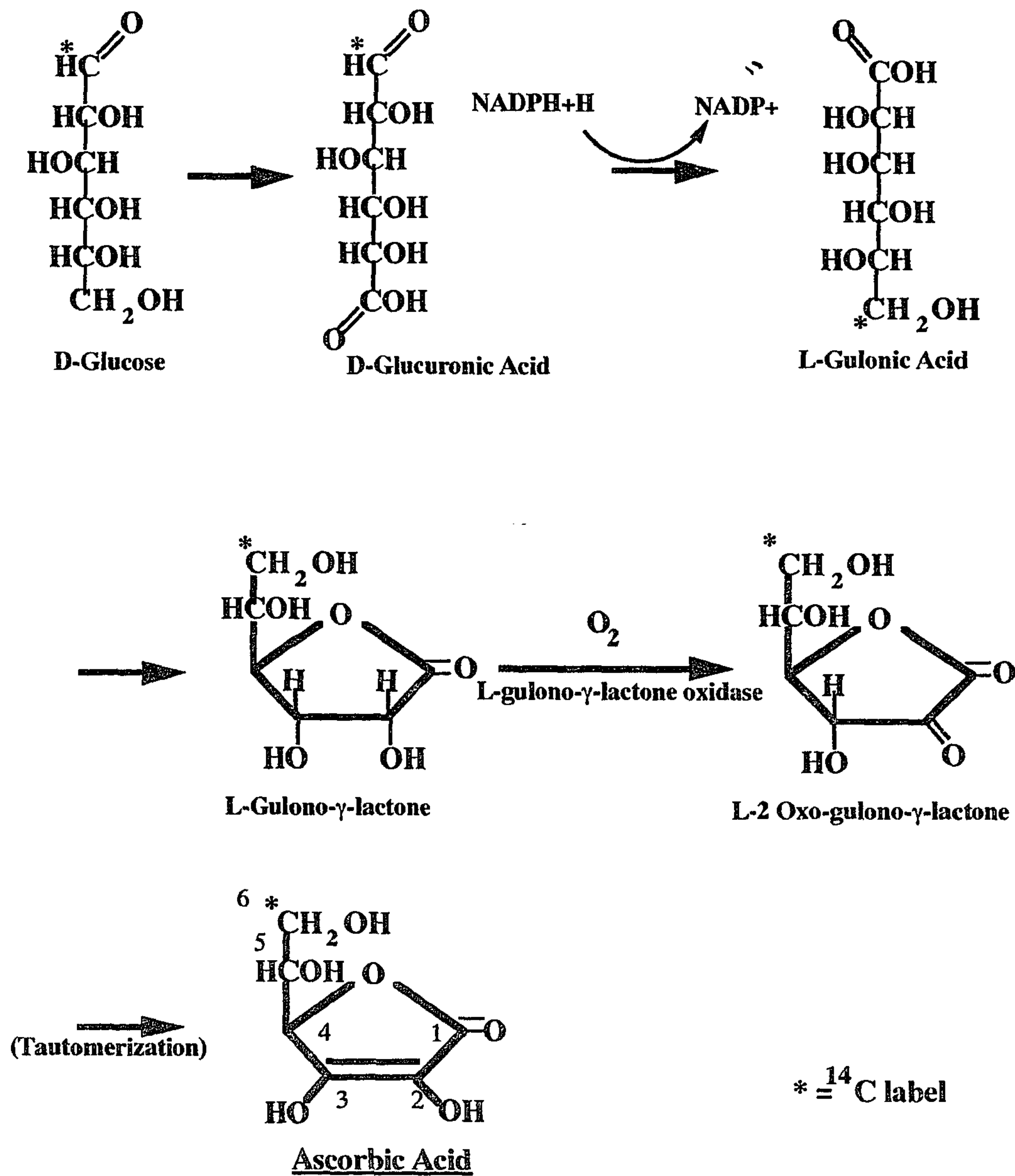
Fig. 2 Ascorbic Acid - biosynthesis



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1.2.2 Ascorbic acid catabolism

The catabolic pathway of AA is shown below in Fig. 3

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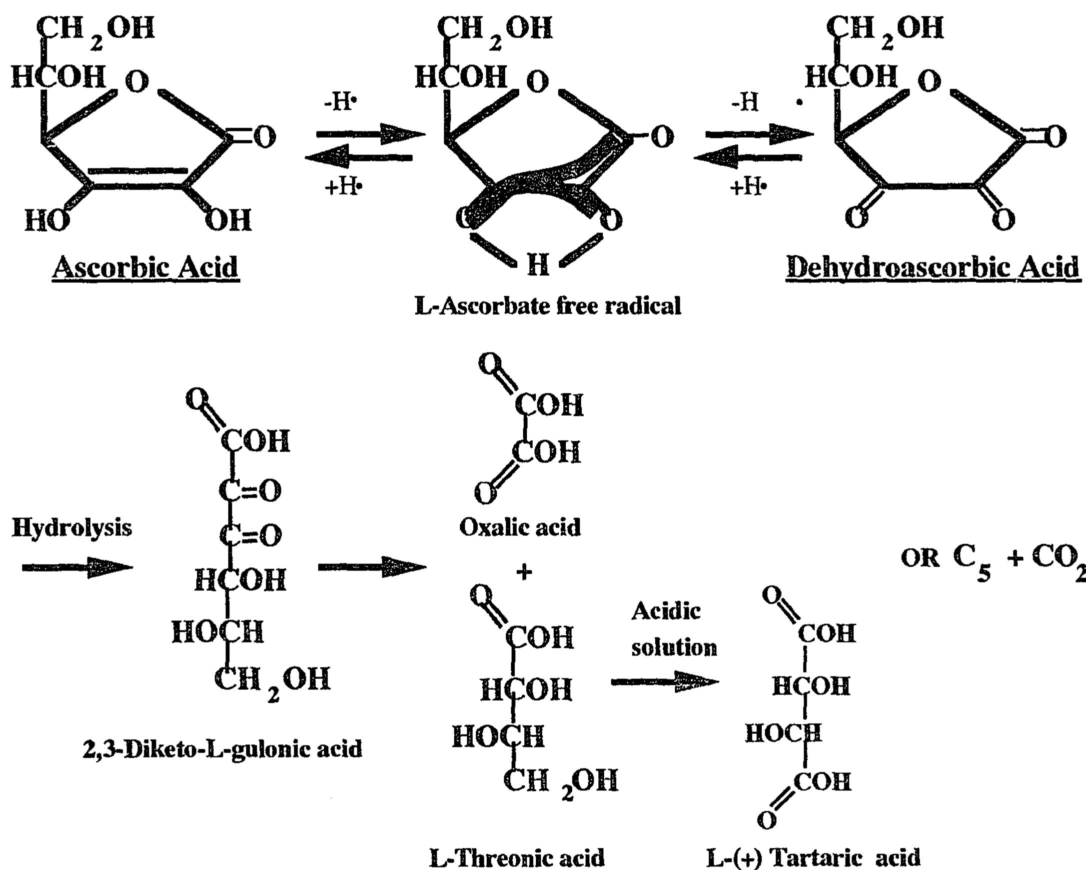
Fig. 3 Ascorbic acid - catabolism



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1.2.3: Ascorbic Acid as an antioxidant

An antioxidant may be defined as a substance which, at relatively low concentrations compared to the oxidizable substrate, significantly delays or inhibits the oxidation of that substrate (Halliwell 1990). In the course of aerobic cellular metabolism, several reactive oxygen species are formed. The reactive oxygen species consist of true radicals, molecules containing oxygen with an unpaired electron, and non-radical oxygen containing molecules with high reactivity, such as H_2O_2. Some are formed as a result of electron leakage from the mitochondrial electron transport chain, while others are formed deliberately-such as $O_2^{\bullet-}$ production by phagocytes (Halliwell 1990; Halliwell and Gutteridge 1990). There are several biologically-relevant reactive oxygen species (Halliwell and Gutteridge 1989):

1. $O_2^{\bullet-}$: Superoxide radical is formed in several ways in the CNS: a. In the mitochondria, as a result of single electron reduction of O_2. b. By oxidative enzymes such as xanthine oxidase (mainly in blood vessel endothelium). c. Through the peroxidase action of PGH synthase d. Catecholamine autooxidation. e. By polymorphonuclear leucocytes using NADPH oxidase.

2. H_2O_2: Much of the hydrogen peroxide in tissues is formed from the dismutation of the superoxide radical by superoxide dismutase: $2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. Some is also produced enzymatically by the divalent reduction of oxygen. Unlike $O_2^{\bullet-}$, H_2O_2 is capable of crossing biological membranes. It is a powerful oxidant which is normally

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broken down to $2H_2O$ and O_2 by catalase or to $2H_2O$ + GSSG (glutathione disulfide) by glutathione peroxidase. Since the activities of these two enzymes in the brain are low, its potential toxicity there is marked.

3. **OH•**: The hydroxyl radical is highly reactive with all cellular components. It is thought to be the major route by which $O_2^{\cdot-}$ and H_2O_2 exert their toxic effects. Its formation may occur via 2 reactions:

a. The Haber-Weiss reaction: $H_2O_2 + O_2^{\cdot-} \rightarrow OH\cdot + OH^- + O_2$, which has a relatively slow rate.

b. The faster Fenton reaction: $H_2O_2 + Fe^{2+} \rightarrow OH\cdot + OH^- + Fe^{3+}$. In the latter reaction, the ferric iron may be recycled to ferrous iron by superoxide thus enabling the continued generation of the hydroxyl radical ($Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$).

4. **Singlet molecular oxygen**: This is an electron-excited state of oxygen, which has considerable chemical reactivity. The changes occurring in the "ground state O_2" which produce it consist of a change in the spin direction of one of the 2p electrons, thus allowing it to occupy the same orbital as the other electron. This, in turn permits the simultaneous acceptance of a pair of electrons rather than one at a time and thus contributes to the marked reactivity of this O_2 species. It is generated via photochemical and lipid peroxidation reactions. Substances capable of neutralizing it by absorbing its excess energy and regenerating the ground state O_2 are called quenchers.

Transitional metals ions, such as iron and copper are able to generate free radicals via Fenton type reactions, and hence are carefully sequestrated by binding to carrier proteins such as

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transferrin for iron and nonspecific binding of copper to ceruloplasmin (Baysal et al. 1989; Halliwell and Gutteridge 1989; Miller et al. 1990; Vile et al. 1987).

AA has long been known to exert powerful antioxidant actions. Because it is water soluble, these actions occur in the aqueous compartment of tissues and are known to complement to a certain extent the function of the lipid-soluble antioxidant α-tocopherol (Vitamin E) (Niki 1987) and other antioxidants such as glutathione (GSH) (Bigley et al. 1980; Trumper et al. 1989; Vatassery et al. 1989). Its presence in relatively high concentrations in various tissues and extracellular fluids makes it a quantitatively significant reducing agent. Its known antioxidant actions are numerous, and include the following (Halliwell and Gutteridge 1989):

a. Scavenging $O_2^{\bullet-}$, $OH^{\bullet}$, and $RO_2^{\bullet}$ (peroxyl) radicals.
b. Inhibiting lipid peroxidation induced by a variety of free radical generating systems (i.e. activated neutrophils).
c. Scavenging and quenching singlet O_2 in aqueous solution.
d. Scavenging thiyl ($RS^{\bullet}$) radicals.
e. Reducing α-tocopheryl radicals in plasma membranes to α-tocopherol.

The relative stability and low reactivity of the ascorbic acid radicals generated as a result of its antioxidant actions (Bielski and Richter 1975) further underscore its usefulness. AA's actions however are not always protective, and depend on interactions with other factors. One of the known hazardous interactions is with transitional metals , in which the AA reducing power drives the recycling of Fe^{3+} to Fe^{2+}

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and thus enable the continued generation of the OH• radical through the Fenton reaction. Recycling copper ions by reducing Cu^{2+} to Cu^{+} enables the continued OH• generation to take place via a Fenton-like reaction: $Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet} + OH^{-}$.

Under such circumstances, AA assumes pro-oxidant rather than antioxidant properties. While iron and copper ions appear to be effectively sequestrated, other transitional metal ions capable of free radical generation by similar mechanisms such as manganese, are not sequestered and are found in the aqueous phase (Donaldson et al. 1982; Halliwell 1984; Parenti et al. 1988). AA's actions here would depend on varied factors such as concentration of the transitional metal, its compartmentalization, contact with oxidizable substrates (i.e. catecholamines), the interaction with other antioxidant systems - such as GSH peroxidase, catalase and superoxide dismutase, and the tissue's capacity to recycle AA by reducing dehydroAA back to AA.

1.3 Dopamine

Dopamine is a neurotransmitter synthesized by dopaminergic neurons from the amino acid L-tyrosine. Tyrosine hydroxylase (TOH) is the enzyme catalyzing the initial hydroxylation of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), which is the rate limiting step. It requires tetrahydrobiopterin as a cofactor (Brautigam et al. 1984). TOH activity has been found to be regulated by a wide variety of factors (Carroll et al. 1991; Joh et al. 1978; Kilbourne et al. 1991; Labatut et al. 1988; Poltorak et al. 1992) some affecting its expression (Fossom et al. 1992; Iacovitti et al. 1992) and others regulating its activity through kinase mediated phosphorylations (Roskoski et al. 1987; Vrana et al. 1981).

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1.3.1: Dopamine synthesis

The DA biosynthetic pathway is outlined in Fig. 4 below.

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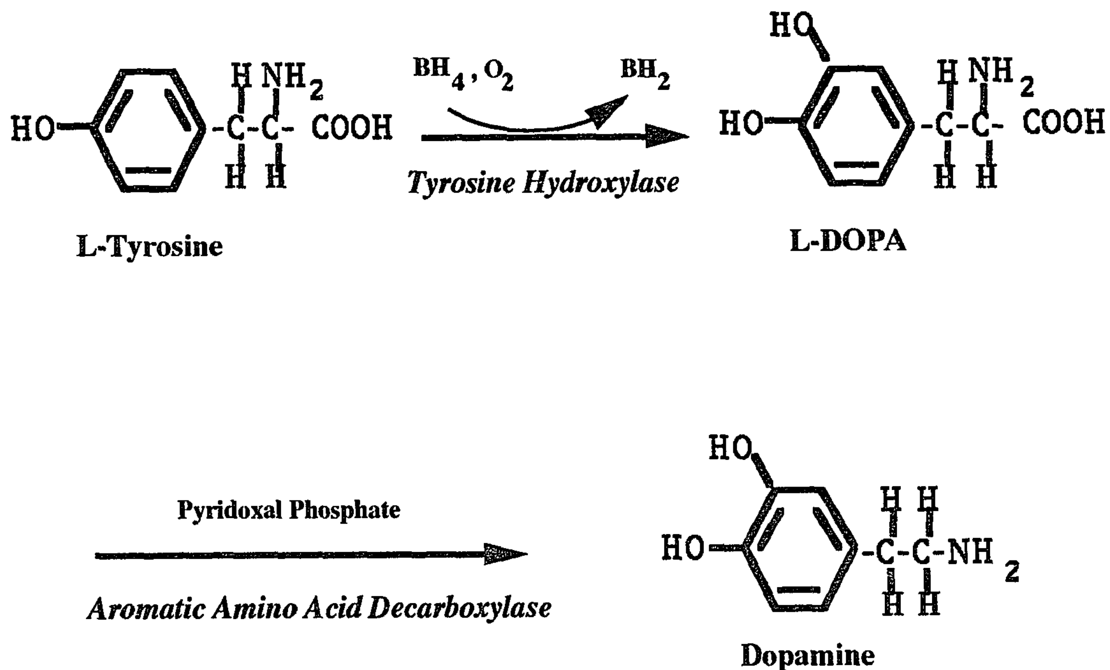
Fig. 4 Dopamine biosynthesis



L-DOPA = 3,4-dihydroxyphenylalanine

BH4 = Tetrahydrobiopterin

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1.3.2: Dopamine catabolism

DA undergoes intracellular oxidative deamination by monoamine oxidase (MAO; mostly the B subtype) present on the outer mitochondrial membrane, while O-methylation by catechol-O-methyltransferase takes place extracellularly (Weiner and Molinoff 1993). See Fig. 5.

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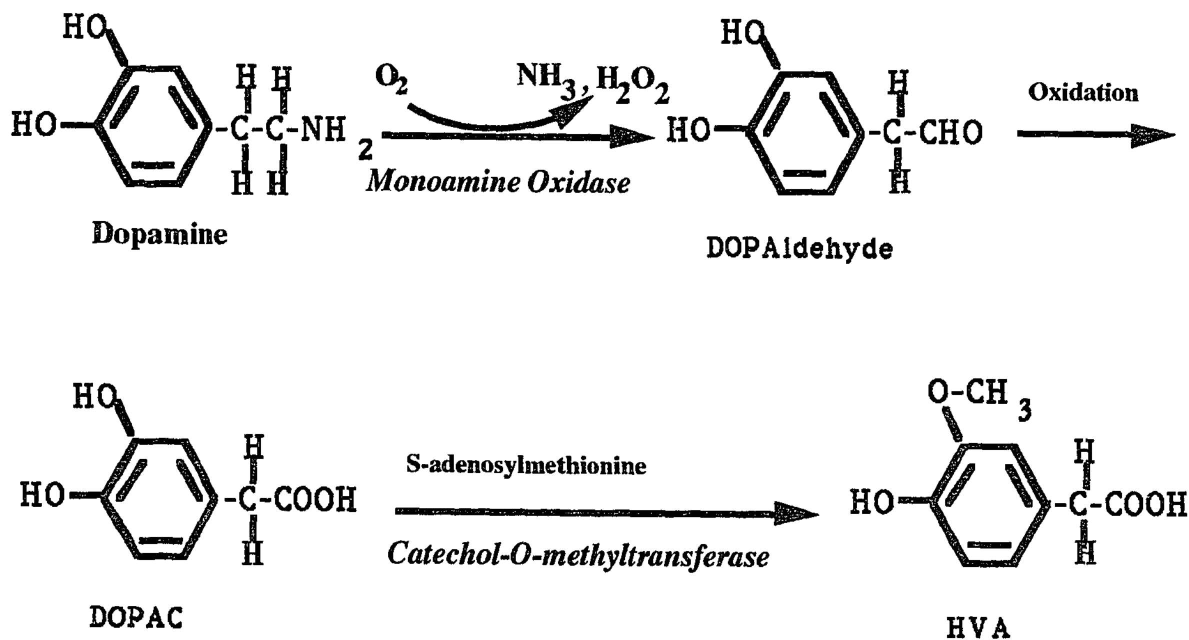
Fig. 5 **Dopamine catabolism**



DOPAC = 3,4-dihydroxyphenylacetic acid
HVA = 3-methoxy-4-hydroxyphenylacetic acid

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1.3.3: Dopamine storage and release

The newly synthesized DA, as well as the DA taken up from the synaptic cleft by a high affinity reuptake system, is stored in vesicles located at the nerve terminal. DA thus stored is protected from degradation by MAO. See Fig. 6.

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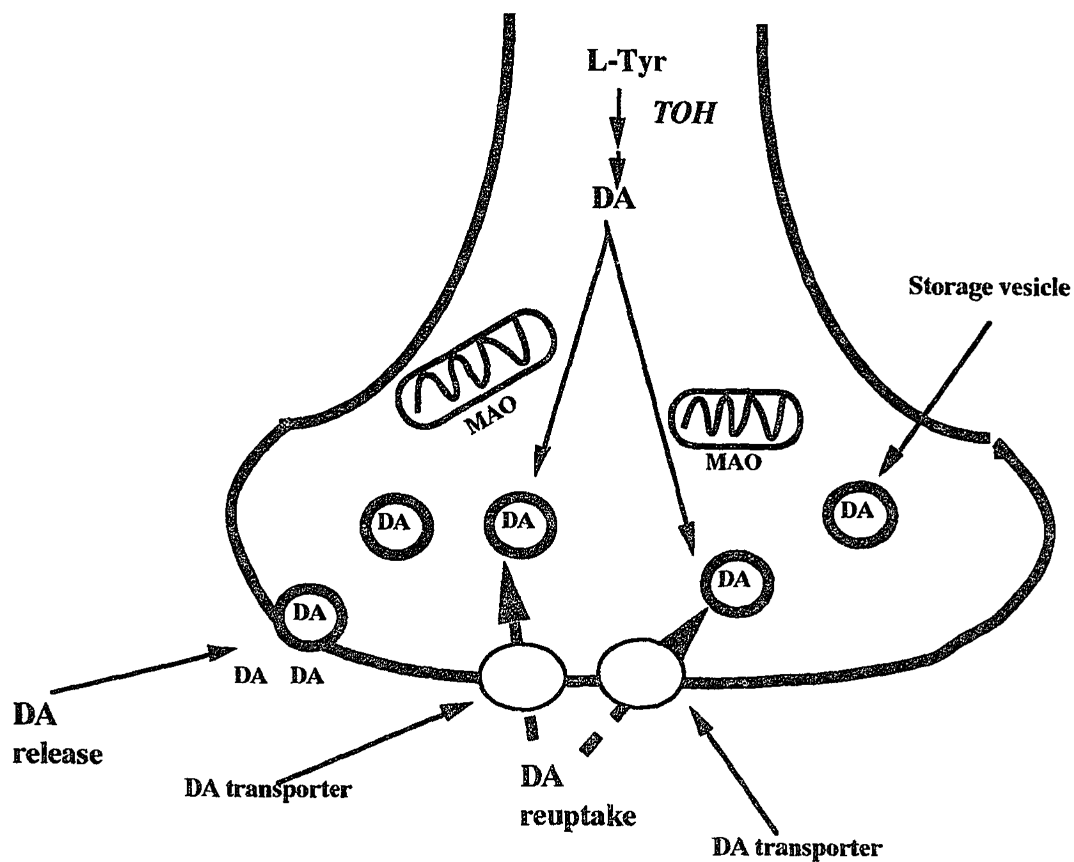
Fig. 6 **Dopamine reuptake and storage in DA nerve terminals**



DA = Dopamine; MAO = Monoamine Oxidase; TOH = Tyrosine Hydroxylase

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Chapter 2: Materials and Methods

2.1 Source of chemicals, drugs and antibodies

Fetal calf serum, horse serum, minimal essential medium (MEM) and balanced salt solution (BSS) were purchased from GIBCO (Grand Island, NY, USA). [^{3}H]DA (37-40 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Mazindol was obtained from Sandoz Pharmaceuticals (Hanover, NJ, USA). Antibodies to tyrosine hydroxylase (TOH) were purchased from Eugene Tech (Allendale, NJ, USA). Antibodies to glial fibrillary acidic protein (GFAP) were purchased from Accurate Chemical and Scientific (Westbury, NY, USA). All other chemicals were of the highest purity available commercially. Chemicals used in the HPLC analysis were of HPLC grade.

2.2: Cell cultures

2.2.1: Cell culture preparation

Timed pregnant Sprague-Dawley rats (Zivic Miller) were obtained and sacrificed on the 14th day of gestation using CO_2. The embryos were rapidly removed and transferred to a laminar-flow hood. Their mesencephalic areas were dissected out and placed in a Stender dish containing ~ 1 mL of Ca^{2+} and Mg^{2+}-free BSS (Dulbecco's). Following the dissection, the fluid was carefully drained, and the collected tissue was minced using fine dissection scissors. The minced tissue was transferred with a pipette to a tube containing the MEM with 10% calf serum and 10% horse serum described below (1 mL per each embryo) and dissociated by gentle trituration with progressively smaller tipped pipettes. 0.5 mL of these mechanically

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dissociated cells was then plated on polyornithine-coated 35-mm plastic dishes (Falcon) containing 1 mL MEM supplemented with glucose (30 mM), sodium bicarbonate (44.6 mM), 10% calf serum and 10% horse serum and having an osmolarity of 325 mOsm. The plating density was 0.6-0.8 x 10^6 cells per dish.

2.2.2: Cell culture maintenance

The cultures were kept at 37 ^oC in a 7% CO_2 atmosphere with 100% relative humidity. After 1 week in vitro, the cultures were treated for 24h with fluorodeoxyuridine (13 μg/mL) and uridine (33 μg/mL) to prevent excessive glial proliferation, and all the dish's medium (1.5 mL) was removed and immediately replaced with the above MEM with 10% horse serum but without the fetal calf serum. Part of the feeding medium (~ 1 mL) was thereafter changed twice weekly. All of these manipulations were carried out in a laminar-flow hood.

2.2.3: Ascorbic acid treatment

a. Chronic treatment: Cultures were initially divided into 2 groups: The AA treatment group received freshly-prepared 10 μl of 0.02 M AA in distilled H_2O per mL medium at plating time (DIV 0). This yielded a final concentration of 0.2 mM AA in the feeding medium. On DIV 5, 8 and 12, the AA treatment group received freshly-prepared 15 μl of 0.02 M AA in distilled H_2O per dish. This too yielded a final concentration of 0.2 mM AA in the feeding medium. The control group was given only the 15 μl of distilled H_2O in an identical feeding schedule, but no AA was given at any time.

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b. Acute treatment: Naive cultures (no previous in vitro AA exposure) were initially divided into 2 groups: the AA treatment group received freshly-prepared 0.02 M L-AA in 15 μl of distilled H_2O per dish which yielded a final L-AA concentration of 0.2 mM. The control group received 15 μl of distilled H_2O per dish, but no AA was given at any time.

Note: 0.2 mM is the AA concentration maintained in the CSF by the choroid plexus (Spector 1989), and is also the highest in which no membrane toxicity would occur even if all the AA were oxidized to dehydroascorbic acid (DHAA) (Bianchi and Rose 1986).

2.3: Ascorbic acid assay

Ascorbic acid in both tissue and extracellular fluid was measured by HPLC with electrochemical detection, according to a procedure described by Lee (Lee et al. 1982) with slight modifications. At the end of each incubation period, the cultures were rapidly washed twice with balanced salt solution (BSS), and the cells scraped off manually with a teflon tissue-scraper and collected in a 1.5 mL microcentrifuge tube (3 culture dishes per one tube). Following centrifugation at 4,700 x g for 10 minutes in the cold, the fluid was removed and 100 μl of 0.1M perchloric acid (PCA) containing 10 mg/L diethylenetriamine pentaacetic acid (DTPA) and 0.3 mM 3,4-dihydroxybenzylamine (DHB; the internal standard) were added. The cells were homogenized by sonication and centrifuged again for 10 minutes at 16,600 x g in the cold. A 20 μl aliquot of the supernatant or the appropriate dilution of it (in the same DHB + PCA and DTPA-containing injection medium) was injected into the HPLC system. Separation was carried out on a reverse-phase paired ion HPLC with

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a Waters C18 column and BAS LC-4B Amperometric Detector set at the 10 - 50 nA range. Cell voltage was +0.7V. The mobile phase consisted of 8 mM sodium acetate buffer, 200 mg/L EDTA and 1 mM n-octylamine (as the paired ion), which was filtered through a 0.22μ filter and degassed. The final pH of the mobile phase was 4.0 and the flow rate was 1.0 mL/min. AA standards consisted of 0.5 mg/L and 1.0 mg/L of AA in the same DHB + PCA and DTPA-containing injection medium. The precipitates were saved for Lowry protein determinations where appropriate. See Fig. 7 for a typical AA chromatogram.

2.4: Catecholamine analysis

The same samples used for the AA analysis were used for determination of catecholamines (CA) and their metabolites. A 20 μl aliquot of the undiluted supernatant was injected into the HPLC system. Separation was carried out on an HPLC system with a reverse phase Biophase column (Bioanalytical Systems, Indiana) and an LC-4B Amperometric Detector (Bioanalytical Systems, Indiana) set at the 1 or 5 nA range. Cell voltage was +0.8V. The mobile phase consisted of a 0.15 M monochloroacetic acid ($CH_2ClCOOH$) buffer with 0.7 mM EDTA, 1.9 mM sodium octyl sulfate (SOS - $CH_3(CH_2)_7OSO_3Na$, Kodak Chemicals) and 5.5% (v/v) acetonitrile (CH_3CN, Fisher Scientific). The final pH of the mobile phase was 3.0 and it was filtered through a 0.22μ filter and degassed. The pump flow rate was set at 1.0 mL/min. Standards consisted of 0.12 to 0.6 mM concentrations of: 3,4-dihydroxyphenylalanine (L-DOPA), norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole acetic acid (5HIAA), homovanillic acid (HVA) and

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dopamine (DA) in the appropriate injection medium described for the AA assay above. See Fig. 8 for a typical CA chromatogram.

2.5: $[^3H]$DA uptake assay

$[^3H]$DA uptake was used as an index of the number of active DA uptake sites, which depends on the number of DA neurons and the extent of DA fiber outgrowth. It was carried out on cultures aged DIV 5 or older as follows: After removing the feeding medium and rinsing twice with Krebs-Ringer phosphate buffer (pH 7.4) containing 5.6 mM glucose, 1.3 mM EDTA and 0.1 mM ascorbic acid, the cultures were incubated with 14 nM $[^3H]$DA for 10 minutes at 37 ^oC. The cells were then washed twice and incubated for 5 min with fresh buffer. In order to release the accumulated tritium from the cells, the cultures were incubated with 1 mL of 95% ethanol for 30 min at 37 ^oC, which was then added to 10 mL of Liquiscint (National Diagnostics, Manville, NJ, USA) and counted for radioactivity in a Beckman LS 3801 liquid scintillation counter. Blank values were obtained by adding 10 μM mazindol - a specific neuronal DA uptake inhibitor - to the buffer and were usually less than 5% of the $[^3H]$DA accumulated in uninhibited cultures. Independent assays demonstrated that the mazindol sensitive uptake measured did not represent uptake into glial cells, since glial cultures showed similar values of $[^3H]$DA uptake in the presence and absence of 10 μM mazindol (Casper et al. 1991).

2.6: Immunocytochemistry assays

These assays were used to directly visualize both the number and morphology of DA neurons, and the amount of glia in the cultures. The cultures were fixed in warm 4% paraformaldehyde dissolved in

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0.1M phosphate buffer at pH 7.4 and made isoosmotic with 2.5% sucrose. Fixation time was 30 min - 10 min at room temp., and 20 min in the cold. The cultures were permeabilized by treating them for 30 min with 0.2% Triton X-100, washed with 0.5 M Tris-saline buffer, incubated with goat serum (block) for 30 min and then with the appropriate dilutions of the primary antibodies (polyclonal raised in rabbits) in block on a shaker at 4 ^{o}C overnight. The peroxidase-coupled avidin-biotin kit (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA) was used for visualization. Diaminobenzidine (DAB) -12.5 mg DAB in 25 mL Tris-saline, was used as a chromogen, 8.3 μl 30% H_2O_2 was added just prior to applying the solution to the cultures. The degree of chromogen development was observed under a microscope and stopped at the appropriate stage by removing substituting it with the Tris-saline buffer.

2.6.1: Tyrosine hydroxylase staining

The above outlined assay was carried out with TOH as the primary antibody, diluted 1:1000 in block.

2.6.2: GFAP staining

The above outlined assay was carried out with GFAP antibodies as the primary antibody, diluted 1:1000 in block.

2.7: GSH assay

GSH and GSSG were measured according to the method described by Tietze (Tietze 1969) to assess the possible interaction between AA and the major intracellular reducing system (Werner and Cohen 1993). The cells were homogenized in 0.4 N perchloric acid and then centrifuged at 16,000 x g in the cold for 10 min. 10-20 μl of the supernatant were transferred to a 100 mM potassium phosphate

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buffer (with 5 mM EDTA and a pH of 7 by KOH titration). To 750 μl of this solution were added 100 μl of 1.7 mM NADPH and 100 μl of 4 mM DTNB [5,5'-Dithiobis-(2-nitrobenzoic Acid)]. The standards contained added GSH only. For GSSG measurements, 25 μl of glutathione reductase in 100 mM potassium phosphate buffer (13 μl/mL buffer) were added. The reaction rates were read on a 340 ATTC microplate reader (Tecan, Austria) using a wide bandwidth filter at 405 nm. The GSSG --> GSH reaction rates of the standards and samples were blank corrected and the GSSG content calculated by subtracting the initial GSH value from the total (post reduction) GSH value.

2.8: Protein Determination

Tissue protein was determined according to the method of Lowry (Lowry et al. 1951).

2.9: Statistical Analysis

AA accumulation, DA and DOPAC levels were analyzed by an ANOVA test, [^{3}H]DA accumulation and TOH-positive neuron counts were analyzed by an unpaired, two-tailed t-test. Values are given as mean ± SEM. All the analyses done used the StatView 512+ program on a Macintosh IIcx computer.

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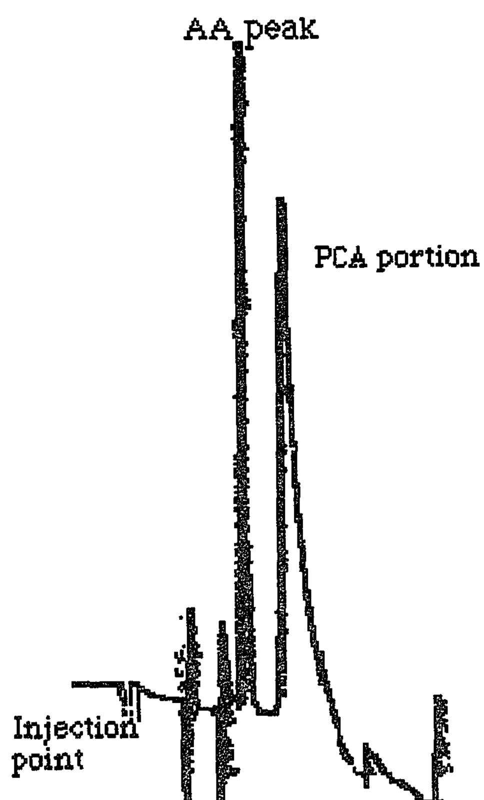

Fig. 7: Typical AA Chromatogram - 0.25 mg/L

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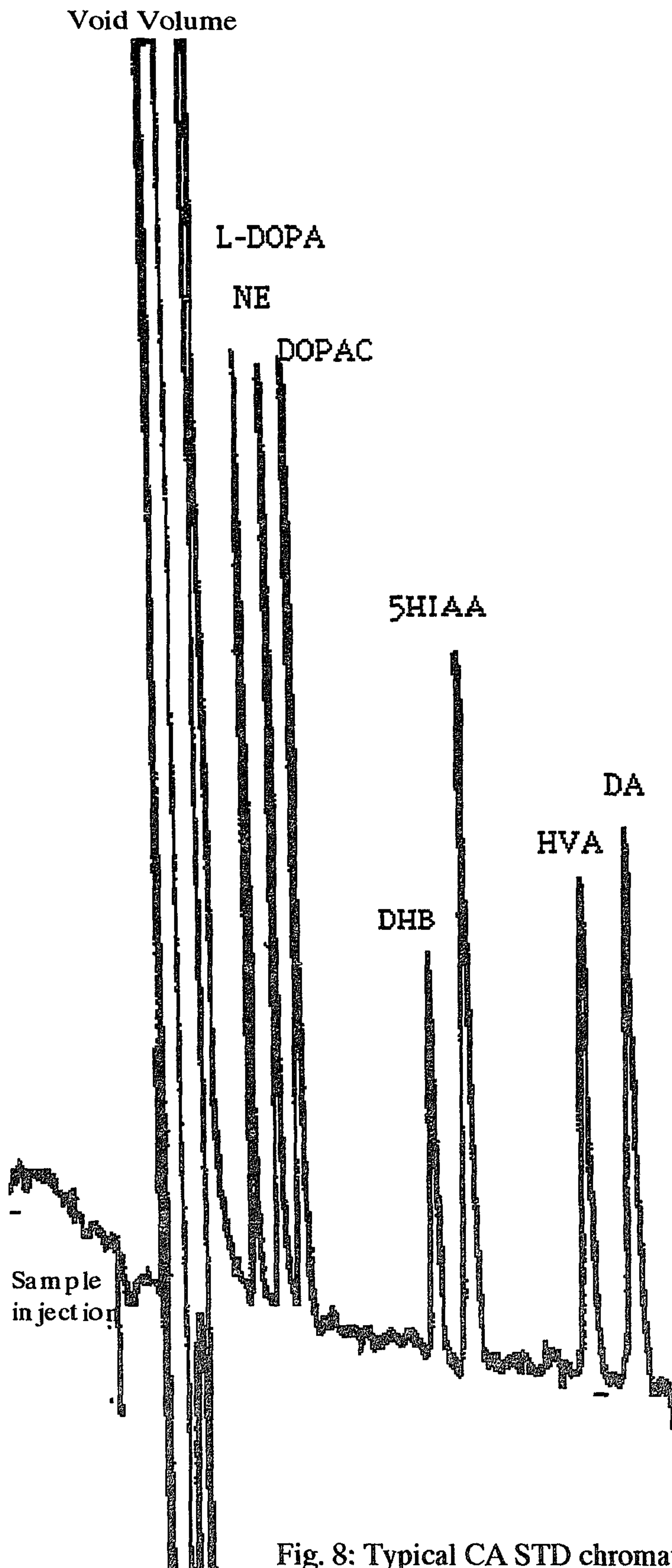

Fig. 8: Typical CA STD chromatogram - 2 μg/mL

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Chapter 3: Experiments: Results and discussion

3.1 Results

3.1.1: Chronic L-AA exposure

Cultures were initially divided into 2 groups: the AA treatment group received freshly-prepared 0.2 mM AA (final concentration) in the feeding medium at plating time (DIV 0) and on DIV 5, 8 and 12. The control group was not given AA at any time.

3.1.1a Effects on culture L-AA content

The rate of decline in the AA content of control cultures, and the ability of the chronically AA-treated cells to accumulate AA is shown in Fig. 9. The AA level of the dissociated mesencephalic cells, measured before plating, was 2 μg/mg protein. By DIV 2, the control cultures had a 100 fold decrease in their AA content, and by DIV 7 had no detectable AA levels (the system's detection limit for AA was 1 ng). The AA-treated cultures, which had been exposed to 0.2 mM AA in the feeding medium at the time of plating and on days 5, 8 and 12, had a 75% decline compared to their initial AA content by the second day (DIV 2), but the AA content increased by DIV 7 and reached the initial DIV 0 levels at the end of 2 weeks (DIV 14). The data in Fig. 9 are from a set of cultures prepared from the same cell suspension. Similar values were obtained in subsequent experiments.

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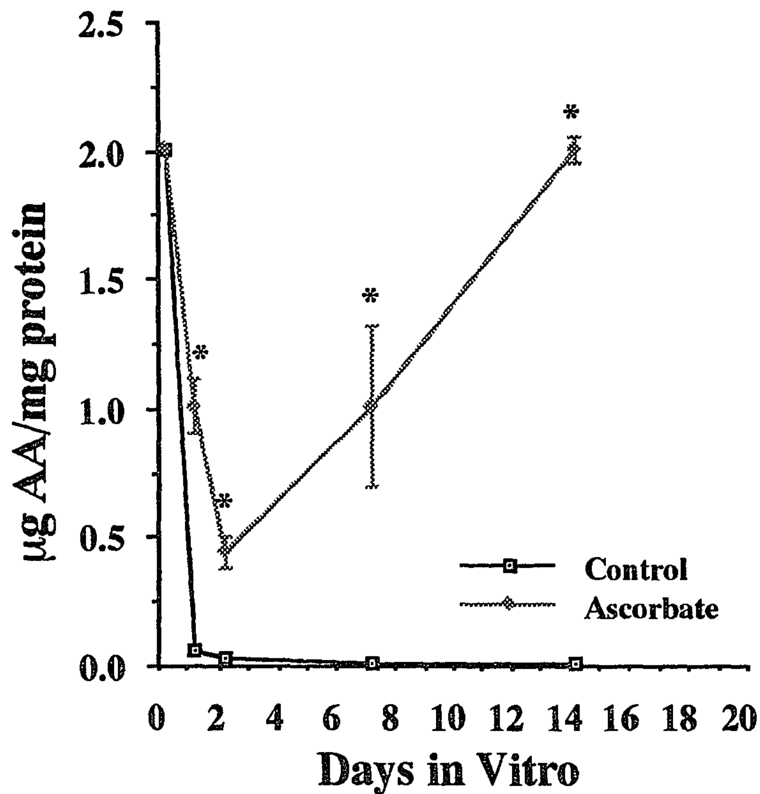

Fig. 9: Cellular content of AA in control and AA-treated dissociated mesencephalic cultures during the first 2 weeks of in vitro development. Each value represents the mean ± SEM of 9 cultures, grouped at 3 cultures per sample. SEM bars shown only when bigger than symbols. Starting with DIV 1, all differences between the two groups were significant at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.1.1b Effects on culture DA & DOPAC content

The DA and DOPAC content of the cultures was measured in the same cell extract used to measure the AA content and are shown as a function of the in vitro age in Figs. 10a and 10b. DA and DOPAC levels in both groups rose over time as the cultures matured, but the AA treated group had higher levels on DIV 7 and 14. The rate of increase in DA and DOPAC content was also higher in the AA treated cultures: For DA - a 1.5 ratio between the AA-treated and control groups was noted on DIV 7, with an increase to a 1.8 ratio on DIV 14. For DOPAC - the DIV 7 and 14 ratios were 2.5 and 2.8 respectively. The DA turnover (DOPAC/DA) was lower on both DIV 7 and 14 in the control group compared to the AA group: 0.082 vs. 0.138 respectively on DIV 7 and 0.075 and 0.116 respectively on DIV 14. The DA and DOPAC values in these experiments are expressed as mg per dish rather than per mg protein because the DA neurons in these cultures are post-mitotic, while the protein values increase mainly as a function of glial proliferation. Similar values were obtained with different sets of cultures.

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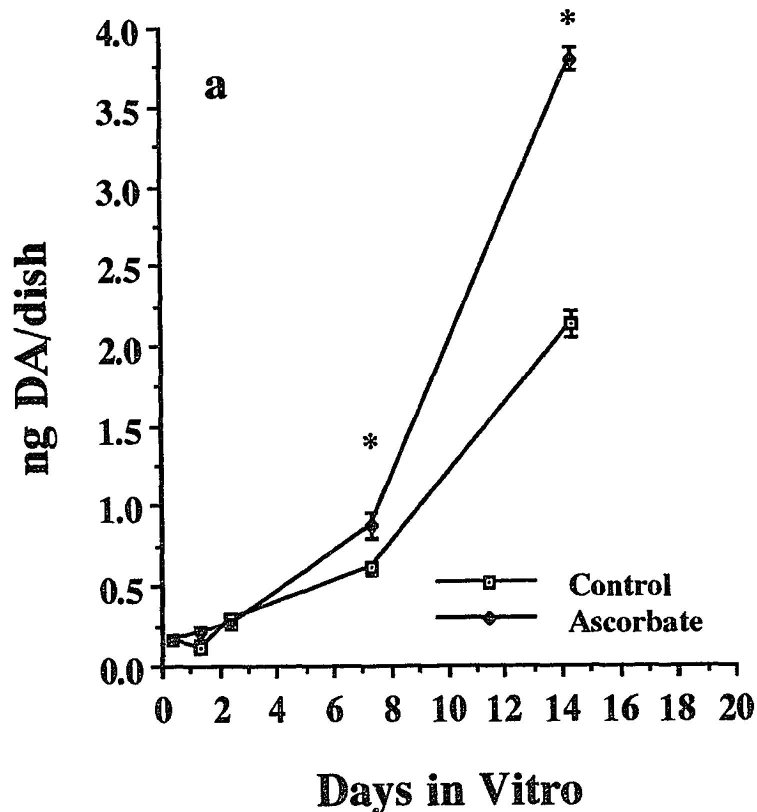

Fig. 10a: The DA in AA-treated and untreated dissociated mesencephalic cultures during the first 2 weeks of in vitro development. Each value represents the means ± SEM of 9 cultures, grouped at 3 cultures per sample. Starting with DIV 7, all differences between the two groups were significant at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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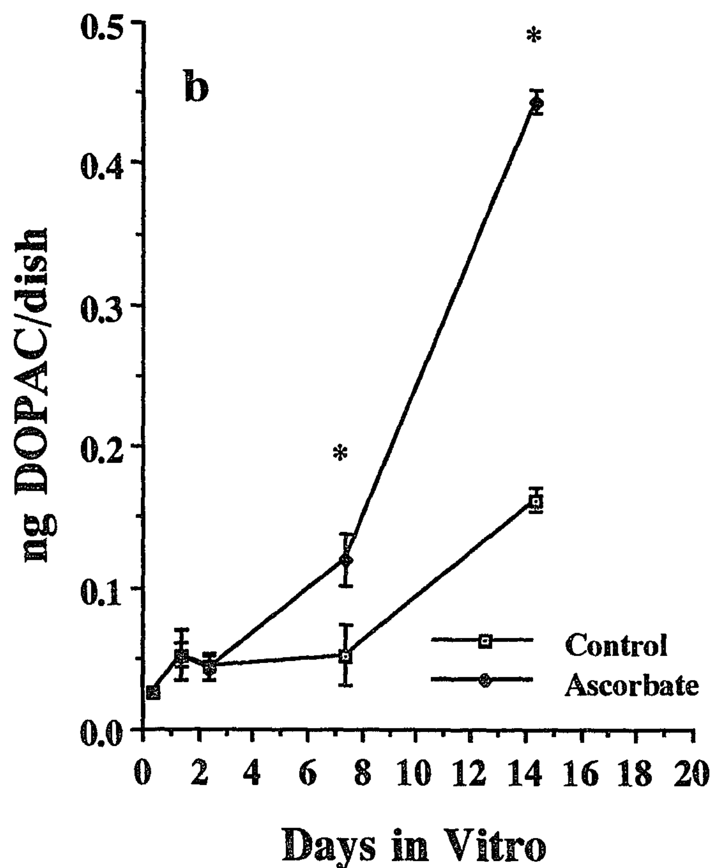

Fig. 10b: The DOPAC level in AA-treated and untreated dissociated mesencephalic cultures during the first 2 weeks of in vitro development. Each value represents the means ± SEM of 9 cultures, grouped at 3 cultures per sample. Starting with DIV 7, all differences between the two groups were significant at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.1.1c Effects on [^{3}H]DA uptake

[^{3}H]DA accumulation was used as an indication of the degree of dopaminergic fiber growth and maturation (in conjunction with direct visualization of DA neurons by TOH immunocytochemistry). [^{3}H]DA accumulation increased linearly over time, as shown in Fig. 11. The ascorbate-treated cultures had a significantly higher accumulation of [^{3}H]DA from DIV 8 onward ($p<0.05$ by t-test). Similar [^{3}H]DA uptake curves were obtained with different sets of cultures.

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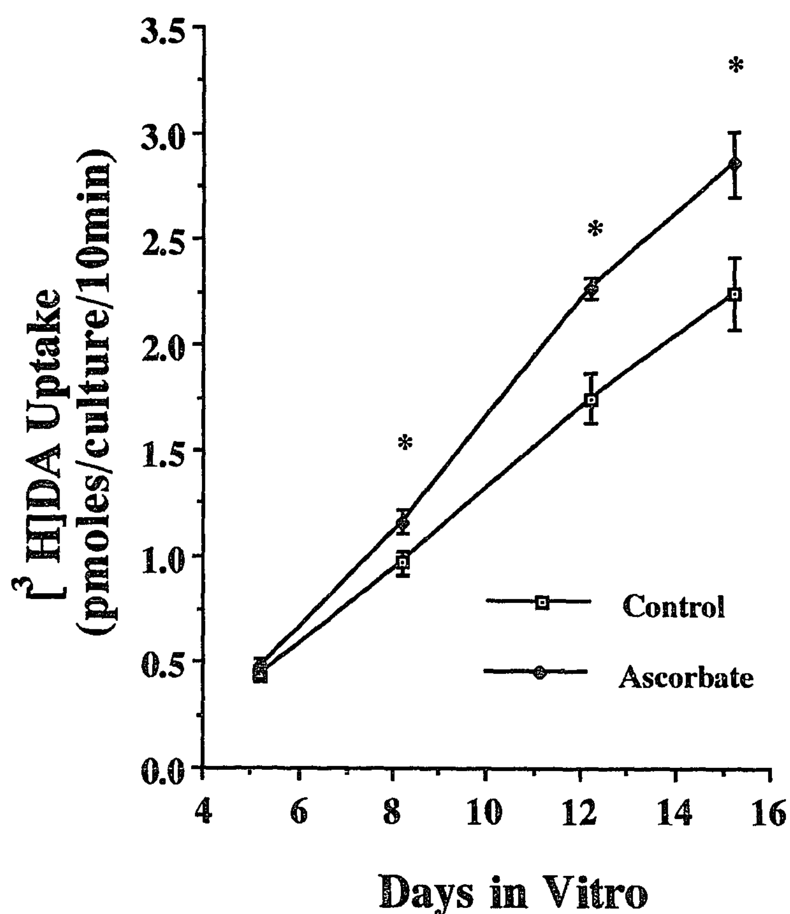

Fig. 11: The development of neuronal [^{3}H]DA accumulation during 2 weeks of in vitro growth of dissociated mesencephalic cultures. The values represent the means ± SEM of 5 cultures per group. Starting with DIV 8, all differences between the two groups were significant at $p<0.05$ on an unpaired, two-tailed t-test and are marked by an asterisk (*).

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3.1.1d Effects on TOH + neurons

Direct visualization of the DA neurons by tyrosine hydroxylase immunocytochemistry showed marked differences between cells in the two groups of cultures (Figs. 12a & b). Neuronal morphological differences consisted of an increase in both the number and length of dopaminergic fibers in the AA group (Fig. 12a) as compared to the control group (Fig. 12b). On DIV 12, the AA group had greater survival of TOH-positive neurons than the control group. The TOH-positive neuronal cell counts per mm^2 were 10.1 ± 0.9 in the AA group, and 6.2 ± 0.6 in the control group. The differences between the two groups were significant at $p<0.05$ on an unpaired, two-tailed t-test. Cell counts of TOH-positive neurons were taken from 3 cultures in each group by 2 observers.

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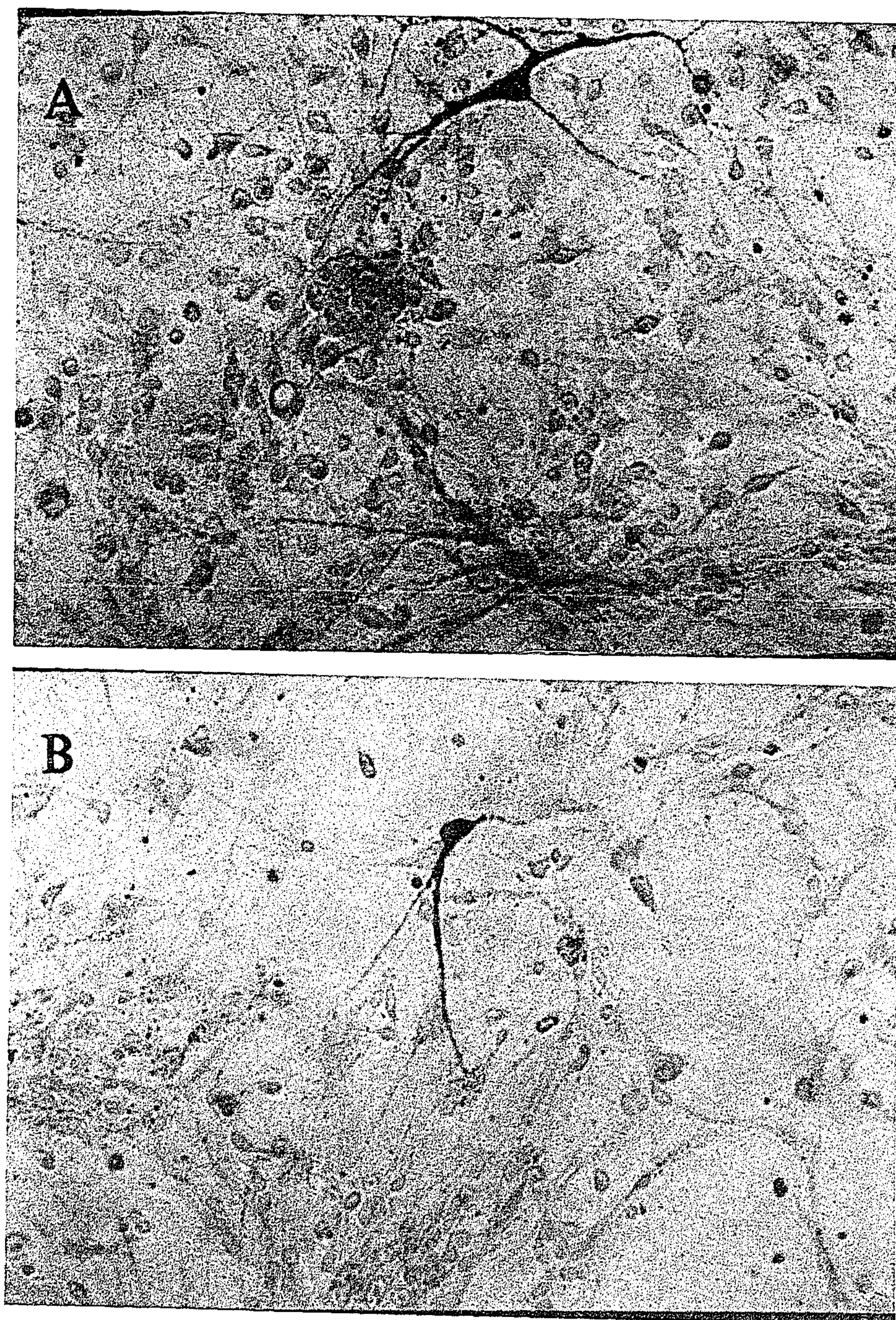

Fig. 12 a&b: Tyrosine hydroxylase staining of DA neurons in AA-treated and control cultures on DIV 12. Note the increased number and length of fibers (Fig. 12a) in the neurons of the AA group as compared to the mostly bipolar shape in the control group (Fig. 12b). x 256 magnification.

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3.1.1e Effects on culture glial cells

When the cultures from the two groups were observed by phase microscopy, increased cell density was noted in the AA group. To determine whether the increase in cell density was due to an increase in glial proliferation, GFAP immunostaining was done (Fig. 13), which showed a marked increase of GFAP-positive cells in the AA group (Fig. 13a) compared to the control group (Fig. 13b).

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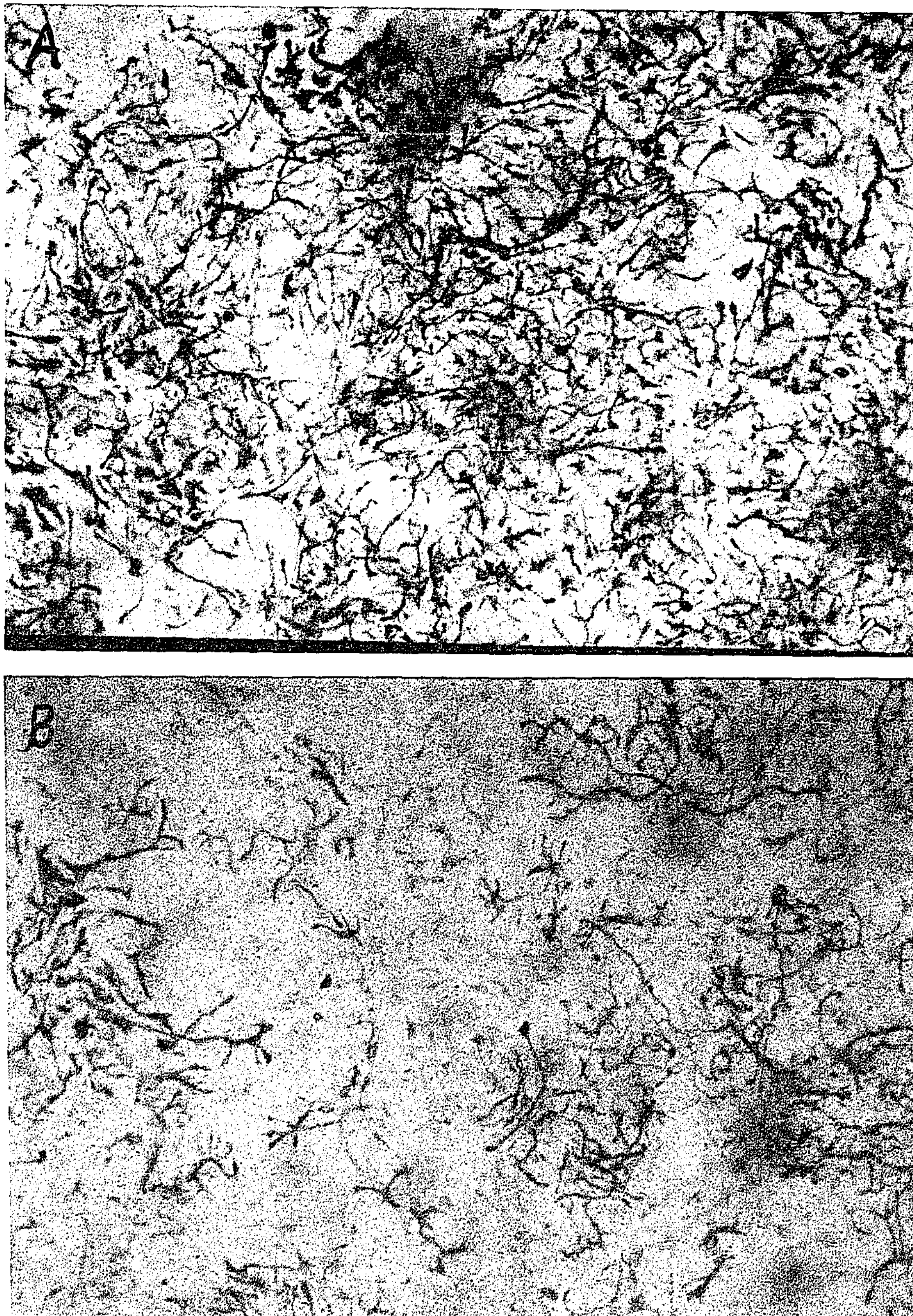

Fig. 13 a&b: GFAP staining of AA-treated and control mesencephalic cultures on DIV 12. Note the increased number of stained glial cells in the AA-treated culture (Fig. 13a) as compared to the control culture (Fig. 13b). x 128 magnification.

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3.1.2 Acute L-AA exposure

Cultures were initially divided into 2 groups: the chronic AA group (A) received freshly-prepared 0.2 mM AA (final concentration) in the feeding medium at plating time (DIV 0) and 15 μl of 20 mM AA 1.5 hrs after feed changes on DIV 5 and 8. The control group (C) was given only 15 μl of H_2O. On DIV 10, no feeding medium change was carried out, and only half of the chronic AA group (A) and half of the control group (CA) received 15 μl of 20 mM L-AA. Half of the control group (C) and half of the chronic AA group (AC) were given only 15 μl of H_2O. The cultures were harvested 4, 24, 48 and 72 hrs after the acute AA treatment.

3.1.2a: Effects on culture L-AA content

The cellular AA content over a 72 hr. period was measured at 4, 24, 48 and 72 hr periods after the acute L-AA treatment. The L-AA levels for the treated (CA) and untreated (C) control groups are shown in Fig. 14a and the L-AA levels for the treated (A) and untreated (AC) chronic AA groups are shown in Fig. 14b. - ~ 60% of the maximal intracellular L-AA level is achieved 4 hrs following the L-AA exposure. At 24 hrs, the cultures reach a maximum L-AA level similar to that found in the in vivo embryonal tissue. A gradual decline of 40-50% occurs thereafter for each 24 hr period. The results demonstrate that the previously unexposed cultures have an L-AA uptake and retention characteristics similar to that of the chronic AA group, and that ~ 60% of the L-AA accumulation has already taken place after 4 hrs.

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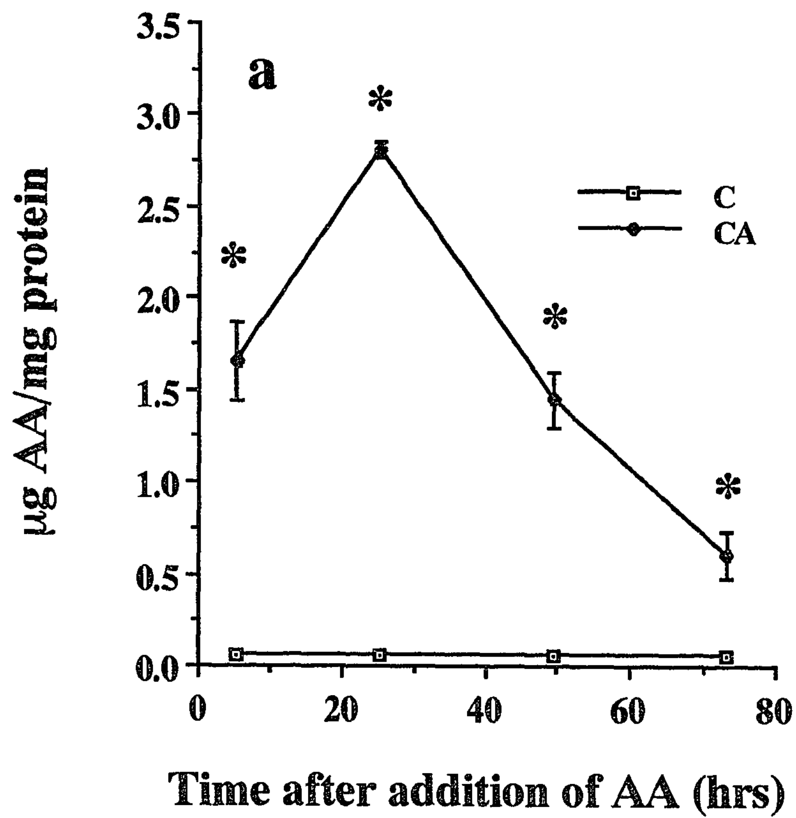

Fig. 14a: See legend under Fig 14b.

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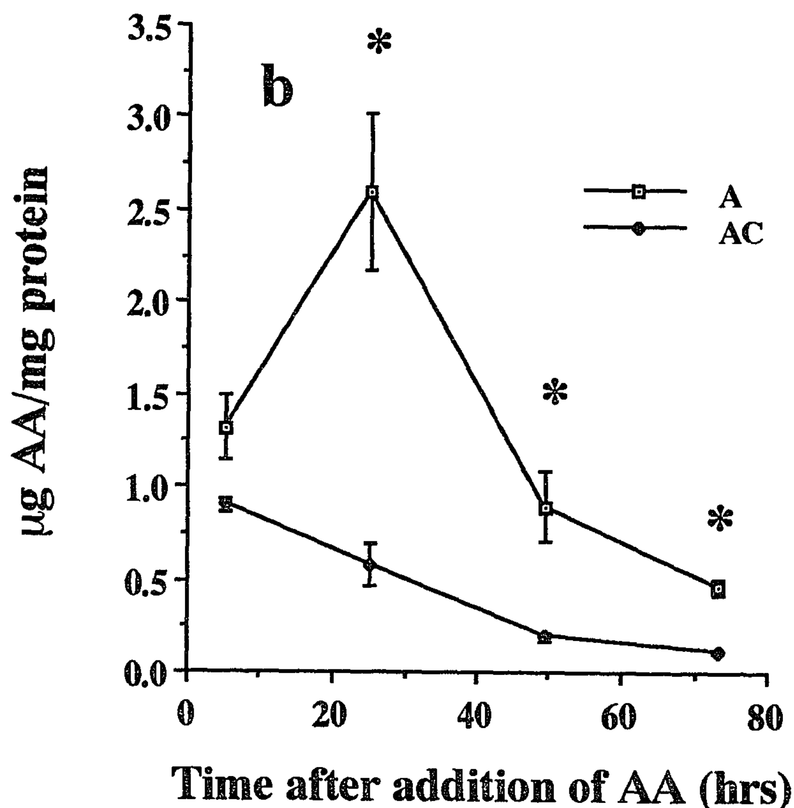

Fig. 14b: See legend below.

Figs. 14 a & b: Cellular content of AA in DIV 10 treated (CA) and untreated (C) controls over a 72 hr period following AA exposure (1A). The same in DIV 10 chronic AA treatment groups (1B) (A)= repeated AA exposure. (AC)= no addition of AA on DIV 10. Note the similar values and retention dynamics between A and CA. Each value represents the mean ± SEM of 9 cultures, grouped at 3 cultures per sample. SEM bars shown only when bigger than symbols. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.1.2b: Effects on culture DA content

The same samples used for the AA analysis at the time intervals described in 3.1.2a above were used for determination of culture DA levels. The effects of L-AA exposure on the DA content as a function of time are shown below in Figs. 15a and 15b. Statistically significant differences in the DA content of the cultures are noted only 48 hrs and 72 hrs after L-AA exposure. The general DA rise in C and AC is due to the maturation of the cultures during that period.

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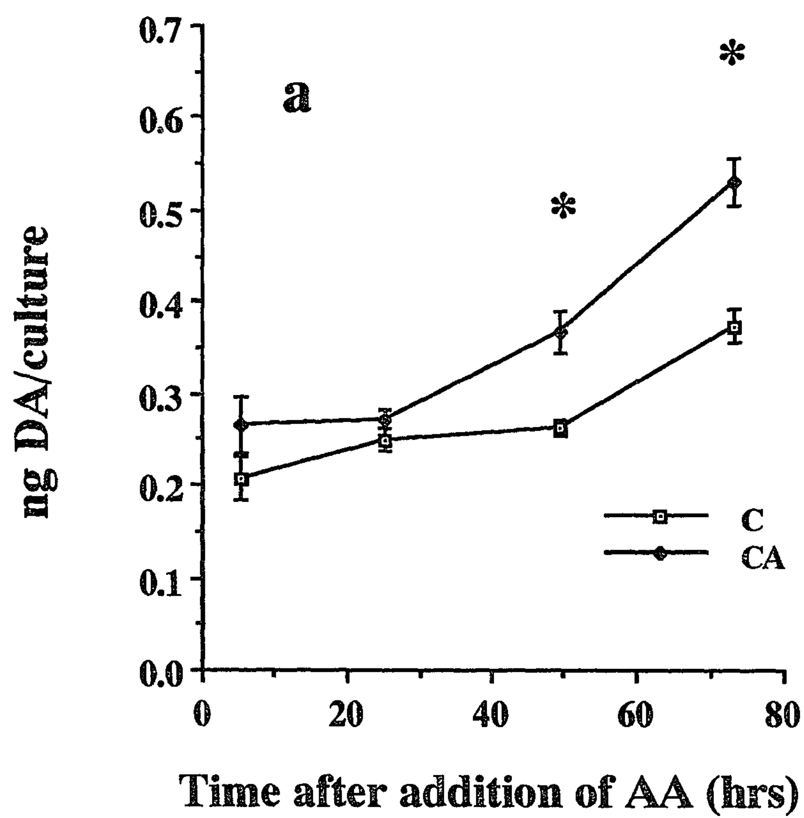

Fig. 15a: See legend below Fig. 15b.

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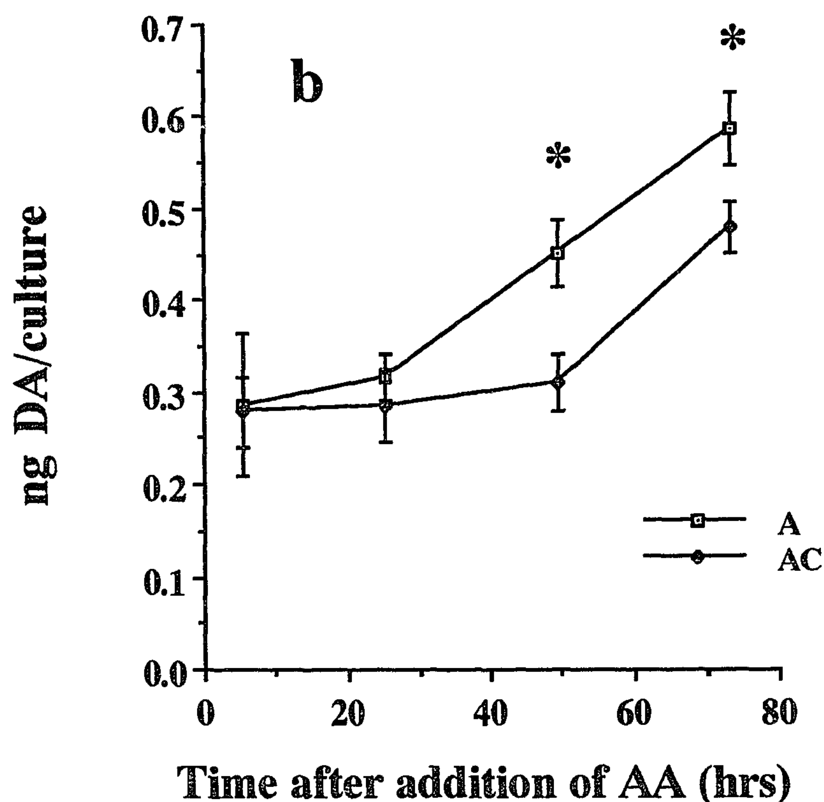

Fig. 15b: See legend below.

Figs. 15 a & b: The DA levels (B) in DIV10 AA-treated (CA) and untreated (C) cultures during the 72 hrs following AA exposure (2A). The same in DIV 10 chronic AA treatment groups (2B) (A)= repeated AA exposure. (AC)= no addition of AA on DIV 10. Each value represents the means ± SEM of 9 cultures, grouped at 3 cultures per sample. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.1.2c: Effects on [^{3}H]DA uptake

[^{3}H]DA accumulation was determined in the naive cultures at 4 hrs, 24 hrs and 48 hrs following the acute treatment with AA. It was used as an indication of the degree of dopaminergic fiber change (growth and maturation). It also served as an independent measure of the number of active DA neurons in the culture, in conjunction with direct visualization of DA neurons by TOH immunocytochemistry, [^{3}H]DA accumulation increased linearly over time, as the cultures matured, but no difference was found between the treated and untreated cultures. This suggests that no changes in the number of DA uptake sites, and presumably in the number of DA neurons, occurred during the 48 hr treatment with L-AA. The results are shown in Fig. 16.

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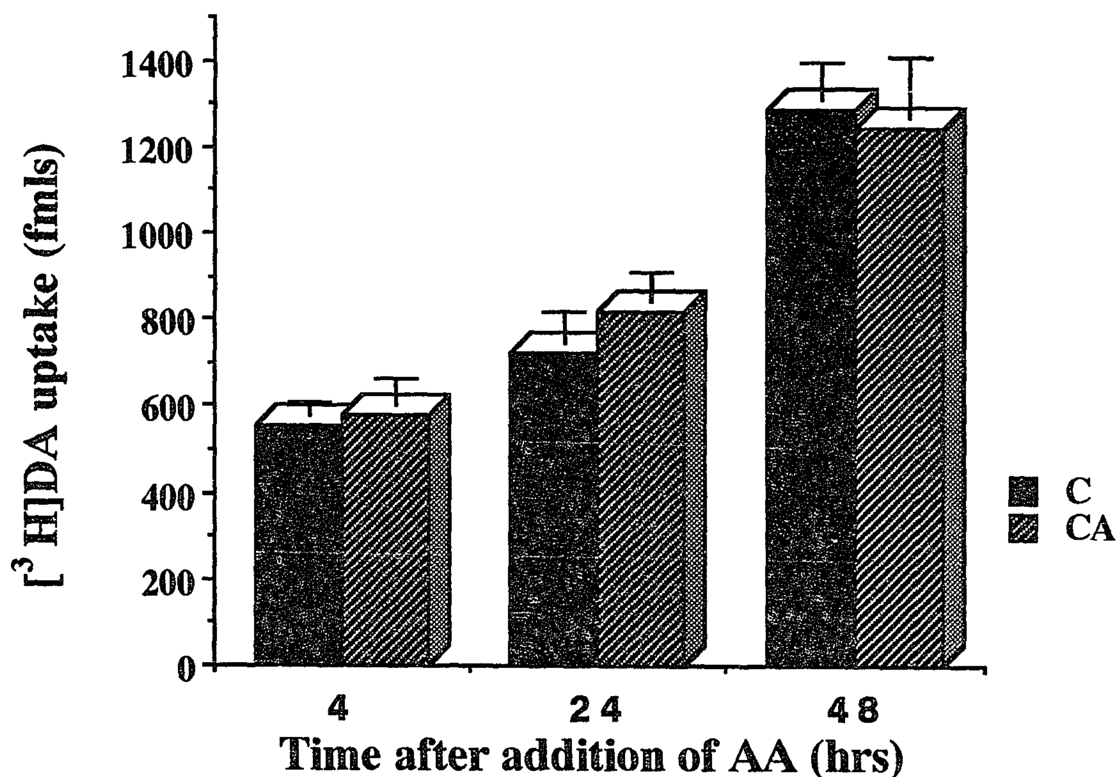

Fig. 16: The [^{3}H]DA accumulation in controls treated with AA (CA) on DIV 10, and untreated controls (C) at 4 hrs, 24 hrs and 48 hrs after AA exposure. The values represent the means ± SEM of 3 cultures per group. No significant differences between the groups at $p<0.05$ on ANOVA were noted. The general rise in accumulation in both groups (C & CA) is due to culture maturation.

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3.1.2d: Effects on TOH + neurons

Staining for TOH was carried out 4 hrs, 24 hrs and 48 hrs following the acute treatment with AA. The staining in general allowed a quantification of the number of TOH-containing DA neurons. The TOH + neuron count is shown in Fig. 17. - a statistically significant increase in the TOH + neuron count in the L-AA treated group is seen 4 and 24 hrs following acute exposure to L-AA. After 48 hrs no statistically significant differences were observed. The photos of the stained neurons are shown in Figs. 18a & 18b. They indicate a greater staining area with a greater number of neurite visualization in the acutely treated groups.

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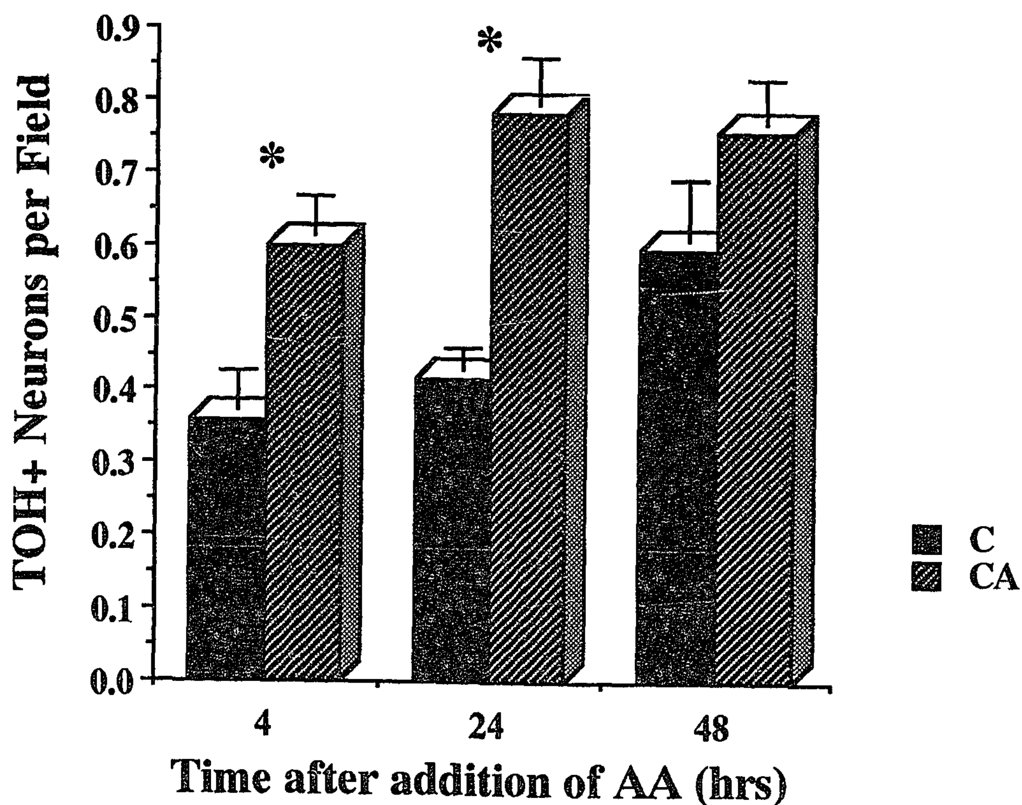

Fig. 17 : TOH+ stained neurons per field in AA-treated (CA) and untreated (C) control cultures on DIV 10, 11 and 12 (4 hrs, 24 hrs and 48 hrs respectively after AA exposure). The values represent the means ± SEM of TOH+ stained neurons per field. Note that the significant differences in count first appear less than 24 hrs following the AA exposure. Compare with the time course for DA in Fig 15a. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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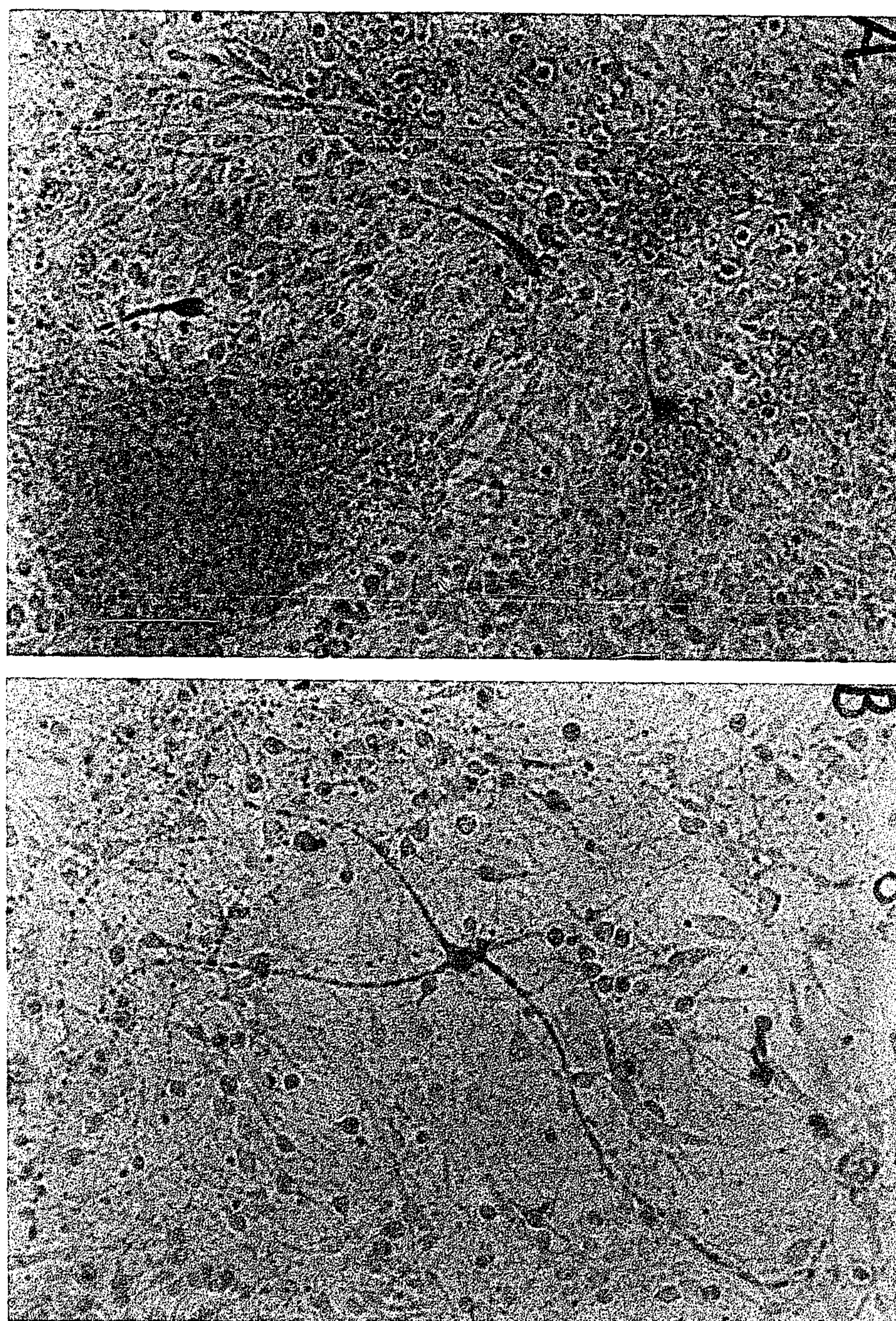

Fig. 18 a & b: TOH + stained cells 24 hrs after AA exposure. a- untreated controls (C). Note the few visible fibers and the relatively low staining intensity. b- AA exposed controls (CA). Note the increase in the number and length of the fibers, as well as the increased staining intensity. x 256 magnification.

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3.1.2e: Effects on GSH content

The GSH and GSSG contents of the cells were measured on DIV 12, 24 hrs. after exposure to 15 μl of H_2O in the control group, and 15 μl of 20 mM L-AA in the control + AA and chronic AA groups. The results showed no statistically significant difference in either the GSH or the GSSG content, although culture variability exists within the groups. The results are shown in Figs. 19 a & b.

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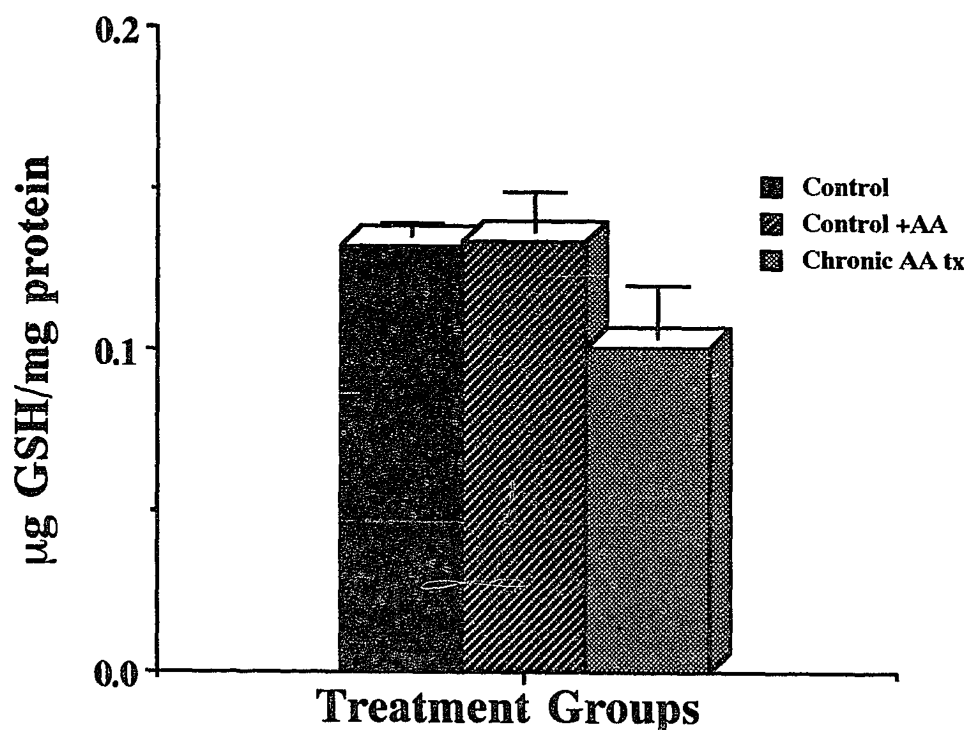

Fig. 19a: The GSH content of 4 pooled DIV12 cultures 24 hrs. following acute exposure to either 15 µl of H_2O in the control group, or 15 µl of 20 mM L-AA in the control + AA and chronic AA groups. The values represent the means ± SEM of pooled 4 cultures. Note the lack of statistically significant difference at $p<0.05$ on ANOVA among all the groups.

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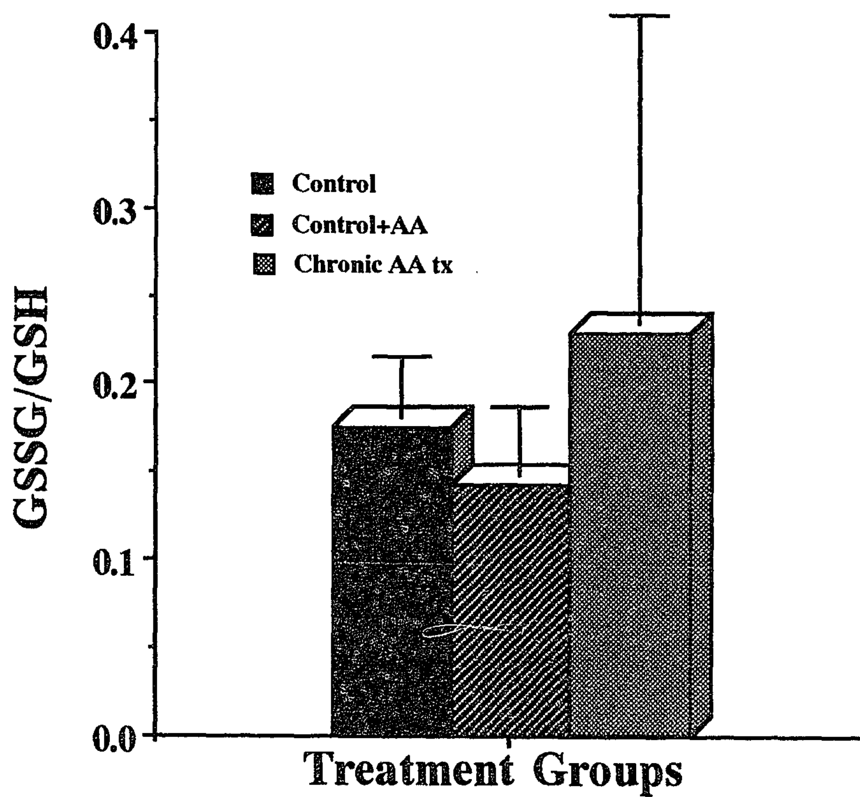

Fig. 19b: The GSSG/GSH ratio of 4 pooled DIV12 cultures 24 hrs. following acute exposure to either 15 μl of H_2O in the control group, or 15 μl of 20 mM L-AA in the control + AA and chronic AA groups. The values represent the means ± SEM of pooled 4 cultures. Note the lack of statistically significant difference at $p<0.05$ on ANOVA among all the groups.

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3.1.3: AA effects on TOH activity

Cultures were initially divided into 3 groups: the AA treatment groups (L=L-AA; D=D-AA) received freshly-prepared 0.2 mM (final concentration) of L-AA (the biologically active isomer) and D-AA respectively in the feeding medium at plating time (DIV 0). The control group (C) was given only 15 μl of H_2O per mL feeding medium at plating time. The cultures were harvested 0 (before AA addition), 24, and 48 hrs. after the acute AA treatment (DIV 0, DIV 1 and DIV 2 respectively). The L-AA content, D-AA content and L-DOPA content (an index of TOH activity) of all 3 groups was measured at each of these points. The results are shown in Figs. 20 - 23.

3.1.3a Cellular accumulation of AA stereoisomers

The L-AA intracellular levels of the 3 groups on DIV 0 through 2 are shown in Fig. 20. They replicated the dynamics previously shown in section 3.1.1a., with the attenuated decline in L-AA content in the L group. The D-AA treatment had no effect on the L-AA content of the D group cells, and their L-AA levels were similar to those of the C group.

Levels of L-AA and D-AA on DIV 0 to 2 for the group treated with D-AA are shown in Fig. 21. The maximal D-AA level was reached on DIV 1 and was similar in value to the L-AA level present in those cultures at that time (note that there is a ten fold difference in the scale of the Y axis for L-AA as compared to D-AA).

The total AA levels of all 3 groups are shown in Fig. 22. The total AA level (D-AA + L-AA) of the D group is now similar to that of the L

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group (which contains only L-AA). Both are significantly higher than the total AA level of the C group (which contains only L-AA remaining from the intracellular stores of the embryonic brain).

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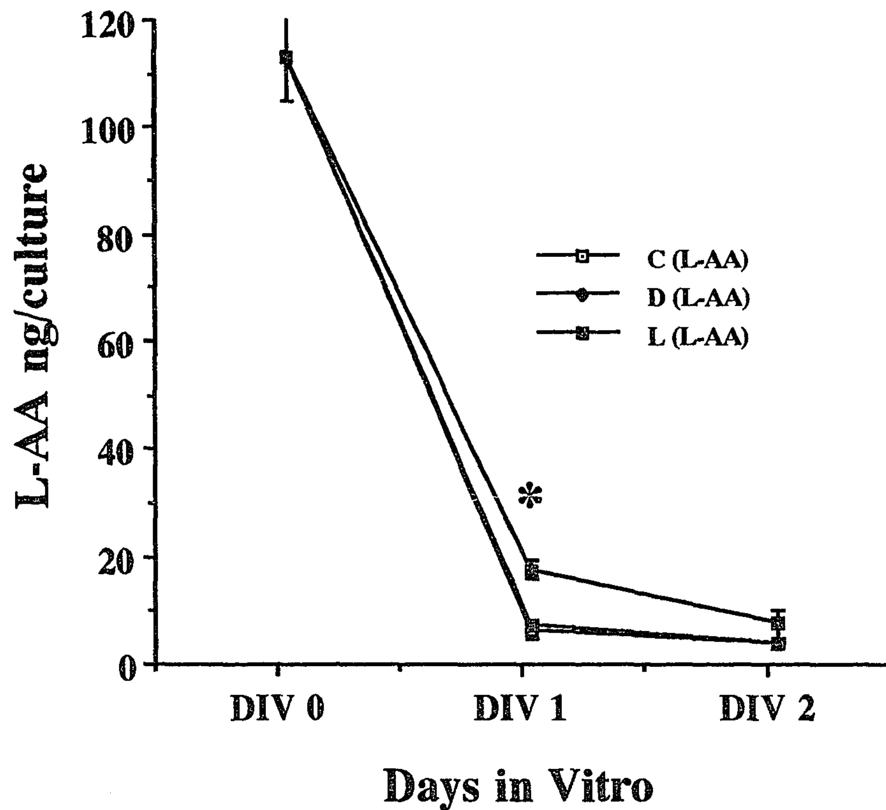

Fig. 20 Cellular content of L-AA in DIV 0, 1 & 2 L-AA treated (L), D-AA treated (D) and untreated (C) controls following exposure at plating time. Note the similar values and retention dynamics between D and C. Each value represents the mean ± SEM of 6 cultures, grouped at 2 cultures per sample. SEM bars shown only when bigger than symbols. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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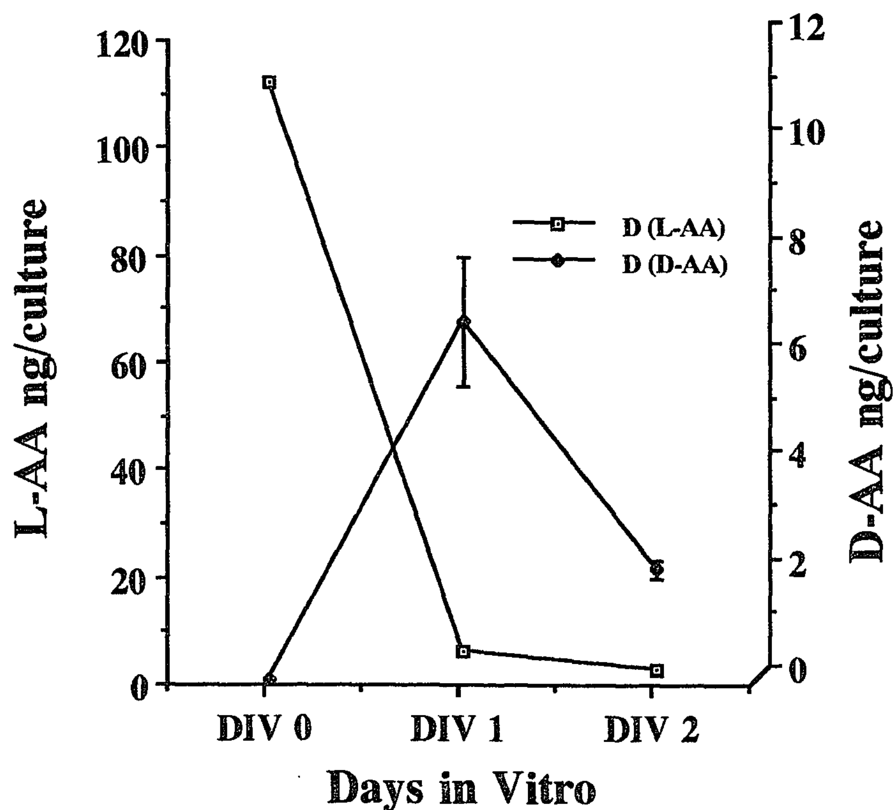

Fig. 21. Cellular content of L-AA and D-AA in DIV 0, 1 & 2 D-AA treated cultures with exposure to D-AA only at plating time. Note that the D-AA levels on DIV 1 are about equal to the L-AA levels. Each value represents the mean ± SEM of 6 cultures, grouped at 2 cultures per sample. SEM bars shown only when bigger than symbols. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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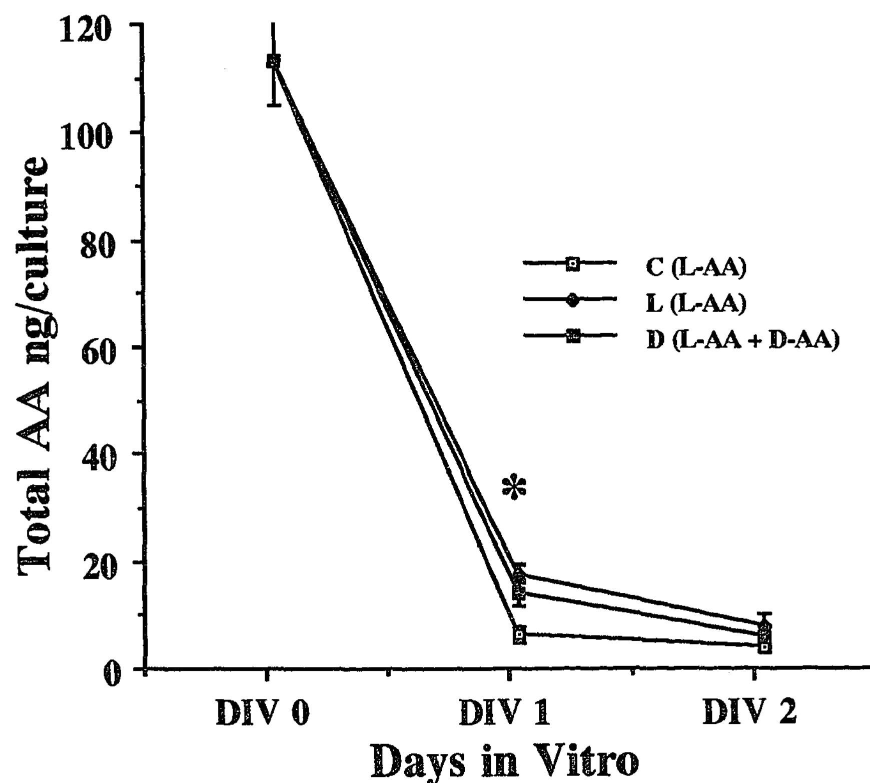

Fig. 22: Combined cellular content of L-AA and D-AA in DIV 0, 1 & 2 D-AA treated cultures with exposure to D-AA only at plating time. Note that the total AA levels (D-AA + L-AA) of the D group on DIV 1 are about equal to the L-AA levels of the L group. Each value represents the mean ± SEM of 6 cultures, grouped at 2 cultures per sample. SEM bars shown only when bigger than symbols. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.1.3b Effects of AA stereoisomers exposure

The L-DOPA levels of the 3 groups (C, D and L) are shown in Fig. 10. A significant (10-fold) increase in the L-DOPA level, which correlates with an increased TOH activity, occurred only in the L group. Treatment with D-AA did not cause any changes in the L-DOPA levels, which remained the same as the levels in the control group. Although there is a decrease to a lower level of L-DOPA on DIV 2, it remains significantly higher than that of the C and D groups.

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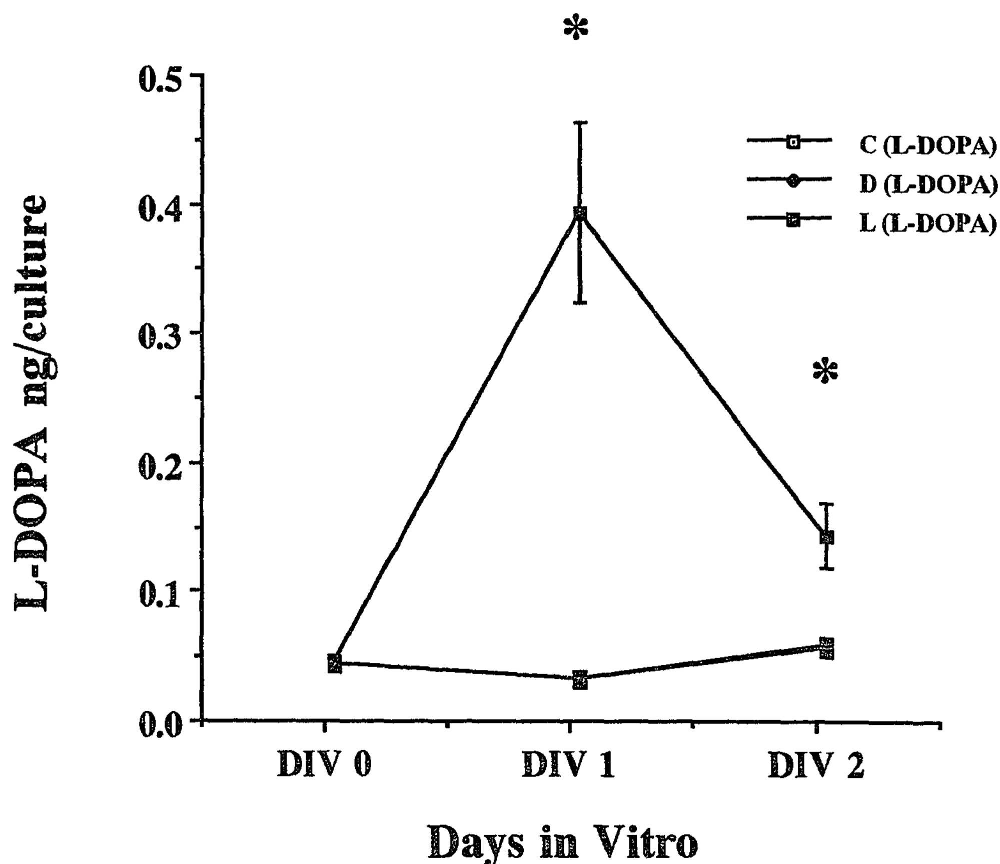

Fig. 23: Cellular content of L-DOPA in DIV 0, 1 & 2 C, D, & L cultures. Note that the D and C groups have similar levels to each other on DIV 1 and 2 without any notable rise, while the L group has a marked L-DOPA increase on DIV 1. Each value represents the mean ± SEM of 6 cultures, grouped at 2 cultures per sample. SEM bars shown only when bigger than symbols. All significant differences between groups at $p<0.05$ on ANOVA and are marked by an asterisk (*).

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3.2 Discussion

This study has confirmed the presence of high AA levels in rat neural tissue, which are consistent with previously published levels for rat brains (Milby et al. 1982). Fetal rat brain has been reported to have higher levels of AA than the adult rat brain (Kratzing et al. 1985). The lack of difference between the fetal brain AA level here - 200 μg/g of wet tissue - compared to the adult brain levels of 159-210 μg/g found by Milby et al. is probably due to the removal of the extracellular AA by washing the dissected fetal tissue with BSS (Kratzing et al. 1982) so that this value reflects the true intracellular AA concentration of fetal rat mesencephalic tissue.

In the absence of an external AA supply (i.e.- adding AA to the feeding medium) the cultures lose their AA rapidly, most of it during the first 24 hrs. By DIV 7 no detectable AA levels are found. Thus, an absolute scorbutic state exists in the cultures by DIV 7. This scorbutic state cannot be produced in the intact animal brain (Hughes et al. 1971; Saner et al. 1975) because of the brain's homeostatic mechanisms such as the choroid plexus' highly specific AA transport system (Spector 1989) and the inability of AA to cross the blood-brain barrier, which effectively isolate the AA content of the intact brain from the rest of the body's AA pool.

Adding 0.2 mM of AA to the feeding medium of the chronic AA treatment cultures at plating time (DIV 0) restores the AA content gradually. At first it only attenuates the decline in culture AA levels during the first 2 DIV relative to the control cultures. By DIV 7 the cultures' AA accumulation and storage capacity is able to offset the

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AA loss, and the AA levels rise to 50% of the embryonic tissue level (DIV 0). Restoration of the initial AA level is complete by DIV 14. This slow increase in AA content of the cultures as they develop is due to the age-related development of the neuronal and glial AA uptake system (Wilson 1989). Further support for this is lent by the similarities in AA accumulation and storage capacity following acute AA exposure seen between the naive DIV 10 cultures and the DIV 10 chronic AA treatment groups (3.1.2a Figs. 14a & 14b). By DIV 10 - 14, the full capacity to retain the AA has also developed, and the AA treated cultures are able to maintain AA at the embryonic tissue level even though the AA exposure is intermittent (AA added to the feed on DIV 0, 5, 8 and 12 and sampling done on DIV 0, 1, 2, 7, and 14) and no AA could be measured in the feeding medium 24 hrs after AA addition. This provides additional evidence for the maturation of the AA uptake and storage system, in conjunction with the capacity to preserve AA through dehydroascorbate reductase (Diliberto et al. 1983).

The initial decline in AA during the first 48 hrs may reflect an immature AA uptake system, as well as an immature dehydroascorbic acid reductase. Under these conditions both AA uptake and intracellular preservation capacity would be lower - further diminishing the AA retention ability. However, some AA uptake and retention capacity do exist even on DIV 0 - immediately following cell plating, since the AA level decline is markedly attenuated (though not abolished) by the addition of external AA (Fig. 9) and the cells are capable of D-AA uptake. Initial cell death during the first two days in vitro and lack of neuronal tissue

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organization during that period may also contribute to the initial decline in AA levels.

Significant DA level increases were a consistent finding in both the chronic AA treatment and the acute AA treatment studies.

In the chronic AA treatment studies, DA and DOPAC levels increased during in vitro growth in both chronic treatment groups of cultures (L-AA treatment and their control groups), which reflects their biochemical maturation (Prochiantz 1987). The significantly higher DA levels in the chronic AA treatment group probably are due to an increased DA synthesis as a result of increased TOH activity by direct stimulation of AA or indirectly through the marked increase in culture glial cells and their trophic effect on the DA neurons (Lieth et al. 1989). The increase in the ratio of DA content of AA cultures over DA content of control cultures, and the similar finding for DOPAC on DIV 14 compared to DIV 7 is probably a result of the greater neuronal survival in AA treated cultures, as well as increased TOH maturation over this period of time. The increased neuronal TOH staining in the chronic AA treated cultures further supports this possibility. The consistent increase in turnover rate on both DIV 7 and 14 suggests that AA's effects on neuronal catecholamine levels are complex and mechanisms other than an increase in TOH activity may be involved. A similar effect of AA on DA turnover has been noted in whole-animal studies (Izquierdo et al. 1968; Saner et al. 1975).

Tyrosine hydroxylase immuno-staining clearly demonstrated the greater morphological maturity of the DA neurons in the AA treated group, with a greater number and length of neurites. The increased

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$[^3H]$DA accumulation served to correlate function with these morphological changes as well as further assess biochemical maturity. The $[^3H]$DA uptake differences here probably resulted from an increase in DA uptake sites in the AA cultures as a result of a greater number surviving neurons. The 30% difference in $[^3H]$DA uptake between the AA and naive chronic treatment groups (3.1.1c Fig. 11) is strikingly less than the 100% increase noted in their DA content (3.1.1b Fig. 10a). This disparity suggests that the increased neuronal survival probably plays only a minor role in causing these differences in DA content.

The acute AA treatment studies help shed further light on the mechanism by which AA brings these DA changes.

In the naive, acute AA treatment group, unlike the chronic AA treatment group, no change in the number of DA neurons takes place as a result of exposure AA. This is demonstrated by the complete lack of change in $[^3H]$DA uptake between the naive, acutely treated groups (3.1.2c Fig. 16). The increase in the number of TOH-stained neurons (3.1.2d Fig. 17), and the greater stained area and staining intensity per cell (3.1.2d Fig. 18 a&b) therefore indicate a possible dual effect on the expression and synthesis of TOH itself (Porter et al. 1991). The time course for the appearance of this increase (4 and 24 hrs. after AA exposure is consistent with studies measuring the time course of forskolin effects on TOH mRNA (Carroll et al. 1991) where TOH mRNA exhibited slight increases within 1 hr of treatment, and reached a maximum 5.5 hrs. later.

The prolonged time course (~ 48 hrs, 3.1.2b Fig. 15a) for the appearance of the DA increase found in the naive, acute AA

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treatment group presents a challenge in itself. This effect of AA on DA levels is unlikely to be due to stimulation of TOH activity since in studies where stimulation of TOH activity was carried out - by exposure to agents which act through a cAMP-dependent protein kinase, such as forskolin or secretin (Roskoski et al. 1989) - the maximal response time for increased TOH activity was 6-15 sec. Another study (Greene and Rein 1978) which compared the effect of AA on the synthesis of DA - where AA is not a cofactor - with that on the synthesis of norepinephrine (NE) - where it is a cofactor of dopamine β hydroxylase (DBH). It showed an exponential change for NE after 20 min., but no change for DA even after 1 hr. Indeed, AA has even been found to produce a dose-dependent inactivation of TOH under phosphorylating conditions (Roskoski and Wilgus 1987). It is also unlikely that AA acts by protecting DA from degradation, since the maximal level of culture AA is reached at 24 hrs. after exposure to AA and, at 48 hrs., is less than that found at 4 hrs (3.1.2a Fig. 14a). Further support for the lack of protective effect on DA precursor degradation was seen in the experiments using D-AA, where no difference in the L-DOPA content was noted between the D-AA group and the controls, even though the D-AA group had a reducing power (measured in terms of the total cellular AA level) which was twice that of the control. The increase in the number of TOH-stained neurons discussed above, possibly indicates that an increase in TOH levels has occurred within a 4 to 24 hr. period, and the L-DOPA measurements in the young cultures (3.1.3b Fig. 23) show that TOH activity increases can occur within a 24 hr. period following the addition of extraneous L-AA (but not D-AA). The

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finding that DA & DOPAC levels are low in those young (DIV 0-2) cultures relative to their L-DOPA level is indicative of the sequential maturity of the enzymes of the DA biosynthetic chain - TOH matures before AADC. Similarly, the delay in DA level changes (24 hrs after the differences in TOH staining have been noted) raises the possibility of a differential effect of L-AA on the enzymes of the DA biosynthetic chain. A differential effect by forskolin, and separately by dexamethasone on synthetic chain enzyme expression has already been noted for TOH and PNMT (Carroll et al. 1991). Thus TOH levels - and as a result its activity - increase within 24 hrs, raising the levels of L-DOPA. This rise (or an unknown L-AA induced factor) might then induce an increase in the levels and activity of AADC within the next 24 hr period, with a resulting rise in the DA levels 48 hrs after the initial L-AA exposure.

Glial proliferation was a notable finding in the chronic AA treatment study. It is therefore of interest that AA has been found to exert an inhibitory effect on the growth of astrocytes in culture (Cheng et al. 1988). This difference may be due to the use of purified glial preparation by these investigators and possibly the consequence of the absence of neuronal interaction. Another reason could be their use of neonatal vs. this study's use of an embryonic source of tissue.

The marked increase in glial proliferation in the chronic AA group, as noted on GFAP staining, may be another mechanism by which AA exerted its chronic effects on mesencephalic cell cultures in the chronic AA treatment studies. Glial-neuronal interactions, both direct and indirect through glial neurotrophic factors (Engele et al. 1991), have been shown to have an effect on neuronal maturation -

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morphologically and biochemically (Lieth et al. 1989; O'Malley et al. 1992; Prochiantz 1987). The fact that the cultures can survive and function without AA reflects the non-obligatory nature of these AA effects.

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Chapter 4: Summary and conclusions

Ascorbic acid (AA) is a ubiquitous compound in living organisms, It has been studied in a variety of non-neuronal tissues and astrocytes, but in the past has been identified only as an antioxidant and as a cofactor in hydroxylation reactions. Its presence in high concentrations in the fetal and adult mammalian brain has been known for some time, and a stereospecific high affinity uptake mechanism for it has been demonstrated in astrocytes, but no studies of its effects on neuronal biochemical and morphological development have been carried out. The presence of a high affinity AA uptake mechanism and the high concentration of AA in the fetal brain, which is higher than that of the adult brain, suggest that AA has a role in neural development. The inability to produce a truly scorbutic state in neuronal tissue of intact animals presented a hurdle to studying this role. As the current study demonstrated, since AA content drops sharply to undetectable levels when no ascorbic acid is added to the medium, the neuronal tissue cultures used here allowed the creation of such a neuronal scorbutic model and enabled the study of AA's effects on its development and function. The absence of a blood-brain barrier and choroid plexus, and the ability to control the extracellular fluid composition with precision - were further factors which facilitated studying the effects of AA on neuronal function and development.

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The results of the experiments presented here, suggest that AA has a stereospecific, regulatory role on the DA neuronal cultures maturation which significantly affects dopamine synthesis, glial proliferation and neuronal morphology.

The capacity to accumulate and retain the intracellular AA developed gradually as the cultures matured. Ascorbic acid reached the embryonic day 14 levels by DIV 10 - 14. The data showed that although neuronal cultures can survive and grow in the absence of detectable levels of ascorbic acid, its chronic presence exerts a broad effect on dopamine neuron morphology and biochemical functioning either directly or through increased glial proliferation, or possibly both.

The data from the experiments measuring the acute and short term effects of AA on naive cultures showed that the AA uptake and accumulation capacity developed independently of any in vitro exposure to AA, and that the naive cultures are capable of AA accumulation and retention in a manner similar to the chronically AA-treated cultures. This finding enabled the isolation of the biochemical effects of AA on DA neurons from the general effects, which might possibly be mediated through cellular proliferation. The acute AA exposure studies strongly suggest that the acute effects on the DA content are most likely to be the result of regulation of TOH activity and not due to an increase in neuronal survival.

The data from the experiments measuring the acute effects of AA stereoisomers on TOH activity (by measuring the levels of L-DOPA) in naive, L-AA and D-AA treated young cultures from DIV 0 through 2, demonstrate the stereospecificty of the stimulation of TOH and, in

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conjunction with the acute AA treatment experiments on naive cultures, suggest that AA may exert a regulatory effect on other enzymes of the DA synthetic and catabolic pathway.

The data presented in this thesis indicate that AA probably plays a significant and specific role in regulating DA metabolism through its effects on TOH activity and possibly other enzymes in the DA synthetic and catabolic chain, and that the current culture model might be useful in further exploring the precise mechanism of these effects.

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