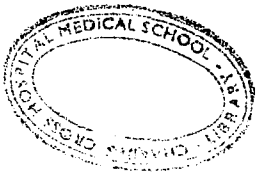
UNIVERSITY OF LONDON

AN INVESTIGATION OF THE MORPHOLOGICAL AND FUNCTIONAL PROPERTIES OF RAT HYPOTHALAMIC DOPAMINERGIC SYSTEMS *IN VITRO*.

HILARY ELAINE MURRAY
Department of Pharmacology,
Charing Cross and Westminster Medical School
London



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ABSTRACT.

Hypothalamic neurotransmitter systems are likely to play an important role in bringing about the changes in neuroendocrine function which occur throughout development. In particular, the dopaminergic neurones intrinsic to the hypothalamus are widely implicated in the regulation of the reproductive and growth axes which exhibit clear ontogenetic changes in their endocrine profiles. Therefore, using foetal rat hypothalamic cells in primary culture, maintained in a serum-free defined medium, we have investigated the morphological and functional development of the intrahypothalamic dopaminergic systems.

Immunocytochemical studies demonstrated the presence of three morphologically distinct sub-types of tyrosine hydroxylase-immunopositive (THIP) neurones. On day three *in vitro* unipolar, bipolar and multipolar cell types were apparent. The latter two sub-types persisted to later stages in culture and increased both in perikarya size and neurite length. All subtypes have been shown to have correlates *in vivo*.

Biochemical studies employing tritiated dopamine ([^{3}H]DA) demonstrated a time- and temperature- dependent uptake mechanism within the cultures which was significantly attenuated by uptake inhibitors such as benztropine and nomifensine in a dose - dependent manner. The degree of uptake into the hypothalamic cells in culture increased as a function with time reflecting a maturation of the uptake mechanism in play. Certain experiments suggest that the uptake may also be influenced by dopamine receptor modulation.

[^{3}H]DA was released under both basal and potassium (56mM) - stimulated conditions and the magnitude of the response was reduced by exclusion of calcium from the release medium. The ability of the cells to release [^{3}H]DA also increased with the age of the culture again indicating a functional maturation of the DA containing neurones within this preparation.

Functional maturation of these systems was also reflected in studies involving the measurement of endogenous DA using high performance liquid chromatography coupled with electrochemical detection (HPLC - EC). The neurones displayed an inherent maturation as shown by an increase in the magnitude of release (basal and potassium - stimulated) of endogenous catecholamine with advancing cultivation time. Potassium - depolarization was seen to enhance the rate of development/improve the efficiency of the release mechanism. In addition, we found that cultures initiated from tissue collected at different gestational ages showed differential behaviour in terms of DA output.

The role of 17 ß - oestradiol (E_2) in regulating hypothalamic dopaminergic function was also investigated both indirectly with the use of [^{3}H]DA and by direct

measurement of endogenous DA. Both uptake and release of [^{3}H]DA and release of endogenous DA were significantly modulated by the concentration of the steroid in the defined medium. The optimal concentration for uptake ([^{3}H]DA) and release ([^{3}H]DA and endogenous DA) were 10^{-10} and 10^{-12}M respectively. These levels of oestrogen are likely to be within the physiological range. The parameters under investigation were also influenced by progesterone, but in a manner which was distinctly different from that of oestrogen.

The effect of prolactin and gonadotrophin - hormone - releasing hormone on the release of endogenous DA was also studied to investigate whether intrahypothalamic dopaminergic systems might be involved in negative feedback regulation of hormone release. While prolactin was ineffective in modulating DA output GnRH was a potent secretagogue for the dopaminergic systems within the culture model.

GABA-ergic neurones within the cultures were also studied in order to test a) the specificity of the results with the DA neurones and b) a functional interaction between hypothalamic GABA-ergic and dopaminergic neurones. Studies on the uptake and release of [^{3}H]GABA in hypothalamic cells in culture highlighted marked differences in behaviour of GABA-ergic and dopaminergic systems both in terms of their pattern of maturation and their responsiveness to gonadal steroids. Bicuculline ($GABA_A$ receptor antagonist) significantly ($p < 0.005$) enhanced the release of endogenous DA at concentrations ranging from 10^{-6} to 10^{-4} M and both $GABA_A$ and $GABA_B$ agonists (muscimol and baclofen respectively) were also found to modulate the release of endogenous DA. Marked differences in the behaviour of [^{3}H]DA release compared with that of the endogenous amine were noted and suggest caution when interpreting pharmacological studies using cells pre-loaded with [^{3}H] - labelled neurotransmitters.

In conclusion, these results demonstrate that hypothalamic dopaminergic neurones in primary culture undergo a pre-programmed morphological and functional maturation which have several correlates *in vivo*. At least one sub-population of these neurones is responsive to gonadal steroids and influenced by hypothalamic GABA-ergic systems. As hypothalamic dopaminergic pathways are essential components in the regulation of neuroendocrine activity we propose that this model may serve as a valuable tool for the investigation of the ontogeny of DA systems intrinsic to the hypothalamus and also their interaction with other hypothalamic neurotransmitters and neuropeptides.

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TO MY FAMILY AND FRIENDS

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GENERAL ABBREVIATIONS.

adr	adrenaline
AgCl	silver chloride
AOAA	amino oxyacetic acid
Arc	arcuate nucleus
aTH	anti - tyrosine hydroxylase antibody
AVP	vasopressin
ß	beta
BSA	bovine serum albumin
CA	conceptional age
$CaCl_2$	calcium chloride
c.p.m.	count per minute
Ci	curies
Cl^-	chloride ion
CRF	corticotrophin releasing hormone
CSF	cerebrospinal fluid
DA	dopamine
DAB	diaminobenzidine
DHBA	dihydroxybenzylamine
DM	defined medium
DIV	days in vitro
DMEM	Dulbecco's Modified Eagles Medium
DMN	dorsomedial nucleus
DNA - ase	deoxyribonuclease enzyme
DOPAC	dihydroxyphenylacetic acid
d.p.m.	disintegrations per minute
E	embryonic age
EBSS	Earles's Balanced Salt Solution
E_2	17 ß - oestradiol
ECD	electrochemical detection
EDTA	ethylenediamine - tetraacetic acid
FCS	foetal calf serum
F	fornix
GABA	gamma aminobutyric acid
GAD	gamma amino decarboxylase
GAP	gonadotrophin associated peptide
GH	growth hormone

GHRH	growth hormone releasing hormone
GnRH	gonadotrophin releasing hormone
Glu	glutamic acid
Gly	glycine
^3H	tritium
5-HT	serotonin
H_2O_2	hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HCl	hydrochloride
HEPES	N -{2 - hydroxethyl} - piperazine - N' - [2 - ethanesulfonic acid]
HP- axis	hypothalamo - pituitary axis
HPLC	high performance liquid chromatography
HS	horse serum
HVA	homovallinic acid
i.c.v	intracerebroventricular
i.p.	intraperitoneal
IP	immnunoprecipitation
i.v.	intraventricular
ICC	immunocytochemistry
ICHDA	incertohypothalamic dopaminergic tract
IgG	immunoglobulin of the class G
K^+	potassium ion
KCl	potassium chloride
K_m	Michaelis - Menten Constant
LH	luteinizing hormone
LPZ	lateral palisade zone
M	molar
µM	micromolar
mM	millimolar
mmol	millimoles
µmol	micromoles
MAO	monoamine oxidase
MBH	mediobasal hypothalamus
ME	median eminence
MFB	medial forebrain bundle
mg	milligram
µl	microlitre
mPOA	medial preoptic area

MPON	medial preoptic nucleus
MPZ	medial palisade zone
NA	noradrenaline
Na^+	sodium ion
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
$Na_2HPO_4.\ 2H_20$	disodium phosphate
$NaH_2PO_4.2H_20$	mono sodium phosphate
NE	Nuclear Enterprise Ltd
NG_0S	normal goat serum
nM	nanomolar
ODS	octadecasilylsilica
6 - OHDA	6 - hydroxydopamine
P	postnatal age
PAP	peroxidase anti peroxidase complex
PBS	phosphate buffered saline
PCA	perchloric acid
PeVN	periventricular nucleus
PIF	prolactin inhibitory factor
pg	picograms
PMT	photomultiplier tube
PRF	prolactin releasing factor
Pro	proline
Prog	progesterone
PRL	prolactin
PVN	paraventricular nucleus
r.p.m.	revolutions per minute
SA	specific activity
SCN	supra chiasmatic nucleus
sEBSS	supplemented Earles' Balanced Salt Solution
s.e.m.	standard error of the mean
SN	substantia nigra
SON	supra optic nucleus
SRIH	somatostatin
Tau	taurine
TH	tyrosine hydroxylase
THDA	tuberohypophyseal dopaminergic system
THIP	tyrosine hydroxylase immunopositive neurones
TIDA	tuberoinfundibular dopaminergic system

Tris	Tris (hydroxymethyl) - aminomethane
VIP	vasointestinal peptide
VMN	ventromedial nucleus
VNAB	ventral noradrenergic bundle
ZI	zona incerta

CHAPTER ONE: INTRODUCTION

1.1. Purpose of the Investigation.

Up until the mid 1960s, interest in dopamine (DA) centred largely on its place in the biosynthetic pathway for the neurotransmitters noradrenaline (NA) and adrenaline (Adr), when it was regarded solely as a precursor in their synthesis. Since then, the acceptance of DA as a neurotransmitter in its own right has led to numerous investigations on the distribution and function of the dopaminergic systems of the CNS (Anden et al 1965, 1966a, 1966b, Bjorklund et al 1970, 1973a, 1973b, 1975 1979).

The majority of central DA containing systems are located within the midbrain and cortex. These pathways have been the subject of close scrutiny, mainly due to their involvement both in psychiatric and neurodegenerative diseases. The mesolimbic pathway has been associated with the development of schizophrenia (Hokfelt et al 1974), while the nigrostriatal pathway is implicated in the aetiology of Parkinson's Disease (Hornkiewicz; 1973, Fuxe & Ungerstedt 1976). Interest arising from the potential use of neural implants to alleviate the symptoms of Parkinson's Disease has resulted in numerous investigations into the function and development of the mesencephalic dopaminergic systems, under both *in vivo* and *in vitro* conditions (Prochiantz et al 1981, Barbin et al 1985).

A significant concentration of dopaminergic neuronal systems is also located in the diencephalic brain region, namely, the hypothalamus. Hypothalamic areas such as the periventricular (PeVN) and arcuate nuclei (Arc) and the zona incerta (ZI), contain dopaminergic perikarya which project locally to innervate areas such as the anterior hypothalamus, preoptic area and median eminence (Bjorklund et al 1973a, 1975, 1979). These anatomical divisions are associated with the control of the neuroendocrine system and their dopaminergic neuronal systems are strongly implicated in many aspects of the regulation of neuroendocrine function. However, despite their likely importance in the maintenance of the body's homeostatic processes, the development of these pathways has received relatively little attention compared with their mesencephalic counterparts.

Throughout life the endocrine system exhibits significant and sometimes dramatic changes in function, which are likely to be pre - programmed to occur at critical stages of development. The late foetal, early neonatal period is one such critical time when the neuronal circuitry influencing adult patterns of endocrine function are established. The mechanisms underlying these events remain unknown, but most certainly include events at hypothalamic level, involving neurotransmitter systems.

Elsewhere in the brain, evidence suggests that the programming of adult behavioural patterns during embryonic development occurs via a process involving DA, (Arevalo et al 1987, Matteson 1988), but a similar role for DA in the neuroendocrine hypothalamus remains unexplored.

The endocrine system, in conjunction with the autonomic nervous system, which is also controlled by the hypothalamus, constitutes the major route whereby the CNS regulates peripheral function and achieves homeostasis. Therefore obtaining further knowledge of the development of the intrahypothalamic neurotransmitter systems and the factors which influence their activity would greatly benefit our understanding of the neural control of endocrine function and ultimately, processes which are important to health and disease.

Access to the hypothalamus is difficult *in vivo*. We have therefore adopted a primary cell culture model to investigate the dopaminergic neuronal systems intrinsic to the hypothalamus in terms of their *in vitro* development and the factors which influence their function.

1.2. The Hypothalamus.

1.2.1. Function.

Amongst its numerous roles the hypothalamus controls both endocrine and autonomic regulatory processes. Growth and reproduction, events related to parturition and milk production in the female and the evolution of the stress response are all intimately associated with hypothalamic function. Furthermore, the ingestion of food and water, and control of respiration and body temperature are all governed at hypothalamic level. The ability of the hypothalamus to integrate the complex array of neurochemical and electrical inputs it receives contributes to its role as a central key to the function of the mammalian CNS. Its complexity has, for many years, hampered investigation into its structure and function, yet advances in technology have allowed the neuronal and neurochemical anatomy of the hypothalamus to be investigated and a considerable amount of detail concerning its neuropeptide and neurotransmitter systems and their receptors has now been elucidated. Despite this, much is yet to be discovered concerning the function and inter-relationships of the many neuronal pathways intrinsic to the hypothalamus and the consequences of their interactions on the activity of the hypothalamo-pituitary (HP) - axis.

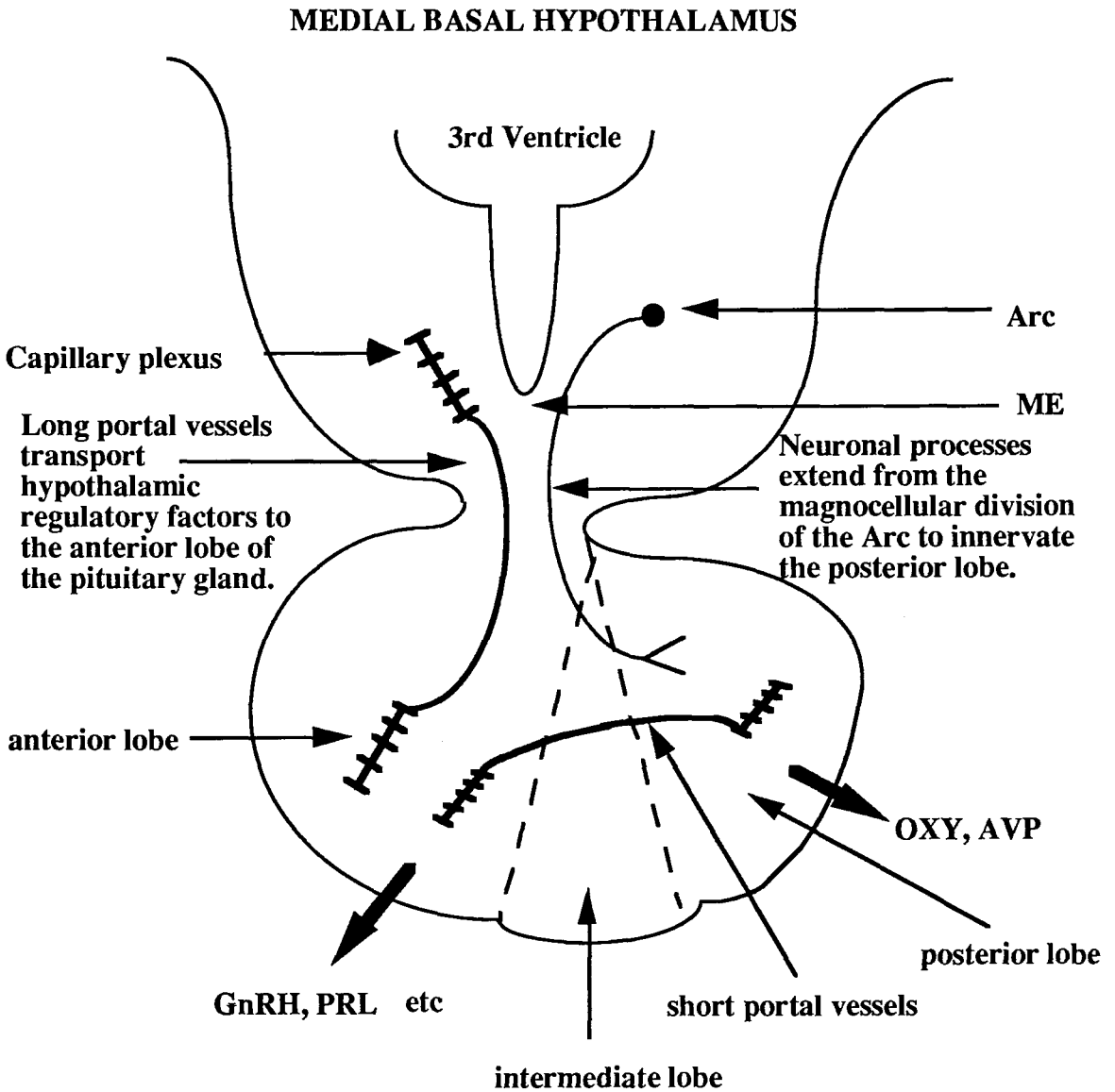
1.2.2. Structure and Development.

The hypothalamus arises from the embryonic diencephalon, a division of the prosencephalon. During early developmental stages, the hypothalamus appears as the lowest of three swellings located laterally in the wall of the third ventricle. During the developmental process the hypothalamic swelling becomes detached from the other two portions, namely the epithalamus and the thalamus and a groove called the hypothalamic sulcus forms between them. The third ventricle forms from the central cavity between the hypothalamus and thalamus and continues to develop within the lateral walls of the diencephalon. The roof of the third ventricle is formed by a monolayer of ependymal cells covered by a layer of mesenchymal cells possessing an extensive capillary system. These capillaries form the choroid plexus of the third ventricle by invaginating locally and connecting with the ependymal cells beneath.

The link between the hypothalamus and the pituitary gland is of special significance to neuroendocrine function and it is important to understand the relationship between these two structures both in terms of their neuroanatomomy and neurochemistry. The neurohypophysis is formed by the downward evagination of a portion of the diencephalon known as the infundibulum and remains in neuronal direct communication with the hypothalamus. By contrast, the adenohypophysis develops from the cavity known as Rathke's pouch, and does not have a neural origin. Instead, the hypophyseal stalk is formed by the infundibulum and the infundibular part of the adenohypophysis. The ependymal cells of the adenohypophysis extend to the pituitary stalk growing along and around it and forming the pars tuberalis. Communication between the hypothalamus and the anterior pituitary is via a highly vascular portal system originating from the internal carotid artery. This portal system consists of primary capillary systems within the median eminence (ME) at the base of the hypothalamus, and also the pituitary stalk and the posterior lobe of the pituitary gland. Blood flow to the ME is provided by the superior hypophysial artery while the pituitary stalk and the posterior lobe are served by the middle and anterior hypophysial arteries, respectively. The blood supply to the anterior pituitary gland originates at the level of the ME and enters the gland via long portal vessels which run along the pituitary stalk. In addition, a secondary portal system, comprising short vessels connects the posterior and anterior pituitary lobes, bridging the avascular cleft of the intermediate lobe (Daniels & Pritchard 1975). Blood drains from the anterior lobe via a secondary capillary plexus which is linked to the systemic venous circulation by Y - shaped pituitary veins. Likewise the blood supply to the posterior lobe re-enters the systemic circulation via venous drainage. A schematic representation of the neurovascular organization of the hypothalamo - pituitary complex is illustrated in Fig. 1.1. At the level of the ME regulatory factors which

influence anterior pituitary function are released into the portal circulation, and this represents the fundamental basis for hypothalamic regulation of neuroendocrine function (Green & Harris 1947).

Fig. 1.1. The neurovascular arrangement of the hypothalamo - pituitary axis.



The hypothalamus is commonly divided into three regions, namely the supraoptic region rostrally, the tuberal region located midway and the mammillary region caudally. The ventral surface of the hypothalamus has three "landmarks" the optic chiasm, tuber cinereum and mammillary bodies which define these three portions of the hypothalamus respectively. Alternatively, it may be considered as divided in a

longitudinal manner into medial and lateral areas. The medial zone has a high cellular content, the neurones forming nuclei of more or less well defined aggregations. In contrast to this, the lateral zone is not easily defined by nuclei, its neurones being more diffuse. The lateral zone is known chiefly for possessing a large bundle of ascending and descending axons which is known as the medial forebrain bundle (MFB) and it is this structure which forms the major input and output pathways of the medial hypothalamus (see below).

1.2.3. Nuclei of the Hypothalamus.

The neuronal cells of the hypothalamus which participate in neuroendocrine function may generally be placed into two categories in terms of their cell body size. The magnocellular division comprises large neurones the processes of which, with few exceptions, terminate on the capillaries of the neural lobe of the pituitary gland and release neurotransmitters and neuropeptides into the circulation. A subgroup of smaller cells, the parvocellular division, project fibres to the precapillary spaces of the hypophysial portal vessels and the neurohormones which they secrete are transported to the anterior pituitary gland where they regulate the release of anterior pituitary hormones.

The cells of both divisions are organised in well defined aggregations (nuclei), situated in several regions of the hypothalamus. Within the supraoptic region of the hypothalamus lie the laterally paired supraoptic nuclei (SON) and the paraventricular nuclei (PVN). Both nuclei, contain magnocellular cells which project axons forming the hypothalamo - neurohypophysial tract, projecting to the posterior pituitary and the PVN has several distinctive parvocellular divisions, namely the anterior, medial, dorsal and lateral divisions as characterised by Van Den Pol (1982).

The paired suprachiasmatic nuclei (SCN) are almost circular in shape and lie in close association at the midline of the basal hypothalamus. They are situated beneath and adjacent to the third ventricle rostrally, just above the optic chiasm. These nuclei are involved with the generation of circadian rhythms and also control daily rhythms of behaviour and hormone secretion (Meijer & Rieveld 1989). Also in the anterior zone, is the medial preoptic nucleus (MPON), a major target for the sex steroids, rich in oestrogen and testosterone receptors. Such receptors are also densely located in the septum with which the preoptic area merges.

The ventromedial and dorsomedial nuclei (VMN and DMN respectively) are found in the medial region of the hypothalamus. Along with the arcuate nucleus (Arc) these cell groups surround the ventral part of the 3rd ventricle. Like the PVN, the VMN is

under the influence of a great number of afferent and efferent connections from and to other regions of the CNS such as the spinal cord and mesencephalic central grey matter (Swanson & Kuypers 1980). The neurones within the Arc project into the ME and their axons terminate in the pericapillary spaces of the portal capillary loops. Thus neurotransmitter pathways emerging from this nucleus have direct access to the portal circulation and from there to the anterior pituitary gland (Swanson & Kuypers 1980). The projections contribute to the parvocellular neurosecretory systems originating from small neuronal cell bodies in the medial portion of the Arc.

The periventricular nucleus, (PeVN), is located at the rostral extreme of the 3rd ventricle. Its function in neuroendocrine regulation is chiefly related to the control of gonadotrophin secretion since discrete lesioning of the nucleus disrupts the pulsatile release of luteinizing hormone (LH). The anteroventral portion of this nucleus exhibits sexual dimorphism regarding the distribution of tyrosine hydroxylase (TH) containing cells, with a 3 - fold greater number of TH immunoreactive neurones contained in the female PeVN than in the male (Simerly et al 1985). In addition, this nucleus is also the location of a population of somatostatin (SRIH) secreting neurones (Herbison et al 1991). The cell bodies of these neurones are large, but, unlike other cells of the magnocellular division, the processes of this cell population terminate at the level of the median eminence and SRIH reaches the anterior pituitary via the portal system.

Finally, there are the mammillary bodies, which are visible on the ventral surface of the hypothalamus. The mammillary nuclei form a complex structure located caudally within the mesencephalic central grey.

1.2.4. Pathways to and from the Hypothalamus.

The medial forebrain bundle (MFB) is the structure through which both the ascending and descending projections of the hypothalamus pass and, in most cases, traverse the lateral portion of the hypothalamus. Immunocytochemical techniques show that a significant percentage of the monoaminergic neurones of the brainstem travel to the hypothalamus via the medial forebrain bundle. The hypothalamus receives a rich noradrenergic input, originating in the ventrolateral medulla oblongata and the nucleus of the tractus solitarius. From the hypothalamus this noradrenergic tract, which is known as the ventral noradrenergic bundle (VNAB), ascends to the MFB. A number of hypothalamic areas receive rich noradrenergic input including the anterior hypothalamic areas, the medial preoptic area and the periventricular zone and these connections are of great neuroendocrine importance and have been the subject of extensive investigation. Other regions innervated by fibres emerging from the

medial forebrain bundle include the PVN, SON Arc and SCN. Adrenergic neurones from the medulla also connect with the medial hypothalamus and like noradrenergic neurones heavily innervate the PVN, although their functional role within the hypothalamus is ill defined.

The 5-HT containing neurones of the mesencephalic raphe nuclei pass through the medial hypothalamus, the mammillary complex, the SCN, the periventricular (PeV) zone and the Arc and also moderately innervate the internal layer of the ME. Noradrenergic, adrenergic and serotoninergic neurones form an extensive network of neurones called the periventricular system which connects the hypothalamus to the mesencephalon. In addition dopaminergic neurones pass through the hypothalamic periventricular and periaqueductal grey and they project to and within the hypothalamus rostrally. More detailed information of the dopaminergic neurones intrinsic to the hypothalamus will be discussed in subsequent paragraphs.

The descending pathways within the hypothalamus also pass through the MFB emerging from the limbic system. This system includes the olfactory areas of the forebrain as well as the septal nuclei and the amygdaloid nuclear complex which are structures of the neocortex. Some of these structures project to the medial hypothalamus, while others terminate within lateral regions of the hypothalamus, innervating the medial hypothalamus via interneurones.

1.3. Anatomy of the Central Dopaminergic Systems.

A summary of all the major dopaminergic tracts may be found in Table 1.0. Catecholamine containing neurones traverse the brainstem from the medulla oblongata to the diencephalon and are also found in the retina and olfactory bulb. The CA-containing systems were first described byDahlstrom & Fuxe (1964) who depicted 12 different cell groups which they designated A1 - 12. Further studies by these workers and Bjorklund et al (1973, 1975) led to the addition of further cell groups, an A13 located in the rostral zona incerta and the A14 in the anterior periventricular hypothalamus and the preoptic region. Tyrosine hydroxylase immunocytochemistry (see Table 1.0) has more recently led to the observation of a system of cells disseminated in the dorsal portion of the preoptic area, the ventrolateral area and the hypothalamus. These cells collectively form the A15 cell group and another cell group, located in the olfactory bulb have been designated to the A16 cell group. An additional cell group exists in the amacrine cells of mammalian retina as observed by Ehinger (1976).

Table. 1.0. Dopaminergic tracts of the CNS.

Name of Tract	Site of Orgin	Innervation Area	Method of visualisation/identification	Reference
Nigrostriatal	A9: substantia nigra	1. dorsal striatum 2. subthalamic nucleus 3. globus pallidus 4. ventral putamen	a. Falck-Hillarp fluoresence b. Glyoxylic -histofluorescence c. Aluminium-formaldehyde histofluorescence d. Immunocytochemistry (ICC) for TH	a. Anden et al 1965 b. Bjorklund & Lindvall 1975 c. Loren et al 1980 d. Pearson et al 1983
Mesolimbic	A10: ventral tegmental area	1. dorsal striatum 2. ventral striatum 3. nucleus accumbens 4. tuberculum olfactorium 5. bed nucleus of the stria terminalis	a. Falck-Hillarp fluoresence b. Multiple fluorescence/retrograde trace studies	a. Ungerstedt et al 1971 b. Albanese et al 1983
Periventricular, hypothalamic and preoptic	A11: mesencephalic peri - aqueductal grey; -periventricular gray of the caudal thalamus **and** A12-A14: periventricular hypothalamus and periventricular preoptic area	1. periaqueductal gray 2. medial thalamus **and** 3. anterior hypothalamus 4. medial preoptic area	a. Glyoxylic histofluorescence	a. Bjorklund & Lindvall 1975
*Tuberohypophyseal	A12-A14: arcuate and periventricular hypothalamic nuclei	1. intermediate lobe of the pituitary gland 2. neural lobe of the pituitary gland	a. fluorescent histochemistry b. microspectrofluorometric mapping c. immunocytochemisty (ICC) d. ICC for TH	a. Bjorklund et al 1973 b. Bjorklund et al 1977 c. Hokfelt et al 1977 d. Daikoku etal 1986
*Tuberoinfundibular	A12: arcuate nucleus and periarcuate nucleus	1. All layers of the median eminence. 2. Pituitary Stalk.	a. fluorescent histochemistry b. microspectrofluorometric mapping c. ICC for TH d. ICC for TH	a. Bjorklund et al 1973 b. Bjorklund et al 1977 c. Hokfelt et al 1977 d. Ugramov et al 1989
Incertohypothalamic	A13: medial zona incerta A11, A14: periventricular hypothalamus	1. zona incerta 2. medial preoptic periventricular hypothalamus 3. septum	a. fluorescent histochemistry b. microspectrofluorometric mapping c. ICC d. ICC for TH	a. Bjorklund et al 1973 b. Bjorklund et al 1977 c. Hokfelt et al 1977 d. Ugramov et al 1989

TH = tyrosine hydroxylase
ICC = immunocytochemistry

* See also: Smith & Fink (1972).

1.3.1. The Mesencephalic DA Cell System.

Dopamine - containing neurones are mostly distributed in the mesencephalon and diencephalon. Others are located in the A2 group situated in the medulla oblongata. By far the greatest population of dopaminergic neurones are found in the cell group known as the mesencephalic DA cell system, which includes the A8, 9 and 10 cell groups. From this cell system originate the neurones which form the extensive mesotelencephalic DA projection system with approximately 15, 000-20, 000 DA neurones emerging from each half on the mesencephalon.

1.3.2. The Diencephalic DA Cell System.

Within the A11-A14 cell groups lie the diencephalic DA cell systems. They give rise to the extensive intradiencephalic projections as well as the diencephalospinal pathway. These are considerably fewer in number than those of the mesencephalon with approximately 500 neurones on each side in the tuberal A12 group which gives rise to the tuberohypophyseal and tuberoinfundibular pathways. An almost equal amount occupies the remaining diencephalic cell groups thus, the diencephalic DA cell population is less than one tenth of that of the mesencephalon.

1.4. Dopamine Containing Fibre Tracts of the Hypothalamus.

In order to appreciate the possible inter-relationships of hypothalamic DA pathways and the likely neuroendocrine functions ascribed to them, it is important to describe briefly their main cytoarchitectural characteristics. The detail so far known concerning the exact location of these tracts has been found by making observations in the rat and primate CNS. Evidence does however suggest that these organisational features hold true for other species and man (Felten & Sladek, 1982, Nobin & Bjorklund, 1973).

1.4.1. The Incertohypothalamic Dopaminergic Tract.

The incertohypothalamic dopaminergic (ICHDA) projection system, (also known as the intradiencephalic system), is comprised of short, locally projecting neurones. The fibres of this tract undergo extensive bifurcation soon after leaving the cell bodies and if visualised by histofluorescent techniques (see Table 1.0) exhibit fine weak fluorescence with varicosities separating the only partially visible intervaricose segments (Bjorklund et al 1975).

The pathway itself may be described in 2 components the caudal and the rostral as defined by Bjorklund et al (1975). The caudal component of the tract extends from an area of dopaminergic cell bodies located in the caudal thalamus, the posterior

hypothalamic area and the medial zona incerta, (groups A11 and A13) and pass upwards to the dorsal part of the DMN as well as the anterior and dorsal areas of the anterior hypothalamus. The rostral component of the incertohypothalamic fibre system originates in the rostral periventricular cell bodies of the A14 cell group and distributes itself in the PeVN of the anterior hypothalamus and in the PVN, SCN and MPON up to the level of the anterior commissure. From here this component of the fibre tract extends to the lateral septal nucleus at its most caudal part.

1.4.2. The Tuberohypophyseal Dopaminergic Tract.

The dopaminergic cell groups from which the tuberohypophyseal dopaminergic (THDA) neuronal system is derived may be divided into 2 groups on the basis of their mode of projection. One cell group within the rostral portion of the Arc projects to all levels of the ME in a diffuse manner, while a second group projects dorsoventrally to connect each portion of the Arc i.e. rostral, middle and caudal with the corresponding segment of the ME situated beneath it. Dopamine containing fibres have also been located around the endocrine cells of the pars intermedia and in the neural lobe (Bjorklund et al 1970). It is considered that the cell bodies of these fibres originate in the rostral portion of the Arc and the cells innervating the pars intermedia lie just rostral to those which impinge upon the neural lobe. The fibres are distributed extensively in the neural lobe, and form a dense network around the endocrine cells of the pars intermedia, some fibres ending as dopamine filled droplets or as a terminal swelling such as those found in peptidergic neurosecretory axons.

1.4.3. The Tuberoinfundibular Dopaminergic Tract.

The tuberoinfundibular (TIDA) neurones originate in the Arc and project to all layers of the ME and hypophysial stalk. In the external layer of the median eminence they form a dense network impinging on the precapillary spaces of the hypophyseal portal vessels. The fibres are varicose and when visualised by histofluorescent techniques (see Table 1.0) fluoresce strongly (Bjorklund, 1973). Within the internal layer of the ME a portion of the fibres are considered to be pre-terminal, en route to the posterior lobe of the pituitary gland or to deeper layers of the median eminence and run parallel to one another in the sagittal and frontal planes. The dopaminergic fibres of the TIDA pathway are arranged in close association with non - catecholaminergic axons and perikarya. On the basis of these structural observations, it is considered that dopamine released at the level of the ME, either enters the portal blood to influence the anterior pituitary gland, or acts on neighbouring axonal terminals. This latter pre - synaptic action may influence the release of hypothalamo - hypophyseal regulatory factors and thus also control anterior pituitary hormone secretion.

The entire dopaminergic input to the ME and to the posterior pituitary gland is considered to originate in the mediobasal hypothalamus (MBH) with additional noradrenergic input coming from the lower brainstem A1 and A2 cell groups as well as from the sympathetic cervical ganglion as detailed by Swanson et al (1981).

1.4.4. Additional Hypothalamic DA Neurone Cell Systems.

Additional cell groups are also found within the hypothalamus. Cell groups are present in the DMN, the area which surrounds the PeVN and also the fornix (F), the supramammillary area, the ventral surface of the hypothalamus and at the border between the medial and lateral hypothalamus.

To date the exact destinations (anatomical and neurochemical identity) of all the projections are unknown although hypothalamic DA projections are strongly believed to make local connections with other neuronal populations within the structure. However, the presence of these large numbers of DA cell groups within the hypothalamus serves to emphasise the important role played by dopamine in the regulation of hypothalamic and pituitary function. Evidence for a neuroendocrine role for DA will be considered in Section 8 of this chapter, but first the methods for establishing the dopaminergic nature of these pathways will be discussed.

1.5. Techniques used for Visualising Dopaminergic Neuronal Pathways.

The first microscopical visualization of catecholamines was within peripheral tissue of sheep and cows, using a chromaffin reaction (Falck et al 1959). With the development of the hot formaldehyde histofluorescence method the demonstration of catecholamine - containing neuronal cells was achieved (Falck et al 1962) and the technique was used subsequently to visualize amine containing neurones in the mesencephalon extending processes to the striatum (Bertler et al 1964) and to the limbic forebrain (Anden et al 1965). Since these early investigations, knowledge of the central dopaminergic systems has increased in line with the development of more sensitive and refined histochemical tracing techniques.

One of the major shortcomings of the original Falck - Hillarp technique was its lack of sensitivity which prevented the visualization of the preterminal portions of catecholamine- containing neurones. Development of the glyoxylic acid histofluorescence method, a more sensitive technique, when used in conjunction with vibratome sections of neuronal tissue, allowed the visualization of fibre networks and

their sites of termination, thus enabling scientists to begin to construct a "map" of the central dopaminergic systems.

A second shortcoming lay in the lack of specificity of the aldehyde histofluorescence methods. It was almost impossible to distinguish between the different types of catecholamine containing neuronal populations by microscopic studies, as a similar fluorescence colour was produced for all the amines. Often, distinction between noradrenergic and dopaminergic populations was made simply on the basis of differences in their morphology but such differences were not true for all catecholaminergic cells. To date, the major method for determining the exact identity of dopaminergic neurones is based to a large extent on the use of pharmacological agents such as α - methyl - ρ - tyrosine which selectively inhibits tyrosine hydroxylase, the rate - limiting enzyme in the synthesis of this catecholamine. Many of the principal dopaminergic systems of the CNS were accurately delineated by using this method. Furthermore, dopamine - ß - hydroxylase inhibitors and depletion of catecholamine stores followed by restoration of intraneuronal DA levels by administration of L- dopa, has further allowed selective visualization of the central DA - containing neurones, although at this stage the "map" remained incomplete (Bjorklund et al 1975).

The next important advancement in visualization methodology involved the introduction of retrograde tracer technology. Axonal tracers such as horseradish peroxidase or fluorescent dyes, when used in combination with histofluorescence provided a greater understanding of the organization of the DA projection systems, i.e. it was possible to identify the location of dopaminergic perikarya and the site of innervation of their processes in relation to other brain structures and autoradiography, involving the uptake of tritiated ([^{3}H]), DA provided further information concerning the topography of central dopaminergic systems. The specificity of this method was provided by the use of uptake inhibitors such as desmethylimipramine which prevented the uptake of [^{3}H]DA into noradrenergic nerve terminals while more recently the use of electrolytic or neurotoxin lesions allowing selective removal of the NA projection systems, have been used to map the cortical DA innervations, (Thierry et al 1973, Fallon et al 1978) and the DA innervations within the diencephalon (Bjorklund et al 1975).

With the purification of TH, the rate limiting enzyme of DA synthesis and the production of antibodies to it, the development of increasingly more sensitive histochemical methods has been achieved. The greater sensitivity provided by immunocytochemistry has allowed the visualization of dopaminergic perikarya

and fibres in foetal tissue which has permitted studies on the embryonic origin and foetal and neonatal development of central dopaminergic systems as discussed below.

1.6. The Development of the Dopaminergic Systems of the Hypothalamus *in vivo*.

The ontogeny of the catecholaminergic systems in the CNS as a whole has been reported in numerous studies (Golden 1973; Specht et al 1981). However, the ontogeny of the catecholaminergic neurones of the hypothalamus per se has received much less attention with most authors looking either only at discrete regions of the structure (Daikoku et al 1986) or conversely at larger portions of the CNS which incorporates the hypothalamus i.e. the diencephalon which consists of the hypothalamus and its surrounding borders (Engele et al 1989). It was a study by Ugramov et al in 1989[a+b] which was the first to characterise, in terms of neurone morphology and location, the differentiation of the catecholamine containing neurones in the hypothalamus (and septal region) during a developmental period from the 12th foetal day (E12) to the 9th postnatal day (P9) in the rat. Using TH antiserum and failing to visualize the enzyme involved in NA synthesis i.e. dopamine ß - hydroxylase, the authors concluded by elimination, that the tyrosine hydroxylase - immunopositive (THIP) neurones which were observed in their study were in fact dopaminergic rather than noradrenergic.

1.6.1. Anatomical Ontogeny.

Ugramov et al (1989a and b) reported that it is possible to detect THIP neurones in the foetal hypothalamus as early as E13 in gestation. In slices of the hypothalamus taken from rat pups, THIP neurones were visualised in the lateral region and anlages of the hypothalamus and septal region. Although initially scattered, the THIP neurones began to migrate during E14 and 15, aggregating in more dorsal regions of the hypothalamus. By E18 DA containing perikarya throughout the hypothalamus, formed dense accumulations which were partially differentiated and by P9, the cells had migrated from their site of origin to their final positions within the hypothalamus. Many THIP cell aggregations occurred forming nuclei in locations corresponding to the hypothalamic nuclei known to exist in the adult structure.

THIP neurones were detected in the region of the SCN in both the foetus and the neonate. The neurones were characteristically bipolar and lay close to the ventral portion of the third ventricle. The cells accumulated in this region prior to spreading dorsally and dorsolaterally during development up to E20, after which time they

occupied positions in the periventricular and preoptic regions. Projections from these dopaminergic perikarya extended dorsally through the subependymal region spreading across the optic chiasma and descending to the medial segment of the optic tracts.

The medial preoptic area was the location of an obvious accumulation of THIP neurones which were even more pronounced in the foetus than in the neonate. This cell cluster consisted of both bipolar and multipolar neurones which projected to the third ventricle and to the optic chiasma. The anterior hypothalamic nucleus had a dense accumulation of THIP neurones and again more were observed in the foetus than in the neonate. They projected in dorsomedial and ventrolateral directions, although the distribution only became obvious during postnatal periods of development.

Still more THIP neurones accumulated from E18 in the dorsomedial, infundibular and postinfundibular regions. Within this area, the dopamine containing cell bodies formed nuclei occupying the PVN, the ZI and dorsomedial hypothalamus, which often overlapped. By P9 these cell clusters were clearly separated and occupied distinct positions which were easily distinguishable.

Within the ventral portion of the developing hypothalamus, an accumulation of THIP neurones were demonstrable by E18 and persisted to P9. There were two notable nuclei in this region, one placed laterally in the ME and a smaller cell cluster also lying laterally. The former nucleus consisted of between 10 THIP cells on E18 and 22 on P9. They comprised both unipolar and bipolar neurones which were smaller than those found in the dorsal region of the hypothalamus. The two nuclei were connected by a chain of single THIP neurones. The nucleus located in the median eminence became displaced by P3 lying close to the Arc. By P9 this nucleus completely occupied the arcuate portion of the hypothalamus sending processes to the median eminence.

The PeVN was first recognisable as a group of THIP neurones from E20. From the arcuate preoptic level, dopaminergic perikarya spread to the retrochiasmatic level. Postnatally, the nucleus became displaced further, to settle in the periventricular area between the infundibular and anterior hypothalamic levels. Characteristically, the neurones within this nucleus were bipolar and projected to the ventrodorsal region along the third ventricle, forming an intricate network along the subependymal layer. By P9 the periventricular accumulation of THIP neurones traversed to the SCN.

The THIP neurones of the SON were only detectable after birth defined as a few bipolar THIP neurones in the lateromedial hypothalamus.

1.6.2. Morphological Ontogeny.

Throughout a 20 day developmental period, *in vivo*, incorporating the latter days of foetal life and the first 9 days of postnatal life, the THIP neurones of the hypothalamus underwent extensive morphological changes which were considered to be a result of cell differentiation. The first THIP neurones to be detected (on E13) possessed either a single thick process or two processes one which was thick and unbranched, and one which was thin. However, by E14, the thick processes underwent extensive bifurcation, giving rise to between 2 and 3 additional processes. By E18, uni- , bi- and less frequently, multi-polar neurones were identified and the latter increased in number to become the predominant type by P9. All THIP subtypes increased in perikarya size and neuronal length during foetal and neonatal development, forming neuronal connections.

1.6.3. Functional Ontogeny.

The technique of high performance liquid chromatography with electrochemical detection (HPLC-EC), has recently been employed to investigate the functional ontogeny of the hypothalamic dopaminergic neurones (Pares - Herbute, 1989; Santana et al 1992). The DA content in the diencephalon followed a biphasic developmental pattern. Levels of DA were first measurable, as early as E13 and increased dramatically by the time of birth (15-fold), after which time there was a less pronounced rise (9-fold) to adult levels by P30. The physiological significance of the high levels of DA found in the developing hypothalamus is poorly understood. In other foetal brain regions, DA is considered to exert modulatory influences on neuronal development and behaviour. For instance, DA in the midbrain and cortical areas was reported to influence neuronal outgrowth (Matteson 1988) and also induces a permanent modification of adult behaviour (Arevalo et al 1987). However, there are relatively few studies which address other aspects of dopaminergic function and investigations into the release of DA from foetal hypothalamic dopaminergic systems and the factors which influence it, are yet to be performed.

1.7. Dopaminergic Neuronal Function.

The recognition of DA as a genuine neurotransmitter followed the isolation of neurones which contained TH but were deficient in dopamine - ß - hydroxylase. Such neurones were unable to synthesise NA/Adr, and DA was proposed to be the end

product of their synthetic pathway. In later studies, DA was found to be located in high concentrations within nerve terminals and the identification of receptors displaying specificity for DA, further substantiated a neurotransmitter role for the amine (Vogt 1973).

1.7.1 Synthesis and Metabolism.

The enzymatic pathway involved in the synthesis and metabolism of DA is illustrated in Fig 1.2. The conversion of tyrosine to 3, 4 - dihdroxyphenylalanine (dopa), serves as the rate-limiting step in DA synthesis, presumably regulating the intraneuronal concentration of the amine. The metabolism of DA is restricted to the cytoplasmic pool of the transmitter,which may represent the storage site for newly synthesised readily releasable pools of DA while the vesicular bound storage site offers protection from intraneuronal enzymatic degradation.

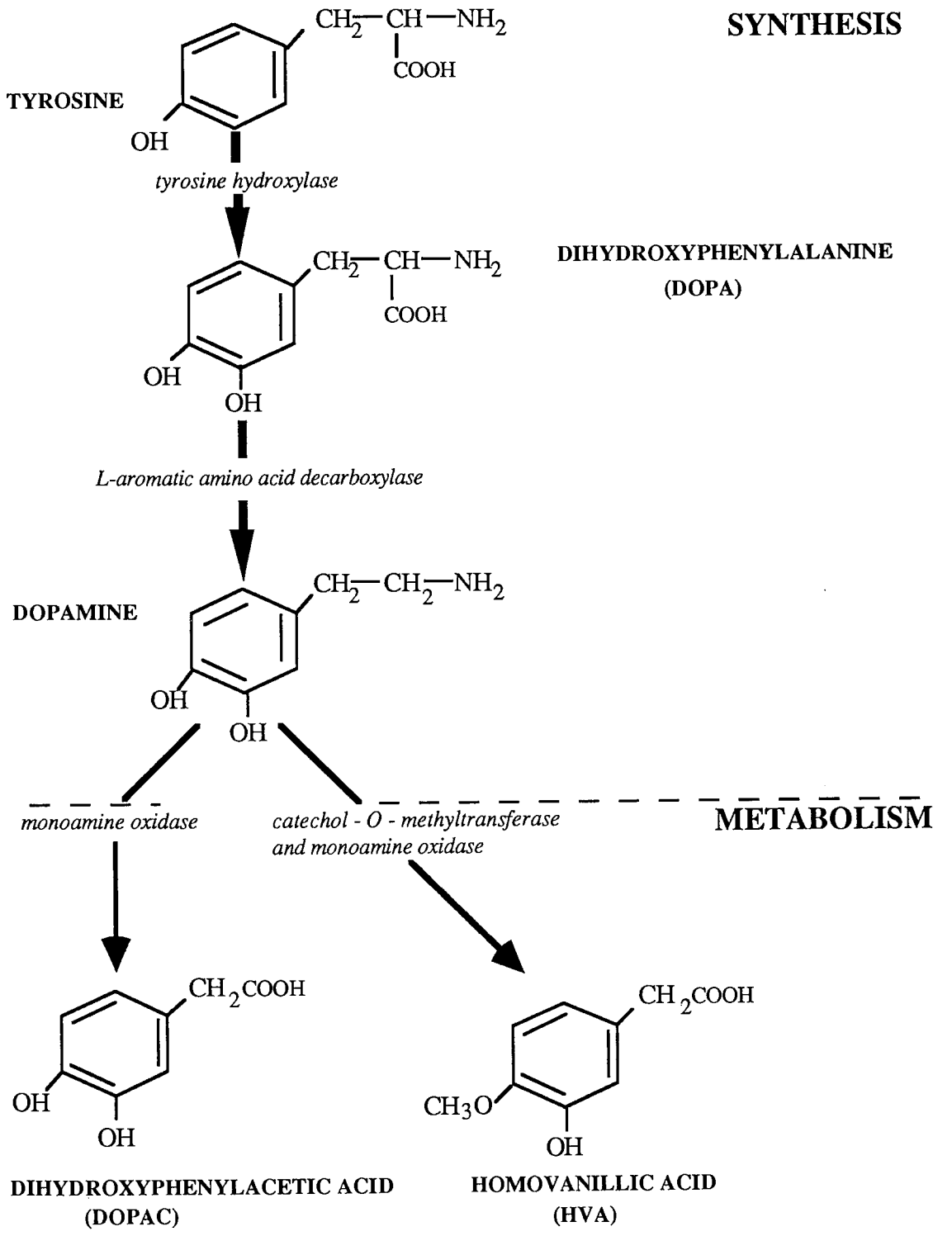
1.7.2 DA Uptake.

Termination of dopaminergic activity is achieved by a combination of re-uptake and enzymatic degradation. The uptake of catecholamines was first perceived in 1932, when it was suggested that NA might be taken up by neuronal and non-neuronal tissue. Small doses of tritiated catecholamines were found to be rapidly removed from the circulation following injection (Axelrod et al 1959; Whitby et al 1961) and the radiolabelled neurotransmitter was subsequently located, in concentrated amounts, in various peripheral tissues which received sympathetic innervation. From such observations, it was postulated that the amines were most likely to accumulate in sympathetic neurones. This theory was substantiated by the observation that physical or chemical destruction of sympathetic neurones totally abolished the uptake of NA into peripheral organs (Iversen et al 1975). Autoradiographic and histochemical studies provided more evidence for this hypothesis and it is now estimated that between 70-80% of released NA may be recaptured under normal conditions. Uptake is now generally considered to be a major mechanism for the termination of activity of aminergic neurotransmitters such as NA, Adr, 5-HT and DA, both centrally and peripherally.

The uptake of DA is inhibited by ouabain an inhibitor of the Na^+/K^+ ATPase pump, suggesting that the uptake mechanism is dependent on the transport of ions across the cell membrane and more specifically the transport of Na^+. The existence of a DA transporter complex was proposed which acts as a mobile carrier with a DA binding site that shuttles from one side of the membrane to the other, transporting molecules of DA and Na^+ with it. The co - transport system is not unique to DA and high affinity uptake mechanisms displaying selectivity for other neurotransmitters and

neurotransmitter candidates such as NA, 5-HT, Glu, Asp, GABA, Gly, TAU, Pro and adenosine are also present in the CNS (Amara 1993).

Fig 1.2. Synthesis and Metabolism of DA.



Within the last few years molecular biological techniques have provided us with further information concerning the transporter molecule. Usdin et al (1991), isolated the cDNA clones encoding the rat and bovine DA transporter as did Shimada et al (1991). Degeneration oligonucleotides corresponding to regions of high sequence identity between NA and GABA transporter molecules served as probes or were the basis of polymerase chain reactions (PCR) to generate probes for screening DNA libraries. The common structural motifs reflect the important generic functions of the protein such as Na^+/Cl^- co - transport which is a vital function to all members of the co-transporter family. The specificity of each transporter, in relation to its affinity to ligands and inhibitors, is provided by the regions of divergence where amino acid sequences between the co-transporters differ.

The DA transporter protein is a potential site for drug action. Inhibitors of the DA uptake mechanism i.e. nomifensine, benztropine, diclofensine, cocaine and manzidol appear to interact with specific membrane sites in a stereospecific and highly selective manner and either inhibit binding of the substrate or prevent translocation of the transporter protein across the membrane. This is considered to be the main mechanism of action of certain anti-depressant drugs which, by increasing the synaptic concentration of DA, augment synaptic transmission. In addition, cocaine and other drugs of abuse act on the transporter complex to mediate their psychotic effects (Ritz et al 1989; Trendelburg et al 1991). All uptake inhibitors have physiological effects which correlate strongly with their ability to block DA uptake and thus they continue to be the subject of much interest to the drug industry as potential therapeutic agents.

1.7.3 DA Release.

The release of DA in rat brain has been studied using *in vitro* preparations such as brain slices (Reisine et al 1982), and synaptosomes (Gregerson & Salmanoff, 1987) and brain homogenates (Plantje et al 1982) either pre-loaded with radiolabelled DA or precursors of DA synthesis (Baldessarini et al 1975). In addition, the release of endogenous DA from brain slices has been investigated by use of radioenzymatic assays (Kant & Meyerhoff 1978) and HPLC-ECD (Sharp et al 1986). The release of DA *in vivo* has also been studied, using the push-pull perfusion technique in both anaesthetised (Gonon et al 1980) and freely moving (Chen et al 1984) animals. A combination of brain dialysis with HPLC-ECD has also been employed to measure extracellular levels of DA and its metabolites and also to monitor drug-induced effects on transmitter release and metabolism (Zetterstrom et al 1983).

It is generally agreed that the release of DA, like that of most transmitters, is dependent on the entry of Ca^{2+}, through voltage dependent Ca^{2+} channels, into the nerve terminals (recent reviews, Szerb et al 1981; Zucker & Lando 1986). The channels are activated by the arrival of a depolarising current which elevates the potential of the presynaptic membrane, a phenomenon known as *depolarization.* The resulting channel opening allows the influx of Ca^{2+}, which promotes a transient fusion of neurotransmitter-packed vesicles with the presynaptic membrane. DA is thus released into the synaptic cleft, which it traverses to elicit a postsynaptic effect on adjacent neurones. Depolarization of the membrane by electrical stimulation or elevation of the extraneuronal concentration of K^+ also induces DA release, the magnitude of release usually being greater than during spontaneous efflux.

From the results of certain investigations of the activity of dopaminergic systems, it appears that DA may be stored in and released from dendritic nerve terminals as well as axonal nerve terminals (Nieoullon et al 1977). Dendritic release of DA may provide tonic inhibition of dopaminergic neurones themselves, with the firing rate of dopaminergic cells being inhibited by the activation of DA receptors on their cell bodies. Furthermore, the synthesis of DA is reported to be inhibited by the activation of DA receptors found either on the dopaminergic cell soma or on the dendrites (Demarest et al 1979b), whilst release is inhibited by activation of DA receptors on dopaminergic nerve terminals (Arbilla et al 1981).

1.7.4 DA Receptor Subtypes.

Spano et al (1978) first proposed a classification for DA receptors based upon their linkage to adenylate cyclase. Activation of the D_1 receptor caused stimulation of adenylate cyclase, whereas D_2 activation either inhibited or was not related to adenylate cyclase activity. Both receptors exist in high and low affinity states with K_d values for DA of 0.8 and 400 nM respectively for D_1 and 8nM and 4, 000nM respectively for D_2 receptors. Pharmacological and molecular biological evidence however, suggests the existence of other D_1 and D_2 - like receptors sub-types and a list of these receptors, their distribution and their respective ligands, may be found in Table 2.0. It is the D_2 receptor, in a high affinity state, which mediates the inhibition of prolactin from the anterior pituitary gland, located on lactotrophs (see below). Furthermore the D_2 receptors located within the mediobasal hypothalamus and the neurointermediate lobe of the pituitary are believed to modulate DA release from TIDA and THDA neurones (Plantje et al 1987; Sarkar et al 1983).

Table 2.0. DA receptors: classification and subtypes.

Receptor Subtype	High Affinity Ligands	Low Affinity Ligands	2nd Messenger Coupling	Site of Distribution
D_1/ D_{1A}	Benzazepine - type drugs e.g. SKF 38393, SCH 23390	Butryophenone - type drugs e.g. haloperidol	Stimulation of adenylate cyclase	Caudate putamen, nucleus accumbens, olfactory tuberculum, cerebral cortex, limbic systems, hypothalamus and thalamus.
$D_{1B/5}$	As for D_1	As for D_1	As for D_1	Mamillary nuclei, parafasicular nuclei of the thalamus and the hippocampus.
$D_{2S/2L}$	butryophenones	benzazepines	Inhibition of adenylate cyclase, activation of K^+ channels.	A9 and A10 regions i.e. substantia nigra and ventral temental area, the mediobasal hypothalamus, the anterior and neurointermediate lobes of the pituitary gland.
D_3	benzamides, e.g. raclopride		Not well defined	Olfactory tubercle, nucleus accumbens, substantia nigra and ventral tegmental area and the hypothalamus.
D_4	clozapine (atypical neuroleptic)	all types of DA antagonists except clozapine.	Not well defined	Frontal cortex, amygdala, medulla and midbrain regions.

1.8. DA and Neuroendocrine Control.

1.8.1 Prolactin.

If the normal connections between the hypothalamus and the pituitary gland are disrupted, whether by lesions to the hypothalamus, sectioning of the pituitary stalk, or *in vitro* incubation of pituitary tissue, there is a significant rise in the release of prolactin, while the release of other pituitary hormones e.g. luteinizing hormone, declines. From such observations it was postulated that the brain, and more specifically, the hypothalamus was the site of synthesis and storage of a prolactin-inhibitory factor (PIF), exerting a tonic inhibition on prolactin release at the level of the pituitary.

It was initially assumed that the PIF, like other hypothalamic regulatory factors identified at that time, was a neuropeptide, but investigations directed at isolating a peptidergic PIF were unfruitful. However, the high concentration of DA located in the mediobasal hypothalamus led scientists to investigate its potential role as a neuroendocrine regulator. It was demonstrated that high concentrations of DA were present in portal blood and that such concentrations of DA were sufficient to inhibit prolactin release from lactotrophs *in vitro,*(MacLeod et al 1970; Shaar & Clemens 1974; Ben-Jonathon et al 1977, Gibbs & Niell 1978). Further evidence was subsequently provided establishing DA as major physiological PIF and, to date, its role has not been successfully challenged. However, the regulation of prolactin release is not determined by the action of a sole factor as the alterations in plasma prolactin levels which are observed during changes in endocrine status, e.g. pregnancy, suckling and the oestrous cycle, could not reasonably be attributed to the activity of a single neurohormone. Gamma-amino butyric acid (GABA), gonadotrophin associated peptide (GAP) and α - MSH have also been implicated in the inhibition of prolactin release. In addition, a whole range of prolactin releasing factors including 5-HT, vasointestinal peptide (VIP), thyrotrophin releasing hormone (TRH) and ß - endorphin, have more recently been reported (Leong et al 1983).

Both the TIDA and THDA neuronal systems are considered to be directly involved in the regulation of prolactin release. A detailed description of these pathways may be found in Sections 1.4.2, 1.4.3 and Table 1.0. It is considered that much of the DA released from the TIDA and THDA hypothalamic systems serves as a neuromodulator rather than as a neurotransmitter. Thus, once released from the dopaminergic nerve terminals at the level of the ME, DA enters the hypophyseal portal system and is transported to the pituitary gland. From here it acts on D_2 receptors located on the lactotrophs of the anterior pituitary gland as shown by

immunocytochemical investigations (DeLean 1982). There is a strong correlation between the potency of D_2 receptor agonists and their effectiveness in suppressing prolactin release. The actual mechanism by which D_2 receptor activation results in suppression of prolactin release is, to date, poorly defined although several theories have been suggested (Barns et al 1978; Gudelesky et al 1980; Heiman & Ben - Jonathon 1982; De La Escalera & Weiner 1988; De La Escalera et al 1988, Murai et al 1989a and 1989b; Mogg & Samson 1990).

Regulation of the levels of DA reaching the anterior pituitary gland is mediated partly by the state of vasoconstriction in the various vascular beds, which determines the blood flow in the portal vessels. Blood flow may be shunted in various directions and such versatility of flow is a vital feature for the necessary exchange of hormonal "signals" between the hypothalamus and pituitary. Furthermore, the activity of the DA nerve terminals at the level of the median eminence may be regulated by pituitary factors, such as prolactin, transported back to the hypothalamus by retrograde blood flow.

A reciprocal relationship exists between the portal blood DA concentration and the plasma prolactin levels during the rat oestrous cycle (de Greef et al 1984), but there are certain instances where the correlation is not maintained. For example, both the peripheral levels of prolactin and the concentration of DA in the portal circulation is higher in female rats that in males and there is no detectable DA in the portal blood of conscious sheep as shown by Thomas et al (1989) suggesting that a direct relationship between portal DA and plasma prolactin is an oversimplification. There are a number of reasons why the direct correlation fails. DA released from the median eminence regulates the release of a number of pituitary hormones, not only prolactin and thus, the level of DA in the portal blood is not influencing plasma prolactin levels alone but the concentration of other plasma hormones as well. Furthermore, as previously mentioned, there are a whole range of PIFs and PRFs, which act in concert to regulate prolactin release, and hence portal blood DA levels alone do not determine plasma prolactin levels. Moreover, the number of lactotrophs which actually express prolactin at any given time will also play a key role in determining the levels of the circulating hormone.

Certain reports state that only between 15-20% of the DA synthesised and transported to the median eminence is actually released into the portal blood (Reymond & Porter 1982). This suggests that the TIDA and THDA tracts are not exclusively involved in the regulation of prolactin secretion and that DA at the level of the median eminence is likely to regulate the release of a number of other hypothalamic and pituitary hormones.

1.8.2. Gonadotrophin - Hormone - Releasing Hormone.

The hypothalamic decapeptide, gonadotrophin-hormone-releasing-hormone, (GnRH), is the major hypothalamic regulator of gonadotrophin secretion from the anterior pituitary gland. Much is known concerning the regulation of the surge in plasma LH levels which is a prerequisite to ovulation, but the neural factors which regulate GnRH release at the level of the hypothalamus are complex and not fully understood.

At present DA is generally believed to inhibit GnRH release and the TIDA neuronal system has been implicated. Histochemical evidence reveals that GnRH and DA nerve terminal networks in the external layer of the median eminence extensively overlap and possibly form synaptic connections (Simerly et al 1987). Furthermore, the DA agonist, apomorphine, reduces plasma LH levels in addition to abolishing the oscillatory release of LH observed in normal cycling rats and selective 6-OHDA lesioning of dopaminergic neurones potentiates LH release (Sarkar et al 1981). Thus, DA released from the TIDA nerve terminals appears to exert an inhibitory influence on GnRH especially at the level of the median eminence, preventing its release into the portal circulation and its subsequent stimulatory effect on gonadotrophin secretion. The fall in DA turnover in the medial pre-optic area observed around the time of the preovulatory LH surge suggests the removal of the inhibitory tone on GnRH allowing its secretion.

DA has also been reported to stimulate GnRH secretion (James et al 1987) as a positive correlation exists between LH levels and hypothalamic DA turnover in the mPOA of rats following steroid priming. This observation implies an involvement of the neurotransmitter in the positive feedback of sex steroids on LH secretion. The stimulatory influence of DA on GnRH release is believed to be exerted by the ICHDA neuronal systems as the activity of dopaminergic neurones at the level of the ZI fluctuates throughout the course of the oestrous cycle in rats (MacKenzie et al 1988) with turnover rising over the second day of dioestrous and the morning of proestrous, being optimal on the evening of that day. This rise coincides with the preovulatory LH surge and there is a strong correlation between the rise in DA levels and the rise in plasma oestradiol levels noted at that time in the cycle. Furthermore, ablation of the ICHDA with the neurotoxin 6 - OHDA induces constant dioestrous in normal cycling rats (MacKenzie et al 1988). It has, however, been reported that differential effects of DA on GnRH release may be demonstrated by modulating the activity of different DA receptor subtypes (Sarkar & Fink, 1981) and this may account for the apparent contradictory role ascribed to the neurotransmitter as found in the literature.

It is also likely that other neurotransmitter/neuropeptide systems are involved in controlling GnRH release such as GABA (see below). Furthermore, the role of the peripheral sex steroids in the regulation of gonadotrophin release is almost certainly directed

albeit not exclusively, at hypothalamic level and several neurotransmitter systems within the hypothalamus are steroid responsive adding yet another factor to the complex regulatory pathway for GnRH secretion.

1.8.3. Growth Hormone.

A number of reports have linked DA with the regulation of growth hormone (GH) release (MacLeod et al 1970, Meister et al 1985). If given i.p. DA, which is unable to cross the blood brain barrier, has no effect on circulating levels of GH, however if given i.c.v., levels of GH in the plasma are elevated. Furthermore, DA agonists, such as apomorphine, which does have central actions, increase circulating levels of GH by either i.p. or i.c.v. routes. Hence the action of DA in regulating GH release is considered to be mediated via a central mechanism.

1.8.4. Somatostatin.

Somatostatin (SRIH) is found in several regions of the hypothalamus as well as elsewhere in the CNS and the periphery. It serves both as a neurotransmitter and neuromodulator and apart from its well established role as an inhibitor of GH release (Brownstein et al 1975) it also inhibits the release of thyrotropin and prolactin (Enjalbert et al 1982) secretion from the anterior pituitary gland. A significant fraction of SRIH nerve terminals within the hypothalamus project to the median eminence and secrete the neuropeptide into the portal circulation and it is this population of SRIH secreting cells which is believed to be influenced by DA.

DA has been ascribed both an inhibitory (Bennet et al 1979) and stimulatory (Negro-Vilar 1978) role in the regulation of SRIH. DA administration was found to stimulate SRIH secretion and cause an increase in the concentration of SRIH in the hypophysial portal blood (Chiara et al 1979) which accords with its reported stimulatory effects on GH secretion. However, DA exerts a stimulatory role on both GHRH and SRIH secretion in a perfusion system of isolated hypothalamic slices (Kitajima et al 1989). The effect on SRIH exerted by this amine may be restricted to a distinct population of SRIH containing neurones within the hypothalamus but it could also act at SRIH nerve terminals rather than cell bodies, as DA stimulates the release of the neuropeptide from ME, but not from mediobasal hypothalamic fragments (Negro - Vilar et al 1979; Maeda et al 1980; Lengyel et al 1985).

As with prolactin, DA is considered to regulate SRIH release via a D_2 receptor mediated process. The D_2 agonists, bromocriptine and LY 171555, increase hypophysial levels of SRIH, an effect which is reversed by D_2 antagonists and a

report suggests that D_2 agonists increase levels of pre-pro mRNA for SRIH (Zorrilla et al 1990).

1.8.5. Other Neuropeptides.

DA has also been implicated in the regulation of other neuropeptides including vasopressin and oxytocin (Lightman et al 1982, Moos & Richard 1982) and corticotrophin-releasing hormone (Fehm et al 1980), although to date, much of the evidence proposed is contradictory and the exact nature of the amines' involvement of their regulation is yet to be clearly defined.

1.8.6. Interactions with Hypothalamic GABA - ergic Systems.

GABA is the major inhibitory neurotransmitter of the CNS and, like DA, is found in high concentrations within the hypothalamus, the highest levels being observed in the preoptic, anterior and dorsomedial nuclei (Tappaz et al 1986).

Within the hypothalamus, GABA and DA neurones interact both anatomically and functionally. Immunocytochemical studies (Oertel et al 1982) show that neurones which are immunoreactive for GAD and TH lie in close proximity within the region of the ZI. GABA-ergic terminals within this area impinge on dopaminergic neurones and also on other GABA-ergic neurones in addition to neurones considered to contain neuropeptides (Leranth et al 1985). Other studies using electron microscopy and ICC to visualize GAD and TH (Horvath et al 1993), provide ultrastructural evidence that GAD-immunoreactive terminals form synaptic connections with TH-immunoreactive neurones in the Arc and evidence exists for the co-localization of the two neurotransmitters within this nucleus, from which both the tuberoinfundibular dopaminergic and GABA-ergic systems are known to arise. Furthermore, electron microscopic studies indicate the presence of axoaxonic and dendrodendritic synapses in the Arc, the PeV zone and within the ME between GABA and DA neurones. Measurements of GABA levels, DA turnover rates and the effects of administered $GABA_A$ agonists such as muscimol, suggest that GABA may exert an inhibitory influence on DA release and especially the activity of TIDA neurones. In addition, GABA is considered to influence dopaminergic activity in a number of other brain regions, including the vertebrate retina (Wulle & Wagner 1990) and the substantia nigra (Smith & Bolam 1990; Lacey et al 1988).

Evidence also exists for a functional interaction between these two systems in terms of their regulatory influence on neuropeptide release. For instance, ICC studies indicate synaptic connections between GABA, GnRH and DA containing neurones

both in the preoptic area and the Arc/ME complex (Van den Pol 1986; Sakaeu et al 1988). A technique combining anterograde tracing coupled with acute neurodegeneration of DA neurones with 6 -hydroxy-dopamine, demonstrate that DA containing cells originating in the ZI terminate on both GnRH and GABA immunoreactive neurones. All three neurochemicals are considered to act in concert to control the episodic release and the pre-ovulatory surge of LH (Fuchs et al, 1984) in a complex manner which is not fully defined. DA and GABA exert opposing effects on GnRH release performing contrasting roles at the same hypothalamic site. GABA inhibits GnRH at the levels of the MPOA (Demling et al 1985), while DA, in contrast, stimulates GnRH secretion in this area (MacKenzie et al 1988). However, in other regions of the hypothalamus, including the Arc/ME complex, GABA stimulates GnRH release while DA exerts an inhibitory influence (McCann et al 1986; Jarry et al 1990). Thus, the two neurotransmitters act either in concert or in opposition to directly or indirectly regulate GnRH secretion and regulate circulating levels of LH. As both neurotransmitter systems are influenced by the activity of gonadal steroids, it is likely that they are also involved in the steroid feedback processes occurring at the level of the hypothalamus, as discussed below.

1.8.7. Interaction with Gonadal Steroids.

Considerable evidence suggests that gonadal steroids influence the release of hypothalamic regulatory peptides. For instance, the regulation of SRIH and GnRH is influenced by the prevailing levels of gonadal steroids which differ between the sexes and during the oestrous cycle. However, oestrogen receptors are not found either in GnRH neurones (Shivers et al 1983) or on the SRIH containing neurones within the PeVN (Herbison et al 1993) and this suggests the existence of an additional, steroid responsive, intermediary neuronal pathway. Hypothalamic dopaminergic neurones have been reported to be directly oestrogen responsive (see below) and DA is thus a likely candidate for these steroid actions.

The activity of the TIDA systems is regulated by circulating levels of oestrogens as well as by prolactin. Oestrogen has been reported to have both stimulatory (Gunnet et al 1986) and inhibitory (Arita et al 1989, 1990; Tong & Pelletier 1991; Pasqualini et al 1993) effects on the activity of the TIDA neuronal systems as assessed by changes in the rate of DA turnover. Furthermore, autoradiographical studies linked with ICC have shown that this population of DA containing neurones accumulate [^{3}H] oestradiol (Sar, 1984). Nevertheless, recent research suggests that oestrogen's role in modulating the activity of TIDA neurones may be mediated via a pituitary factor. Hypophysectomy significantly attenuates the stimulatory effect of E_2 on dopaminergic activity (Eikenburg et al 1987) and *in vivo* and *in vitro* studies have

implicated prolactin as a likely mediator of these actions of E_2 (Labrie et al 1980; Lookingland & Moore 1984a, Gunnet et al 1986). Alternatively, an as yet uncharacterized cytotropic factor isolated from the pituitary gland has been shown to increase tyrosine hydroxylase expression in cultured brain cells, (Gonzalez et al 1989; Porter et al 1990) and may also possibly mediate the modulatory effect of oestrogen on TIDA activity.

A number of reports have also documented a modulatory effect on ICHDA turnover by oestrogens both *in vivo* (Gunnet et al 1986) and *in vitro* (Sanghera et al 1991) and the DA containing neurones within this tract also accumulate [^{3}H] oestradiol (Sar 1984). In addition, the rostral and caudal portions of the ICHDA tract have been demonstrated to be differentially but directly modulated by oestrogens (Gunnet et al 1986; Lookingland and Moore 1984b), although to date, the consequence of this on neuroendocrine function is poorly understood.

In the studies to be reported in this thesis we have aimed to investigate both the morphological and functional development of the intrahypothalamic dopaminergic systems, employing a primary cell culture model. Previous studies within this laboratory have shown that this experimental preparation is useful for the investigation of hypothalamic neuropeptide systems both in terms of their development (Davidson & Gillies 1993) and their function (Gillies & Davidson 1992). The culture model is advantageous in that it allows the observation of changes in dopaminergic activity occuring at the perinatal period, a time at which many of the homeostatic and behavioural patterns expressed in the adult are established. Furthermore, accurately controlling the environment to which the cultures are exposed, may allow us to elucidate the role played by a range of physiological factors on the behaviour of these dopaminergic neuronal populations. As DA neurones are so prevalent in the hypothalamus and have been ascribed a number of regulatory roles in the control of hypothalamo - hypophyseal hormone secretion, we believe that an investigation into the ontogeny of these pathways, the identity of substances which modulate their function and the relationship existing between DA and other hypothalamic factors, will enhance our understanding of the importance of this neurotransmitter to both the developing and the adult neuroendocrine system.

CHAPTER TWO: METHODOLOGY

2.1. Initiation of Primary Hypothalamic Cell Cultures.

In the present study we have employed a primary cell culture model to investigate the morphological and functional ontogeny of the dopaminergic neurones intrinsic to the hypothalamus, as well as their interactions with other hypothalamic neurotransmitter and neuropeptide systems and gonadal steroids.

2.1.1. Dissection.

Female Wistar rats, which were timed 18 days pregnant, were anaesthetised under diethyl ether and the uterus was removed, aseptically, by Caesarean section, into a 10 ml sterile petri dish containing saline, supplemented with penicillin (100IU/ml) and streptomycin (0.1mg/ml). The foetuses were excised using sterile dissection scissors and forceps which were heat sterilized prior to use. The pups were decapitated and the heads placed on filter paper soaked in saline. Curved forceps were used to hold the foetal head in position while an incision was made from the base of the skull to the tip of the snout with iridectomy scissors, exposing the dorsal surface of the brain. Curved forceps were placed under the frontal lobe and the brain was inverted to expose its ventral side. The hypothalamus of the foetal brain is a prominent structure, 2-3mm across, and using the curved forceps, the hypothalamus was removed as a "pinch" and transferred to collection buffer (Table 3.0). The foetal hypothalamic tissue gathered from a number of pregnant rats (usually 10) was pooled for each set of investigations.

Table. 3.0. Collection Buffer. The following constituents were added to 100mls of Hanks Balanced Salt Solution (Ca^{2+}/Mg^{2+} free).

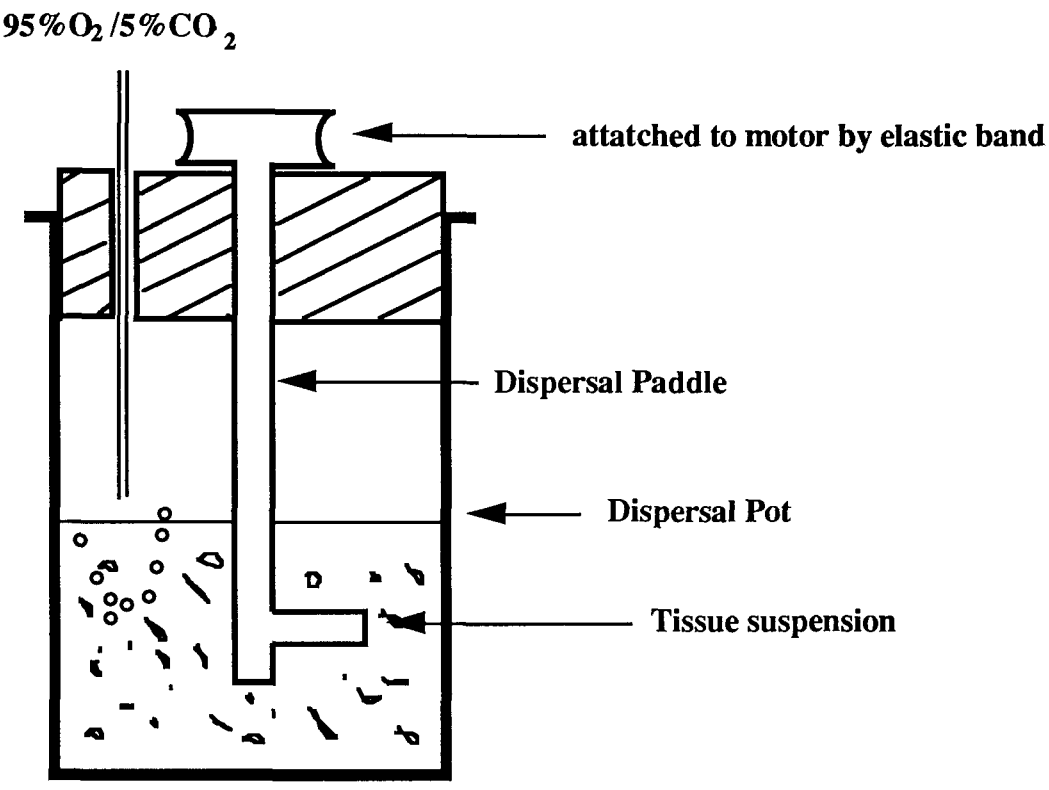
CONSTITUENT	CONCENTRATION
PENICILLIN	100IU/ml
STREPTOMYCIN	0.1mg/ml
BOVINE SERUM ALBUMIN	0.4% $^{w}/_{v}$
HEPES	4.8mg/ml

2.1.2. Cell Dispersal.

The cells were dissociated using a combination of mechanical agitation and enzymatic disruption of the hypothalamic tissue as described by Clarke et al (1987). Essentially, hypothalamic tissue was pooled (approximately 100 hypothalami per experiment) and suspended in collection buffer in a 30ml universal container. Gentle trituration was performed using a fire - polished, siliconized pasteur pipette with an internal diameter of approximately 1.5mm. The tips of the pasteur pipettes were "flamed" using a bunsen burner to produce pipettes with varying bore sizes. As the dissociation procedure proceeded, pipettes with progressively smaller internal diameters (< 0.5mm), were used to aid the dispersal process. After the first trituration the tissue fragments were collected by centrifugation (using a bench top centrifuge) at 900 r.p.m. for 3 min. The supernatant was discarded, and the pellet re-suspended in the enzyme solution (Table 4.0) before transferal to the dispersal pot (Fig 2.1) which was placed in a water bath at 37°C. The dispersal paddle was attached to an electric motor and gently rotated for 30 min to aid cell dispersal. After the initial incubation the large tissue fragments were allowed to settle and the cell suspension was decanted to a universal container. The larger fragments were re-suspended in 2-3mls of enzyme solution and gently triturated using a pasteur pipette with a smaller bore size (< 1.0mm internal diameter).

A further 18mls of enzyme solution was then added to the dispersal pot which was returned to the water bath for a further 15 min incubation. After a final trituration virtually no tissue fragments remained and the cell suspensions derived from both the 15 and 30 min incubations were centrifuged at 1000 r.p.m. for 10 min. The supernatant of each was discarded and the pellets were re-suspended and pooled in 2 mls DNA -ase solution (Table 5.0) using minimal trituration. After the addition of a further 18mls of DNA- ase solution, the cell suspension was allowed to stand for 15 min at 37°C. Following a further pelleting the cells were re-suspended in a small volume of collection buffer, triturated a final time and layered onto 20 mls of debris removal buffer (Table 6.0). Centrifugation was again performed at 1000 r.p.m. for 10 min and the resulting pellet was re-suspended in defined medium (Table 7.0). After checking the cell yield, the final volume was adjusted to give a plating density which was consistent for all batches of cultures (see below).

Fig. 2.1. Apparatus for the dispersal of hypothalamic cells.



2.1.3. Plating and Maintenance.

This method of culture preparation consistently produced a yield of 0.7 - 0.8 x 10^6 cells per hypothalamus with a viability in excess of 90% (Clarke et al 1987). The final cell suspension was, if necessary, adjusted to the equivalent of 3 hypothalami per ml of defined medium and in all instances the cells were plated at an equal density. Depending on the nature of the experiment (see later) 1ml of cell suspension was plated in each 35mm well or 180µl per 15mm well to achieve equal plating density, i.e 2.5 x 10^3 cells per mm^2. Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2. The incubation medium was first changed on day five (unless an experiment was performed on day 3) and thereafter on alternate days throughout the experimental period with the day of initiation of the cultures being designated as day 0. Under these conditions the cells remained viable for several weeks (Clarke & Gillies 1988).

Table. 4.0. Enzyme solution. The following constituents were added to 100mls of Hanks Balanced Salt Solution (Ca^{2+}/Mg^{2+} free).

CONSTITUENT	CONCENTRATION
PENICILLIN	100IU/mL
STREPTOMYCIN	0.1mg/ml
BOVINE SERUM ALBUMIN	0.4% $^{W}/_{V}$
HEPES	4.8mg/ml

Dispase, (0.2mg/ml) and Deoxyribonuclease I (DNA-ase I, 0.5mg/ml) was added to 40mls of the above solution and filter sterilized to give the final enzyme solution used for cell dispersal.

Table. 5.0. DNA -ase Solution. The following constituents were added to 100mls of Hanks Balanced Salt Solution

CONSTITUENT	CONCENTRATION
PENICILLIN	100IU/ml
STREPTOMYCIN	0.1mg/ml
BOVINE SERUM ALBUMIN	0.4 % $^{W}/_{V}$
HEPES	4.8mg/ml

Deoxyribonuclease - I (0.5mg/ml) was added to 20mls of the above and filter sterilized to give the final DNA -ase solution used for cell dispersal.

Table. 6.0. Debris Removal Buffer. The following constituents were added to 20mls of Collection Buffer.

CONSTITUENT	CONCENTRATION
BOVINE SERUM ALBUMIN	0.8 % $^{W}/_{V}$

Table 7.0. Constituents of the defined medium (DM). The following constituents were added to a 1:1 mixture (final volume, 100mls) of Dulbeccos Modified Eagles Medium (DMEM) and Ham's F12.

CONSTITUENTS	FINAL CONCENTRATION
PENICILLIN	100 IU/ml
STREPTOMYCIN	100µg/ml
HUMAN TRANSFERRIN	100µg/ml
FUNGIZONE	0.5µg/ml
INSULIN	5µg/ml
PUTRESCINE	0.1mM
SODIUM SELENITE	30nM
17ß - OESTRADIOL *	1.0pM
PROGESTERONE *	20nM
TRI-IODOTHYRONINE *	1.0nM

Sterile aliquots of the supplements were stored at -20°C. The medium was pre-warmed to 37°C prior to use. * Stock solutions of these constituents were dissolved and stored in ethanol.

2.1.4. Preparation of Tissue Culture Plastics.

Poly - L - Lysine (10µg/ml in sterile water) was added to the tissue culture wells (15 or 35mm in diameter, as stated), for 30 min. After rinsing three times with sterile distilled water, the wells were incubated in a medium containing 10% foetal calf serum (FCS Table 8.0) for at least one hour and then removed before seeding of the cells. These procedures improved the attachment of the cells to the plates and provided a suitable matrix on which the cells could establish themselves and grow.

Table. 8.0 Coating medium. The following constituents were added to 50mls of Dulbecco's Modified Eagles Medium.

CONSTITUENT	CONCENTRATION
PENICILLIN	100IU/ml
STREPTOMYCIN	0.1mg/ml
FUNGIZONE	0.5µg/ml
FOETAL CALF SERUM	5mls
HORSE SERUM	5mls

2.1.5. Preparation of Glass Coverslips.

For the immunocytochemical studies the cells were grown on 15mm coverslips placed in multiwells so that they might be easily manipulated through subsequent procedures. The coverslips were first washed in a mild antibacterial detergent and rinsed thoroughly. Following this, they were rinsed in high grade methanol and placed individually on fluff - free gauze sheets to dry. Sterilisation of the coverslips was performed by placing them (typically in groups of 30), in glass bottles, sealed with aluminium foil and placed in an hot oven at 180°C for three hours. Prior to use, the coverslips were soaked overnight in a sterile solution of poly -L- lysine (100µg/ml) then rinsed extensively in the sterile atmosphere of the microbiological safety cabinet, in sterile water. The coverslips were propped vertically against the side of the multiwells and allowed to dry and then laid flat on the bottom of the well and left in the coating medium for 1 hour prior to cell seeding.

2.2. Immunocytochemical Studies.

Immunocytochemistry (ICC) was employed in the present study to visualize specific neuronal systems within the hypothalamic preparation. Both dopaminergic and GABA-ergic systems were visualized using ICC according to the following procedures.

2.2.1. ICC for Tyrosine Hydroxylase. Fluorescence Method.

Indirect fluorescent immunocytochemical studies were performed on hypothalamic cells grown on glass coverslips as outlined above (Section 2.1.5) and all stages prior to mounting the cells were carried out in the multiwells. Cells were initially washed three times with a 400µl volume of Earles' Balanced Salt Solution (for constituents see Table 9.0), prior to a 30 min fixation period in a modified Zamboni fixative (a 4% w/v paraformaldehye, 1% (v/v) picric acid solution in phosphate buffered saline, pH 7.6). The cells were incubated overnight, at 4°C, with an anti-tyrosine hydroxylase (aTH) mouse monoclonal antibody (ascites fluid). The specificity of the antibody was confirmed by immunoprecipitation of the phosphorylated enzyme (Semenenko et al 1986). The antibody has been reported to react with TH immunoreactive sites in the known catecholaminergic neuronal systems of the rat brain, visualized using fluorescence and PAP/DAB immunocytochemistry (Semenenko et al 1986; MacMillan et al 1986; Melander et al 1986). Following three sequential washes in phosphate bufferered saline (PBS) containing 2% Triton - X - 100 (PBS + TX, pH 7.6), to remove excess primary antibody, the cells were incubated in a 10% solution of normal goat serum (NG_oS), made up in PBS, for 30 min, which provided a blocking stage reducing non-specific signal. The antigen was then visualized by exposing the cells (for 90 min) to a Lissamine - rhodamine - conjugated second antibody directed against mouse IgG and diluted in PBS containing 2% NG_oS. Thereafter the processed coverslips were mounted on ethanol washed glass microscope slides in a commercially available mountant (Citifluor) and the tyrosine hydroxylase immunopositive (THIP) neurones were viewed by fluorescent microscopy. Controls involved the omission of the primary antibody. Optimisation of the conditions are discussed in 3.1.1.1.

2.2.2 ICC for TH. PAP/DAB Method.

In addition to the fluorescence visualization system, the peroxidase - antiperoxidase (PAP) technique was also used in the present study with certain modifications of the procedure as follows.

The cells were washed, fixed and rinsed as outlined in section 2.2.1. Following rinsing the cells underwent 10 min washes in 100% ethanol, 50% ethanol and 100% ethanol again, in order to remove endogenous peroxidase which would otherwise interfere with the visualization system. The cells were then processed as outlined above in terms of the treatment with the primary antibody and blocking stage. However, following the blocking stage the cells were exposed to an unlabelled second antibody (raised in a goat against mouse IgG) for an incubation period of 90 min. After three washes in PBS the cells were incubated with a third antibody layer, the PAP complex, for a further 90 min (Fig 2.2). All excess antibody was removed by three sequential washes in Tris HCl, (0.78G of Tris hydrochloride in 100 mls of distilled water, pH 7.4), for 10 min. Visualization was achieved by incubating the cells in 200µl of a solution consisting of 5mg of diaminobenzidine (DAB free base), and 3.3µl of 20% hydrogen peroxide (H_2O_2) in a 10ml volume of Tris HCl. Development of the colour reaction was observed under light microscope and as the brown stain began to appear (typically between 7 and 10 min), the reaction was terminated by rinsing the cells in fresh Tris HCl. It was necessary to dehydrate the cells by passing them through an ethanol gradient from 70% to 100% before dipping the coverslips in xylene and mounting them on ethanol washed glass microscope slides in a commercially available mountant (Ralmount). THIP neurones were visualized by light microscopy.

2.2.3. ICC for GABA.

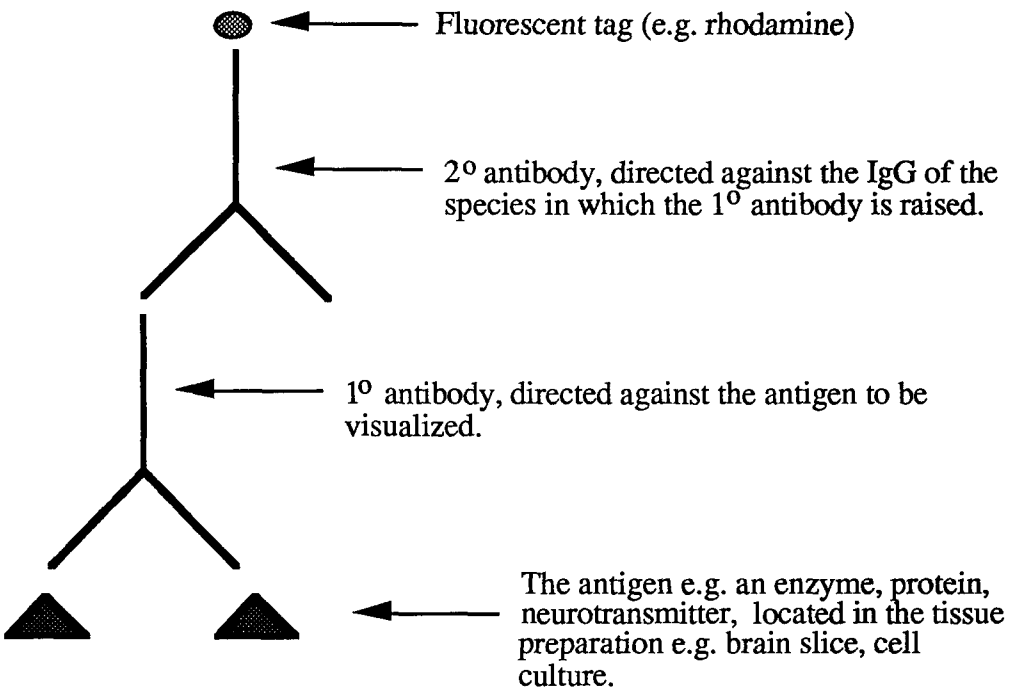
For GABA ICC cells were processed as detailed in Section 1. The fixative consisted of the modified Zamboni fixative supplemented with 1% glutaraldehyde. The anti - GABA polyclonal antibody was generated to recognise GABA conjugated to a glutaraldehyde moiety (Somogyi et al 1985) and tested negative for cross reactivity with L-glutamate, L-aspartate, D-aspartate, glycine, taurine, L-glutamine, L-Lysine, L-threonine, L-alanine, α–aminobutyrate, putrescine, δ-aminolevulinate, and positive for cross reactivity with γ-amino-β-hydroxybutyrate, β-alanine,δ-aminovaterate, ε-aminocaproate. The primary antiserum was used at a dilution of 1:3000 and the cells were incubated overnight at 4°C. Normal goat serum (10%), was again used as the blocking stage. The second antibody was a fluoroscein (FITC), conjugated antibody raised against rabbit IgG, to which the cells were exposed for 90 min at a dilution of 1:100. Mounting was performed as described above (section 2.2.1.) and controls included the replacement of the primary antibody with normal rabbit serum.

2.2.4. Dual Immunocytochemical Studies.

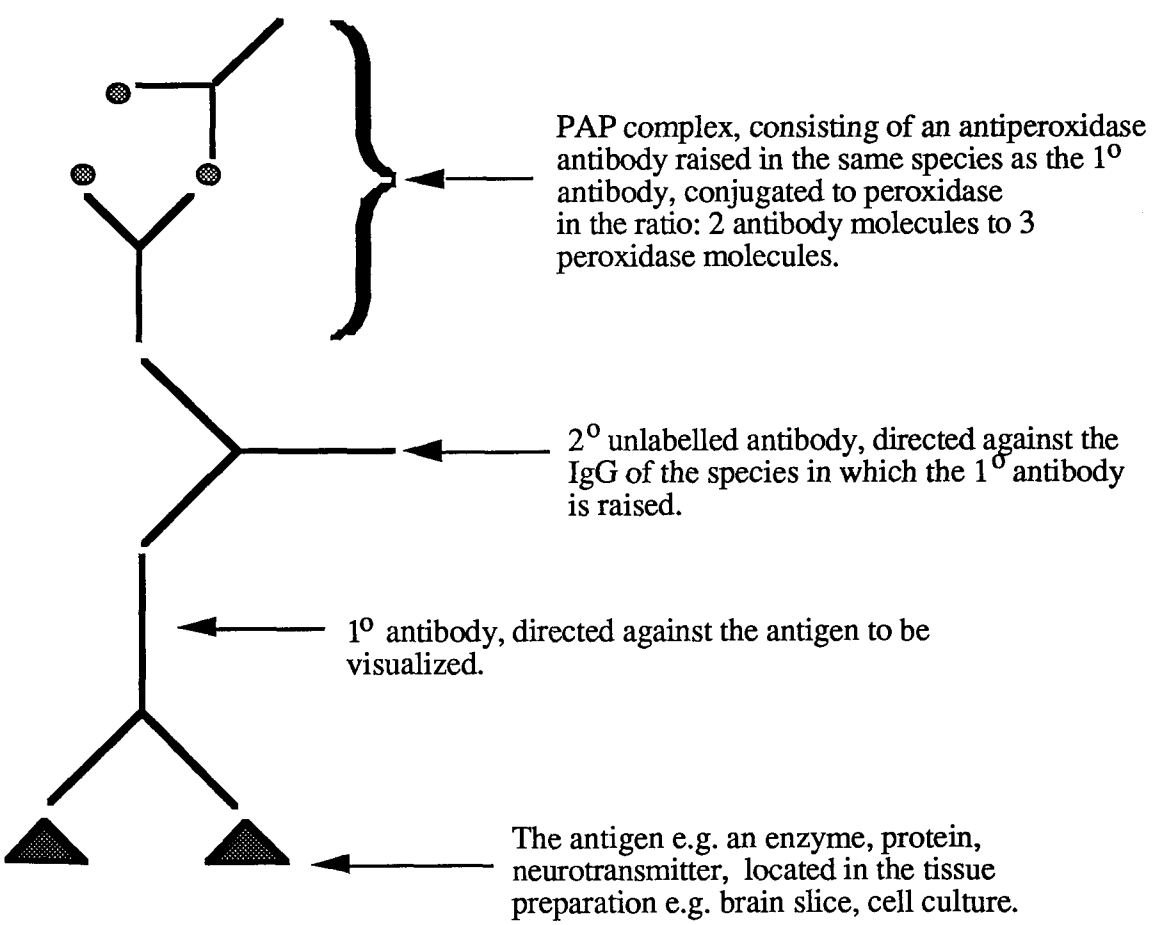
In order to investigate the possible interactions between the dopaminergic and GABA-ergic systems within the cultures, double staining experiments were performed. Cells were processed initially for TH immunocytochemistry followed immediately by visualization of GABA - immunoreactivity. As different fluorescent labels were employed i.e. rhodamine (red) for DA and fluoroscein (green) for GABA, it was possible to distinguish the immunofluorescence by observing the mounted cells through filters providing different wavelengths (400 - 490nm for FITC; 550 - 650 for rhodamine).

Fig. 2.2 Principle of ICC Reactions.

a). Indirect (Fluorescent) Method of ICC



b). Third Layer Antigen Method of ICC (peroxidase antiperoxidase).



2.3. Studies with Radiolabelled DA and GABA.

Cells grown in 15mm diameter, 4 x 4 multiwell plates were used to investigate the uptake and release of [^{3}H]DA and [^{3}H]GABA into the hypothalamic cells in primary culture and the levels of tritium labelled neurotransmitters accumulated and released were measured using liquid scintillation spectrophotometry.

2.3.1. The Principle of Liquid Scintillation Spectrophotometry for detection of [^{3}H]DA and [^{3}H]GABA.

Liquid scintillation spectrophotometry relies on the conversion of kinetic energy into light energy. When a molecule of tritium [^{3}H] decays a ß - particle is emitted from its nucleus, which collides with the aromatic solvent molecules within the scintillation fluid (e.g. toluene, benzene or xylene). The kinetic energy produced by the collision is not readily detectable and is rapidly dissipated. However, the scintillation fluid also contains solutes known as scintillators, the molecules of which become excited and when their orbital electrons return to their ground state, energy emission occurs as a photon of light.

There is, to a first approximation, a linear conversion of energy generated to photon light emitted and hence the intensity of the light produced by scintillation is directly proportional to the initial energy of the ß - particle. The generated photons of light are detected by the photomultiplier tube (PMT), and a small electrical current is produced, with an amplitude directly proportional to the number of electrons detected by the cathode. The electrical pulse is in turn analysed by means of integrated electronic circuits and recorded as discrete counts or as a count rate. Absolute values of radioactivity are attained by the construction of an internal quench curve, which corrects for the efficiency of the analyser (in this instance 40%) and converts the subsequent counts per minute (c.p.m.), to disintegrations per minute (d.p.m.).

2.3.1.1. Calibration.

In order to investigate the linear relationship between the concentration of the radiolabel in the sample and the resulting electrical impulses generated following scintillation, a standard curve was constructed. To produce this a range of concentrations of radiolabel (i.e. [^{3}H]DA) from 5 to 150µCi/ml) were added to 10mls of the scintillation cocktail and counted (each standard was counted in triplicate). When a graph was plotted depicting concentration of radiolabel v's d.p.m. (Fig 2.3), a

linear relationship was observed which was conserved over the range being investigated hence proving that d.p.m. was a reliable indicator of radioactivity.

2.3.1.2. Counting Efficiency.

A portion of radioactivity decayed in a liquid scintillation solution is always lost in the system. This is due to interferences with the production or transmission of light emitted by the scintillators and different sample preparations will affect the efficiency of the counting procedure, i.e. causing quench. The degree of quenching in the system was thus determined by repeating the procedure above by preparing [^{3}H]DA standards (over the same concentration range) containing the tissue solubilizer, Triton - X- 100 and/or solubilized cells, in amounts comparable to those used in the investigation procedure. Standard curves were again plotted and the degree of quench produced by the presence of the solubilizer and cells was represented by a change in the gradient of the standard curves (Fig 2.4). The percentage difference in the gradients of the curves achieved by counting in the absence versus the presence of the solubilized cells reflects the degree of quench in the sampling system and was accounted for in subsequent data analysis.

2.3.2. [^{3}H]DA Uptake Studies.

The method of the uptake studies followed a method similar to that reported elsewhere (Prochiantz et al 1979). The culture medium was discarded and the cells were rinsed three times with a 200µl volume of pre-warmed Earles' Balanced Salt Solution (EBSS - Table 9). Following this, the cells were pre-incubated for 15 min in 200µl of supplemented EBSS (sEBSS, Table 10.0) containing the monoamine oxidase inhibitor, pargyline, (10^{-5}M). The cells were exposed to [^{3}H]DA at a concentration of 2µCi per well, (3.3×10^{-7}M) initially diluted in sEBSS plus pargyline as detailed above. The specific activity for the radiolabelled DA altered from batch to batch and ranged from 43 - 60Ci/mmol.

The cells were exposed to [^{3}H]DA for 1 hour at 37°C (or a time and temperature specified by the experimental design). Uptake of [^{3}H]DA was terminated by rinsing the cells in ice-cold sEBSS prior to a further three washes to remove excess and non-specific, extracellularly bound label. Cells were solubilized in a 50% solution of Triton - X - 100 and the "cell suspension" collected into scintillation vials containing 5ml of NE 260, a scintillation cocktail. The accumulated radiolabelled neurotransmitter was estimated by liquid scintillation spectrophotometry. In those investigations employing pharmacological agents, such as uptake inhibitors or

receptor antagonists, the drugs were added to the [^{3}H]DA prior to incubation to produce the desired final concentration.

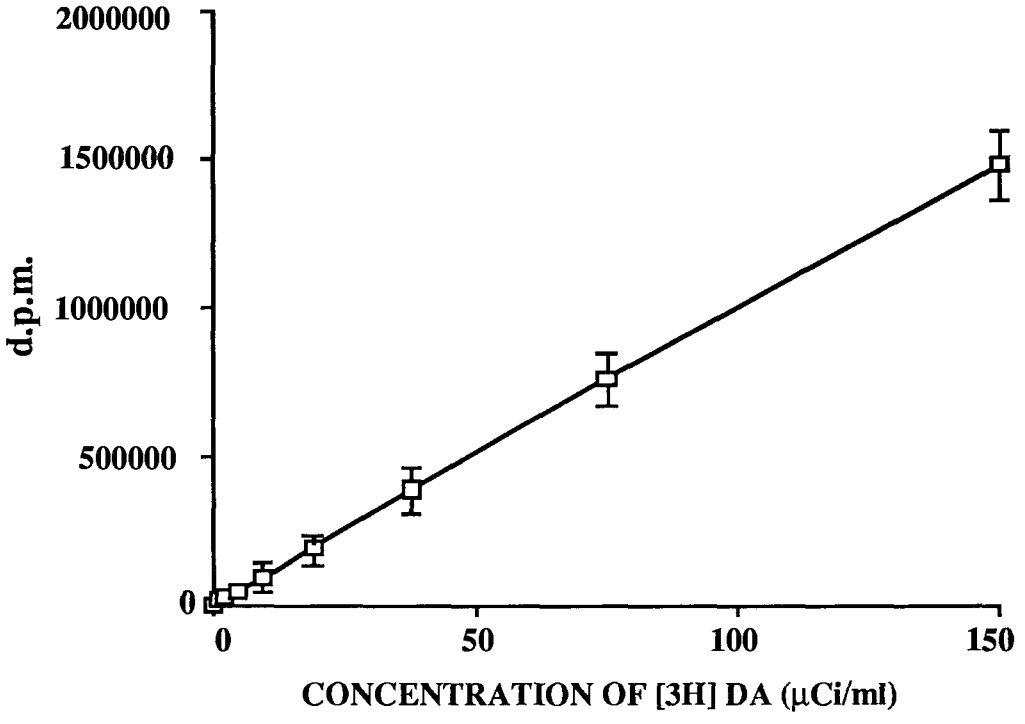


Fig. 2.3. Illustration of the linear relationship between the concentration of the radiolabelled neurotransmitter [^{3}H]DA and d.p.m.. The linear relationship was conserved over a range of concentrations of [^{3}H]DA from 5 to 150μCi/ml. (S.A. = 60Ci/mmol).

2.3.2.1. Kinetic Analysis.

Kinetic analysis of [^{3}H]DA uptake was performed by loading the cells with one of a range of concentrations of [^{3}H]DA from 0.3nM to 30.0μM. The cultures were then treated as outlined in section 2.3.2. In order to determine the Michaelis - Menten constant (K_m) and the maximum velocity for uptake (V_{max}) a double reciprocal plot was constructed of V(in pmoles/min/well of cells) and the concentration of [^{3}H]DA (in μM). The rate of uptake was expressed as "per well of cells" rather than as "per mg of protein", as protein estimation studies (performed using the method of Bradford 1976) revealed increases in protein content within the cultures between DIV 3 and 7 followed by a decline in content on DIV 14 (0.175 ± 0.015 on DIV3, 0.215 ± 0.003 on DIV 7 and 0.05 ± 0.005 on DIV 14). The rise in protein content is widely

considered to be the result of glial proliferation within the cultures, whereas the decline is considered to be due to both neuronal and non - neuronal cell death. Thus relating the data derived from kinetic analysis to protein content may give misleading K_m and V_{max} values, especially when attempting to relate these values to increasing culture age. This argument is in agreement with issues which have been discussed elsewhere (Ahnert - Hilger et al 1986) and other workers have expressed affinities and rates of [^{3}H]DA uptake in terms of amount per well of cells (Valchar & Hanbauer 1993). We therefore adopted this method for our analysis. Each concentration was measured in quadruplet and the reciprocal of each concentration was plotted against the reciprocal of the corresponding value for the rate of uptake expressed as µmoles of DA/min/well of cells. Each point was plotted to give a line of best fit using a computerized least square method and linear regression analysis. The value of K_m was determined from the intercept in the x - axis (i.e when y = 0), whereas the value for V_{max} was measured by the intercept on the y - axis (when x = 0). A schematic representation of the plot is illustrated in Fig.2.5.

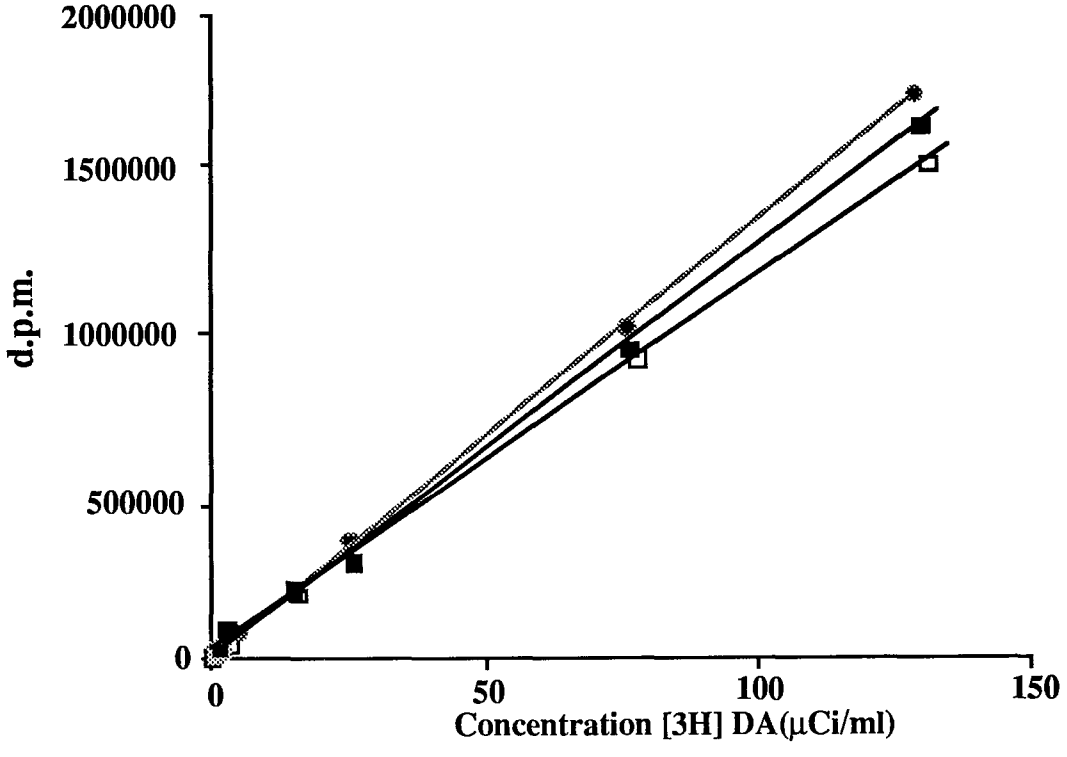
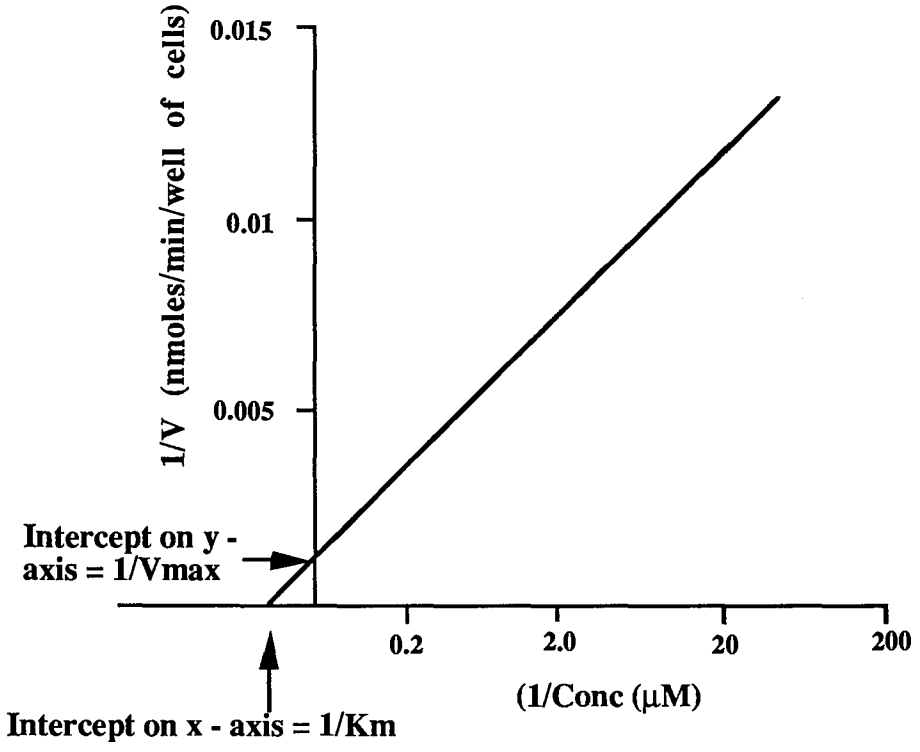


Fig. 2.4. Effect of the tissue solubilizer Triton - X - 100 and solubilized cells on the counting efficiency of the system. The phenomenon of quench was not observed either when standards were prepared and counted in the presence of by Triton - X - 100 (—✹—), in the presence of the solubilized cells (—■—) or in their absence (—□—). There was no significant difference in the gradients of the standard curves constructed under the three conditions and hence the characteristics of the sample did not alter the efficiency of the counting system. (SA = 60Ci/mmol)

Fig. 2.5. A diagramatic representation of the double linear regression plot used to determine the K_m and V_{max} values for [^{3}H]DA and [^{3}H]GABA uptake into hypothalamic cells in primary cell culture.



2.3.3. [^{3}H]DA Release Studies.

For release studies, the cells were loaded with [^{3}H]DA in the manner outlined in Section 2.3.2 above. However, instead of terminating the uptake by rinsing the cells in ice-cold buffer, the cultures were washed sequentially (3 x 200μl), in sEBSS, pre-warmed to 37°C, to remove excess label and attain a steady state (Fig. 2.6). The cells were then subjected to 7 min incubation in sEBSS under basal conditions or in the presence of 200μl EBSS containing 56mM potassium (56mM K^+, Table 9.0). The sEBSS was removed to scintillation vials containing 5ml of scintillation cocktail and the amount of radiolabelled DA releasedwas estimated using liquid scintillation spectrometry. Additional investigations were performed to verify the Ca^{2+} - dependency of the observed release by repeating the experiments omitting Ca^{2+} from the incubation medium.

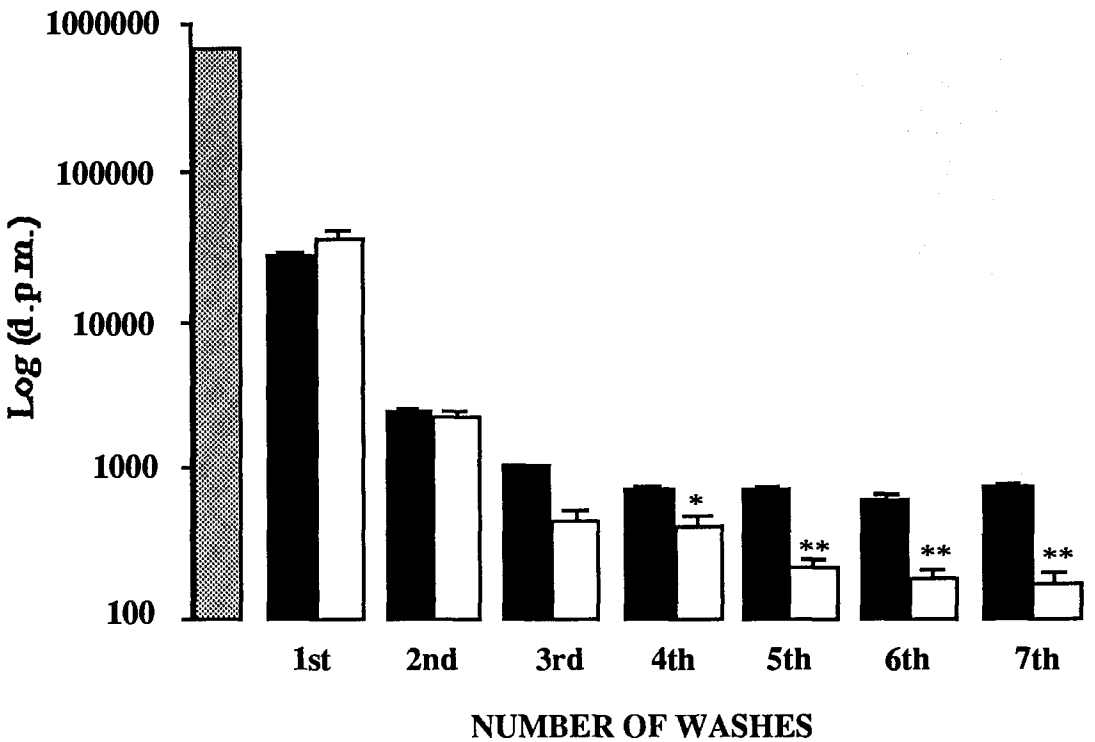


Fig. **2.6**. The attainment of steady state levels of [^{3}H]DA release by rinsing of the hypothalamic cells. After loading the cells as described in Section 2.3.2 the excess label was removed (▒) and the cells were rinsed with sEBSS either at 37°C (■) or at 4°C (□) and all washes were counted by liquid scintillation spectrophotometry. After the third wash the release of [^{3}H]DA became stable depicting that all excess label had been removed and that a steady state of basal release had been attained. When the cells were rinsed at 4°C, release followed a similar pattern but at a significantly (* $p < 0.02$, ** $p < 0.005$) lower level. This was considered to represent non-specific release.

Table. 9.0. The constituents of Earles' Balanced Salt Solution (EBSS). The following constituents were added to 100mls of distilled water and filter sterilized.

	BASAL RELEASE EBSS		STIMULATED RELEASE EBSS	
CONSTITUENTS	WEIGHT (mg)	FINAL CONC (mM)	WEIGHT (mg)	FINAL CONC (mM)
NaCl	680	120	370	65
KCl	41.7	5.6	417	56
$CaCl_2$	36	2.0	36	2.0
$MgSO_4$	20	0.8	20	0.8
$Na_2H_2PO_4.7H_2O$	15.8	1.0	15.8	1.0
$NaHCO_3$	220	26	220	26
D - GLUCOSE	100	5.4	100	5.4

Table. 10.0. Supplements to the Earles's Balanced Salt Solution. (sEBSS). The following constituents were added to 100mls of EBSS.

CONSTITUENTS	CONCENTRATION
PENICILLIN	100 IU/ml
STREPTOMYCIN	100µg/ml
BACITRACIN	30µg/ml
ASCORBIC ACID	30µg/ml
HEPES	10mM
APROTININ	80 KIU/ml

Studies with [^{3}H]GABA.

Uptake and release studies were performed to investigate these properties in the GABA-ergic systems within the cultures using a method which was similar to that for [^{3}H]DA.

2.3.1. [^{3}H]GABA Uptake Studies.

In uptake studies the GABA - transaminase (GABA-T) inhibitor, amino-oxyacetic acid (AOAA), at a concentration of 1 x 10^{-5}M, was included in the sEBSS throughout the experimental procedure. AOAA prevents intraneuronal degradation of GABA (Drejer et al , 1987) by inhibiting pyridoxal phosphate (Kruk & Pycock 1991), which acts as a co-factor for GABA-T. The cultures were rinsed and pre-incubated as outlined in the [^{3}H]DA studies before being exposed to [^{3}H]GABA at a concentration of 0.1µCi (2.5 x 10^{-8} M; specific activity 91 Ci/mmol), for 10 min. The remainder of the procedure was identical to that outlined in Section 2.3.2.

2.3.4.1. Kinetic Analysis.

Kinetic analysis of [^{3}H]GABA uptake was performed by loading the cells with one of a range of concentrations of [^{3}H]GABA from 0.54nM to 0.54μM. The cultures were then treated as outlined in section 2.3.1. Values for K_m and V_{max} were calculated as outlined in Section 2.3.2.1.

2.3.5. [^{3}H]GABA Release Studies.

The release studies for [^{3}H]GABA followed the protocol described for [^{3}H]DA as described in Section 2.3.3.

2.4. Investigations with Gonadal Steroids.

2.4.1. 17ß - oestradiol.

Investigations were performed to determine the effect of the gonadal steroid 17 ß - oestradiol (E_2) on dopaminergic neurone function. Batches of cells, prepared as outlined in Section 1 were divided into groups and maintained in defined medium (Table 7.0), containing one of a range of concentrations of E_2 from 10^{-14}M to 10^{-6}M, from the time of plating i.e. DIV 0. All other components of the defined medium remained unchanged. On specified days uptake and release studies for [^{3}H]DA and [^{3}H]GABA were performed as described above (Section 2.3).

2.4.2. Progesterone.

Experiments were performed to determine the effect of the gonadal steroid, progesterone (Prog), on the uptake and release mechanisms displayed by the dopaminergic neurones within the culture model. Cultures were divided into groups and maintained in DM containing one of a range of concentrations of Prog from 10^{-12} to 10^{-8}M, from DIV 0. All other components of the DM, including E_2, remained unchanged. Uptake and release studies for [^{3}H]DA were performed as detailed in Section 2.3.

2.5. Studies with Endogenous DA.

The release and content of endogenous DA in the hypothalamic cultures was measured using high performance liquid chromatography coupled to electrochemical detection.

2.5.1. Principle of High Performance Liquid Chromatography with Electrochemical Detection.

The technique of High Performance Liquid Chromatography (HPLC) is based upon the separation of a mixture of compounds (analytes) in solution on the basis of their hydrophobic or lipophilic properties. This method was first introduced by Refshauge et al (1974) and, although initially of limited use due to the high cost of the equipment, it is now the method of choice for the analysis of catecholamines, other related amines and a host of other related and non-related compounds in a range of biological fluids including plasma, CSF and urine. Refinements of the technique rely partly on the development of more efficient hardware, such as pumps, autosamplers and detectors, and partly due to the development of more refined columns and packing materials which allow efficient through-put of samples while retaining high specificity and sensitivity. The principles which aided in the improvement of HPLC-ECD may be found in a review by Kilpatrick (1986).

Widely speaking there are two methods by which separation may be achieved, either using ion-exchange chromatography or reverse phase chromatography. The former is employed for the measurement of ionised material and the stationary phase i.e. the column has a more polar nature than the mobile phase. In the latter case the situation is reversed in that the solid phase of the system is less polar than the mobile phase. In fact under normal conditions of reverse phase chromatography the material which forms the column usually consists of a non-polar compound such as C_{18} silica or ODS. Such material provides a lipophilic phase which serves to attract analytes within the solution which are of a non-polar nature and, combined with other factors such as adsorption, will influence the degree to which the constituents within a solution are separated. Rapid mass transfer of substances in solution between the stationary phase and the mobile phase is provided by the structural characteristics of the packing material. ODS columns available commercially consist of very small diameter, spherical particles (usually between 3 and 5μm diameter) which by nature of their size and spacial arrangement provide a large surface area over which the mobile phase will flow. This mass transfer determines the degree of resolution achieved i.e. the smaller the particles of the packing material, the greater the resolution between the analytes.

It follows that analytes with very differing pKa values (i.e. that at which 50% of the compound is in an ionized form), will be separated with a greater degree of resolution. However, other factors such as the width of the peaks (as shown on the resulting chromatogram) and the capacity factor (retention time) of the analyte on the column also play a major role in determining the separation power of the chromatography system.

The separation of catecholamines cannot be based solely on the degree of ionization as all catecholamines are ionized at a range of pHs from 1 - 7. Instead, separation is dependent on differences in the lipophilicity of the amines. DA is the most lipophilic (or hydrophobic) catecholamine due to a lack of an hydroxyl (OH) moiety on its catechol ring. The next most hydrophobic catecholamine is Adr due to the presence of an alkyl group in its structure and the least hydrophobic amine is NA. It is for this reason that the retention time for NA is often very low and this amine elutes rapidly from the column and is lost in the solvent front making its detection and quantification very difficult and unreliable. Ion pair reagents such as the aryl and alkyl sulphonates and sulphates (e.g. octyl sulphonate) are often added to the mobile phase and attach themselves to the column. They attract the ionized analytes which then become more firmly held by the stationary phase thus increasing the retention time. Conversely, the addition of organic modifiers such as methanol or acetonitrile - shorten the length of time that analytes are retained on the column by competing with the column for the non-polar moieties of the analytes, the result of which is that they are attracted away from the column and are thus more rapidly eluted.

Kissinger (1977) was the first to introduce the method of electrochemical detection, a technique by which the oxidation of a compound leads to the generation of electrons and hence a small but detectable current. This current may then be amplified to a level which may be recorded. The current generated by oxidation is considered to be directly proportional to the number of molecules which have passed over the electrode and thus the method allows quantitative as well as qualitative analysis to be carried out. The relationship between the analyte concentration and the generated potential is affected by such external factors as the temperature, pH and ionic strength of the mobile phase and these parameters must be kept constant if quantitative determinations are to remain reliable.

Detection of the generated current is produced by a carbon working electrode which supplies the current for the oxidation of the electro-active analytes as well as measuring the extent of the oxidative process. The potential is applied via an auxilliary electrode made of platinum or steel. This potential causes oxidation of the

analytes and is kept at a steady, workable level by a reference electrode which is usually composed of Silver/Silver Chloride (Ag/AgCl) The measured potential is then transferred to an external recorder where it is displayed by pen deflection as a peak. The concentration of the analyte in the original sample may thus be expressed either as peak height, area or width.

2.5.2 The HPLC - System.

A description of the HPLC -ECD hardware used in the investigation may be found in Table 11.0 and Fig. 2.7.

2.5.2.1. The Mobile Phase.

The mobile phase into which the analytes were dissolved and delivered to the system was prepared from HPLC grade reagents and only acid washed glassware was used in the preparation procedure. It was important to eliminate any risk of contamination to the mobile phase as this would have been severely detrimental to both the chromatography and detection apparatus. Hence all water used in preparation of the mobile phase and rinsing of glassware was at least double glass distilled, de-ionized and underwent purification by reverse osmosis prior to filtration in a Milli - Q water purification system.

Table. 11.0. The HPLC - EC hardware.

COMPONENT	SPECIFICATION	COMMENTS	MAINTENANCE
Solvent delivery system	PM-48 pump, operated at 0.8ml/min	Gives reproducible flow rate, minimal noise due to incorporated pulse dampner.	Frequent cleaning of pump head and replacement of piston seal to increase pump life and prevent leakage of mobile phase.
Sample loop injector	Rheodyne 7125 50µl capacity.	Ensures delivery of constant volume of sample to the system with good reproducibility.	Replacement of rotor seal every 6 - 12 months, removal of all abrasive particles from the sample to prevent scratching of the rotor seal surface.
The Guard Column	MF - 6026, pellicular particles of ODS - 53 µm diameter.	Protects analaytical column by removing insoluble contaminants from samples and/or mobile phase.	Replacement after every 200-500 samples, dependent on their prior preparation.
Analytical Column	MF - 6213 Phase II, ODS 3µ diameter spherical particles, 15cm x 46mm external diameter.	Allows separation of analytes with good resolution and reproducibility.	Cartridge seal replacement prevents leakage from the column, peak splitting is often due to a void in the column requiring repacking or replacing.
The Detector	LCB - 4 amperometric detector, set at a potential of 0.7mV in relation to the reference electrode (see below).		
a. Working Electrode	MF - 1000, glassy carbon.	Composed of chemically resistant, highly conductive material, provides the surface for electrolysis.	Loss of efficiency due to build-up of oxidised material on the surface. Frequent cleaning with alumina polish removes deposits and increases the sensitivity of the cell.
b. Reference Electrode	Model TL 5A, Ag/AgCl	Provides a stable, reproducible voltage to which the working potential may be referenced.	Stored in 3M KCl, in light proof container, when not in use, to increase life expectancy.
c. Teflon Cell Gasket	MF - 1046	Determines the dead space volume within the thin-layer cell and thus determines the speed with which the sample passes through it.	
Chart Recorder	HP 3396 A	Illustrates the generated current as a peak which is directly proportional to the amount of analyte detected at the electrode.	

Fig. 2.7. SCHEMATIC DIAGRAM OF THE HPLC-ECD SYSTEM

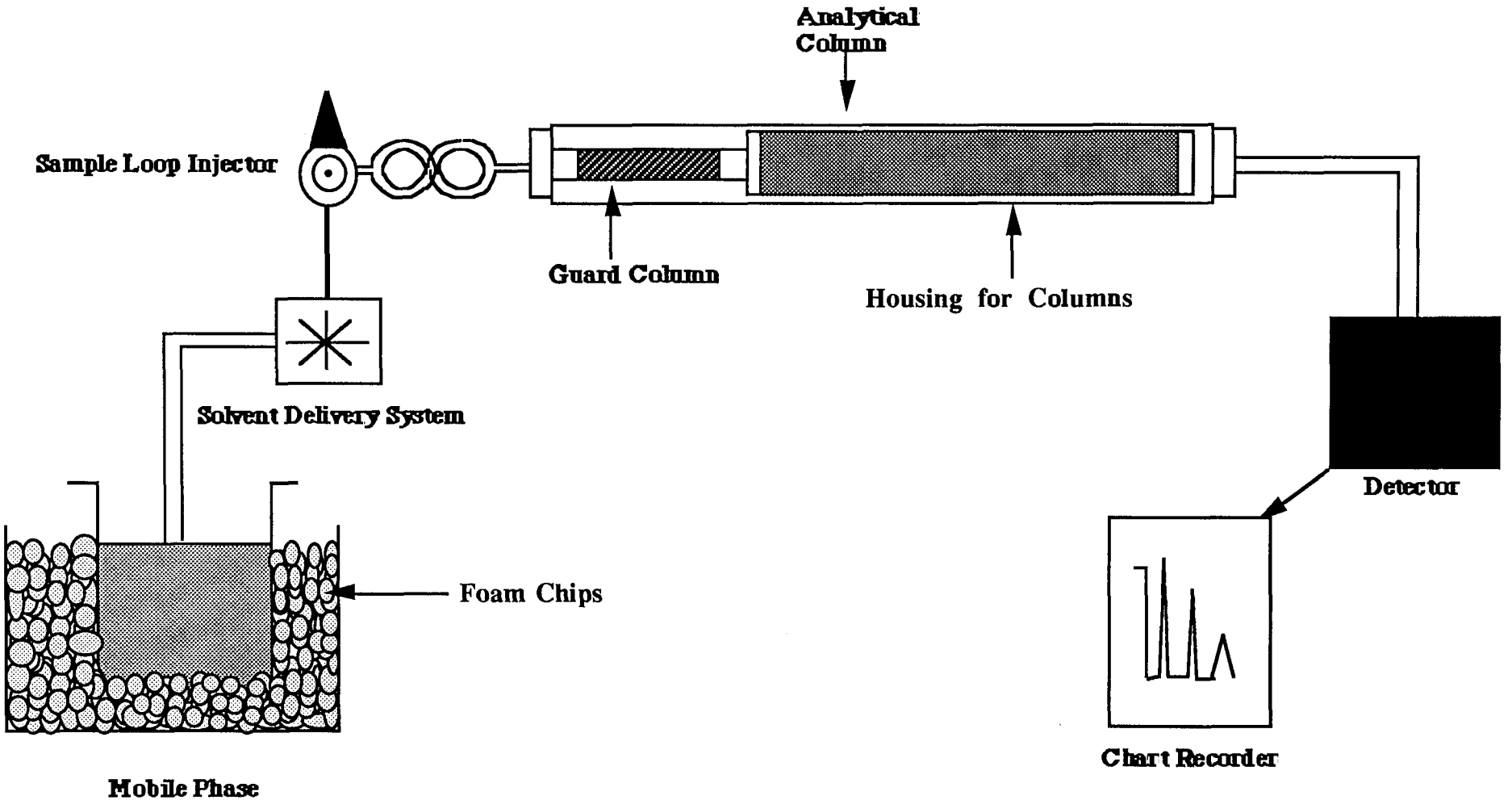


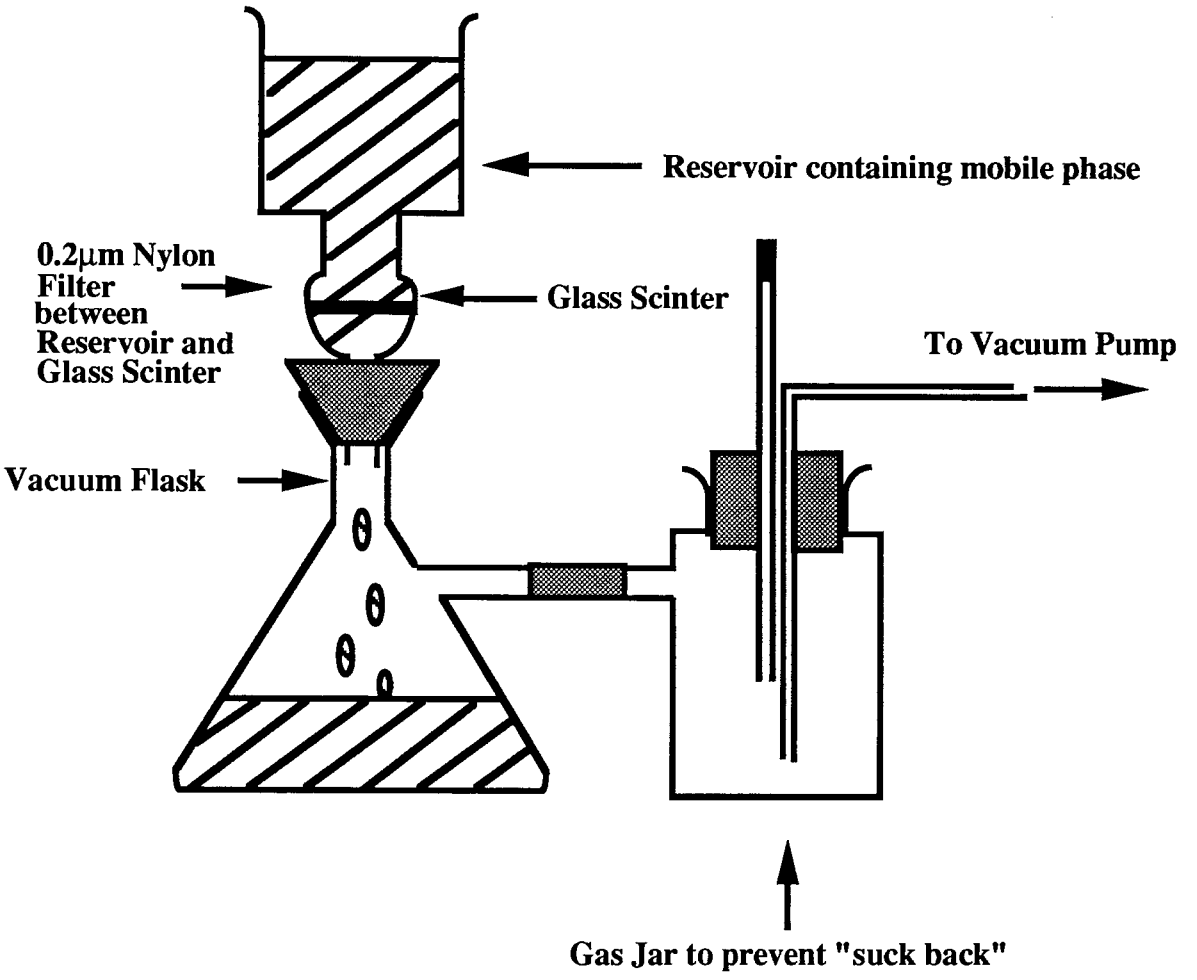
Table 12.0. Mobile phase was prepared 1 litre at a time by dissolving the following amounts of each reagent in 900mls of H_20.

REAGENT	WEIGHT (G)
CITRIC ACID	21.01
SODIUM HYDROXIDE	0.25
ETHYLENEDIAMINE-TETRA - ACETIC ACID (EDTA)	0.37
OCTYL SULPHONATE	0.216

The pH of the buffer was then measured and, if necessary, adjusted to 2.55. The organic modifier was then added (40mls of acetonitrile) and the mobile phase was made up to volume.

It was vital to remove all air and any possible insoluble contaminants from the mobile phase by de-gassing and filtration using the apparatus illustrated in Fig 2.8. A glass scinter covered with a 0.2μm filter was attached to a vacuum flask connected to a gas jar which prevented "suck -back" of the water from the vacuum system and hence contamination of the mobile phase. The mobile phase was then transferred to a glass bottle wrapped in aluminium foil (as the mobile phase is light sensitive) and kept at ambient temperature surrounded by foam chips for insulation against temperature fluctuations. An inlet tube was placed in the mobile phase which was connected to the solvent delivery system and thus pumped the mobile phase into the apparatus. When not measuring samples, the solvent was recycled over short periods of time. The mobile phase was typically replenished every week, but was occasionally changed more frequently depending on the nature of the investigation being carried out.

Fig 2.8. Apparatus for the De-gassing of the Mobile Phase.



2.5.2.2 Sample Injection.

The sample/standard (at least 100μl) was injected (using a flat needled Hamilton syringe), via the needle port, into the loop while the injector valve was in the "load" position. In this position the mobile phase which flows through the system continuously, was redirected to bypass the injector loop. Once the loop was filled, the injector valve was turned to the "inject" position thus bringing the loop into series with the column, resulting in the sample being flushed onto the column as the mobile phase passed through the sample loop. This ensured that the volume of sample

introduced to the system was constant for each injection and thus improved the reproducibility of the resulting chromatograms.

2.5.2.3. Calibration/Standardization.

In the present investigation the **internal standard** used was dihydroxybenzylamine (DHBA). This was chosen as it behaves like the catecholamines, eluting within the same time frame but not at the same time as other amines. It is however, resistant to enzymatic breakdown and is therefore stable. A known concentration of the internal standard, which would produce a peak of a pre-calculated height, was added to each sample (or standard), prior to extraction, and served as an indicator of amine recovery. From this it was possible to estimate absolute recovery and correct the values of the other amines accordingly.

In order to calibrate the system and to determine the limit of detection, standards of the catecholamines, NA, Adr, DA, DOPAC and the internal standard DHBA, were made up in 0.1M PCA. A stock solution of each amine (1mg/ml) was made in the first instance, which was subsequently diluted in 0.1M PCA to give a top standard of 5ng/ml, (equivalent to 250pg per 50µl injected onto the column). Serial dilutions were made to the top standard and the lowest standard used was 0.3ng/ml (equivalent to 15.625pg per 50µl injected onto the column). Each catecholamine was first injected individually, starting with the lowest concentration to construct a standard curve and also to determine the retention time for the amine. Each standard was measured in triplicate. Finally, all five amines were made up together in a single solution (from here on referred to as an amine mix) and injected in order to determine whether each individual retention time was conserved or whether they interacted. Standard curves were thus plotted of amount of amine versus peak height for each analyte and these were linear over a range from 10 - 250pg. Figs 2.9, 2.10 and 2.11 illustrate standard curves for DA, DHBA and DOPAC respectively. The limit of detection for the HPLC system was defined by a signal:noise ratio of greater than 3:1. For DA, DOPAC and DHBA, the limit of detection was 0.2ng/ml (10pg), 0.1ng/ml (5pg) and 0.3ng/ml (15pg), respectively.

2.5.2.4. The Chromatogram.

Examples of typical chromatograms for the amine mix are shown in Fig 2.12. The top chromatogram (A), illustrates the blank i.e. injection 50µl of 0.1M PCA alone onto the column, thus the vehicle for the amines was not in itself significantly electro-active. Chromatogram B depicts injection of the lowest concentration of the amine mix (0.3ng/ml) and chromatograms C and D illustrate the amine mix concentrations of 2.5 and 5.0ng/ml respectively. NA, Adr, DHBA, DOPAC and DA had retention

times of 1.95, 2.5, 3.0, 3.9 and 4.66, respectively. The integrator recognized the peaks by their respective retention times allowing for 0.5 time units on either side of the "standard" time. The detector current was set at 1nA.

Fig 2.13. shows examples of chromatograms for samples collected from the hypothalamic cultures. Chromatograms A and B illustrate the injection of release medium from basal and stimulated incubations respectively (see below). Chromatogram C (with the detector current set at 0.5nA) illustrates the injection of a purified homogenate of hypothalamic cells in order to determine amine content. Such samples contained a number of uncharacterized substances which could not be removed by the extraction procedure.

On occasions the amines, when injected as an amine mix, co - eluted or eluted with retention times which were very close resulting in partially merged peaks. This naturally hampered quantitative analysis and the mobile phase was subsequently modified by use of either an ion pair reagent such as octyl sulphonate, or an organic modifier such as acetonitrile, to increase or decrease the retention times and this was sufficient to alter the retention time and thus separate individual analytes. In some instances the peaks were only just merging and at such times, merely altering the flow rate slightly was sufficient to separate two peaks and allow their individual detection.

For quantitative analysis the chromatograms were recorded by an on - line integrator (HP 3396 A, Hewlett Packard) and the height of the peak was taken as a measure of the quantity of analyte oxidised at the working electrode. This served as a quantitative indicator of the amount of the analyte present in the injected standard (or sample) as the amount of current generated is directly proportional to the pen deflection.

The peaks obtained on injection of the sample were identified by comparing the retention times with those of the standard amine mix.

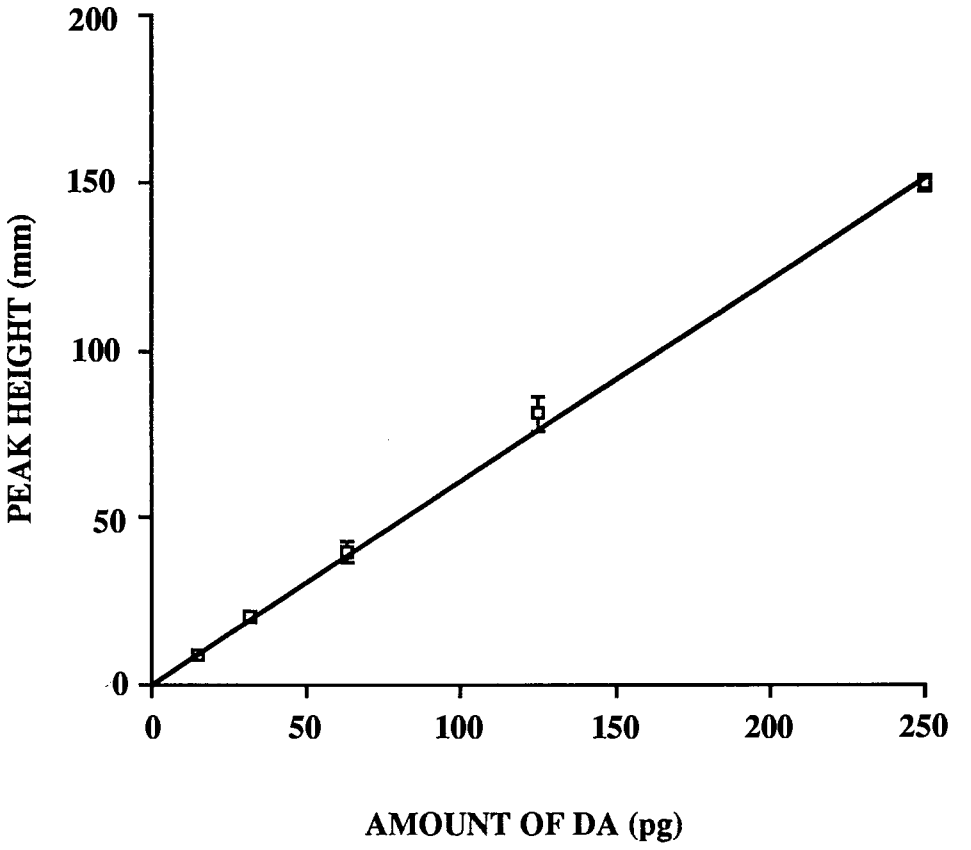


Fig. 2.9. Linear relationship between the amount of DA and the peak height as measured from the chromatogram. Linearity was conserved over a range of DA levels from 15 - 250pg injected onto the column. Each amount was measured in triplicate and a calibration curve was constructed prior to measurement of each batch of samples and also after maintenance of the HPLC hardware.

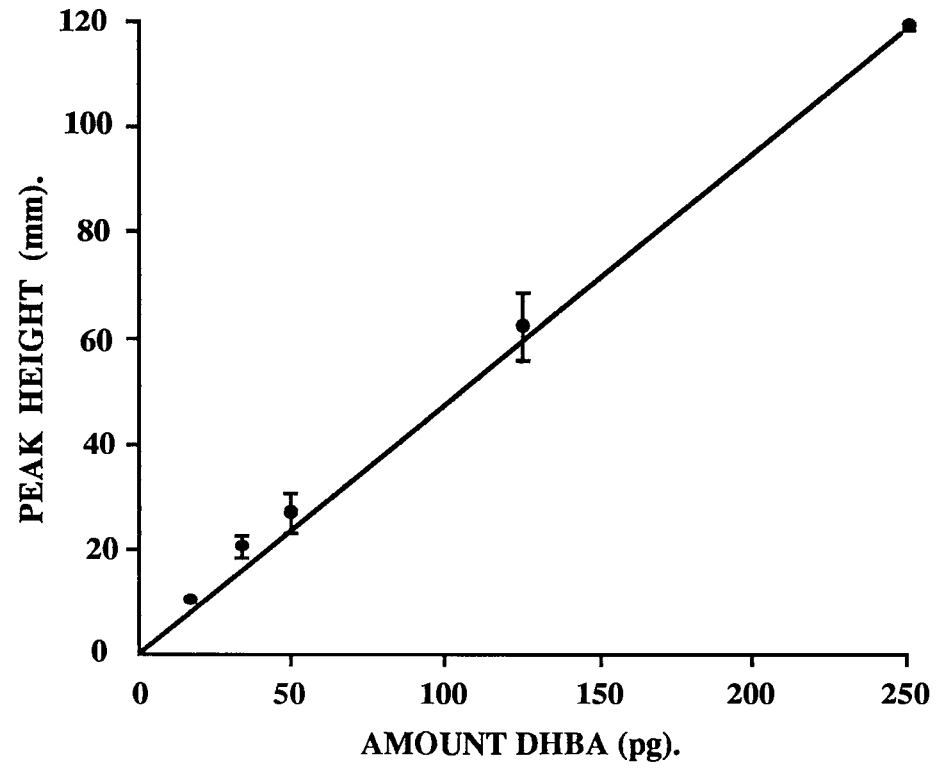


Fig. 2. 10. Linear releationship between the amount of the internal standard, DHBA and peak height as measured from the chromatogram.

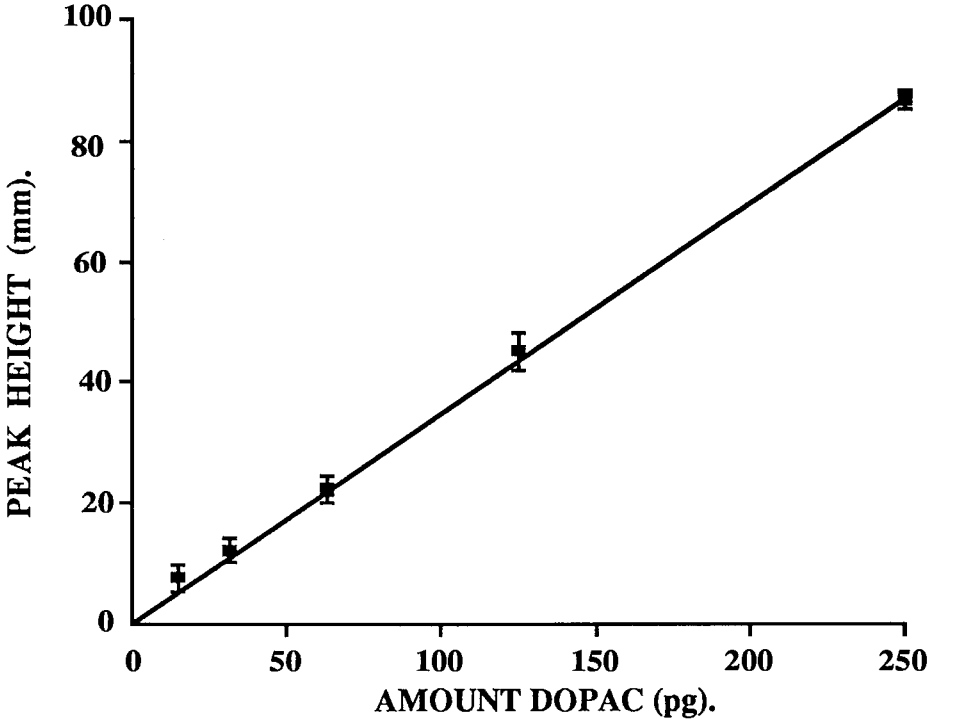


Fig. 2.11. Linear relationship between the amount of DOPAC and the corresponding peak height as measured from the chromatogram.

Fig. 2.12. Chromatographic analysis of the amine mix.

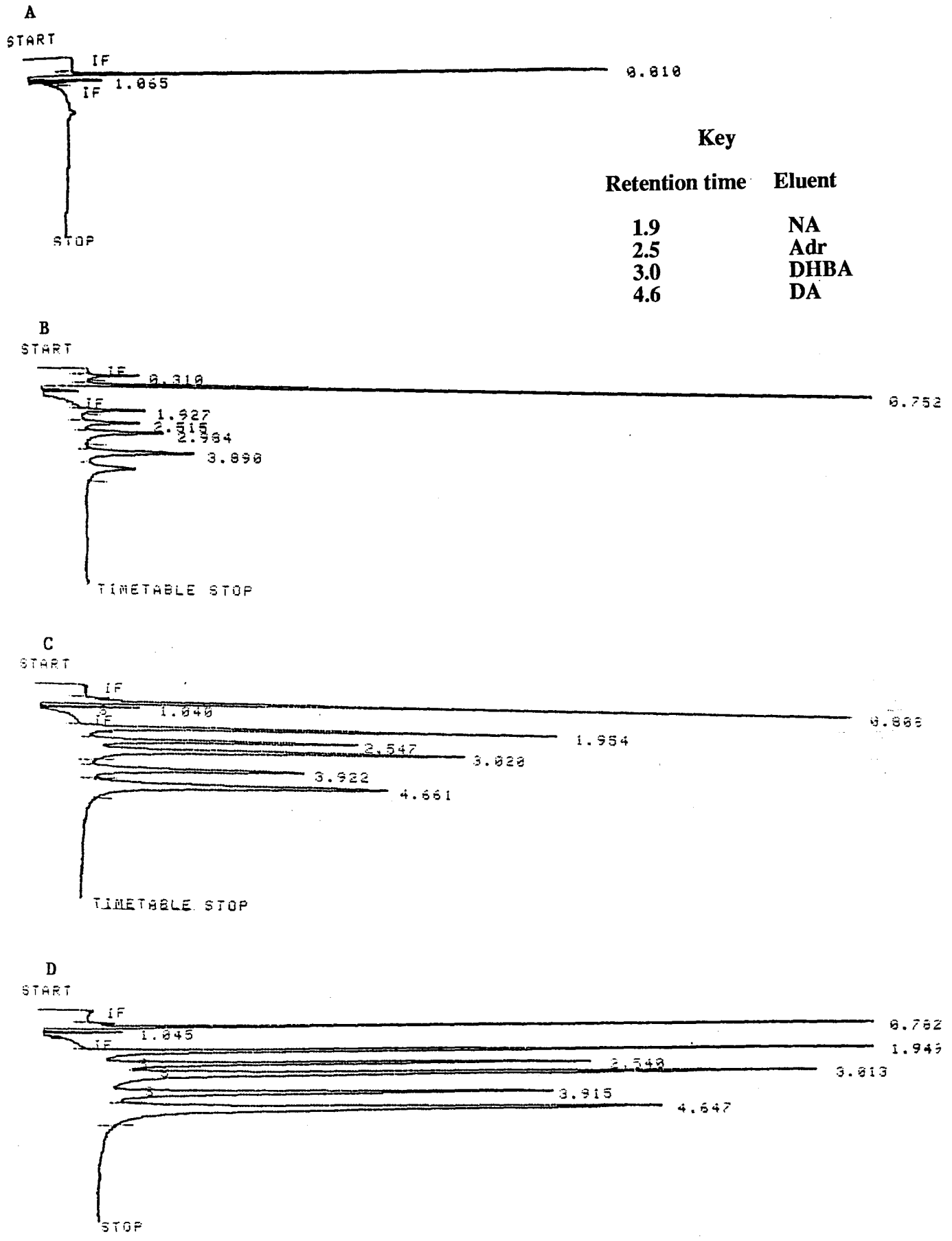
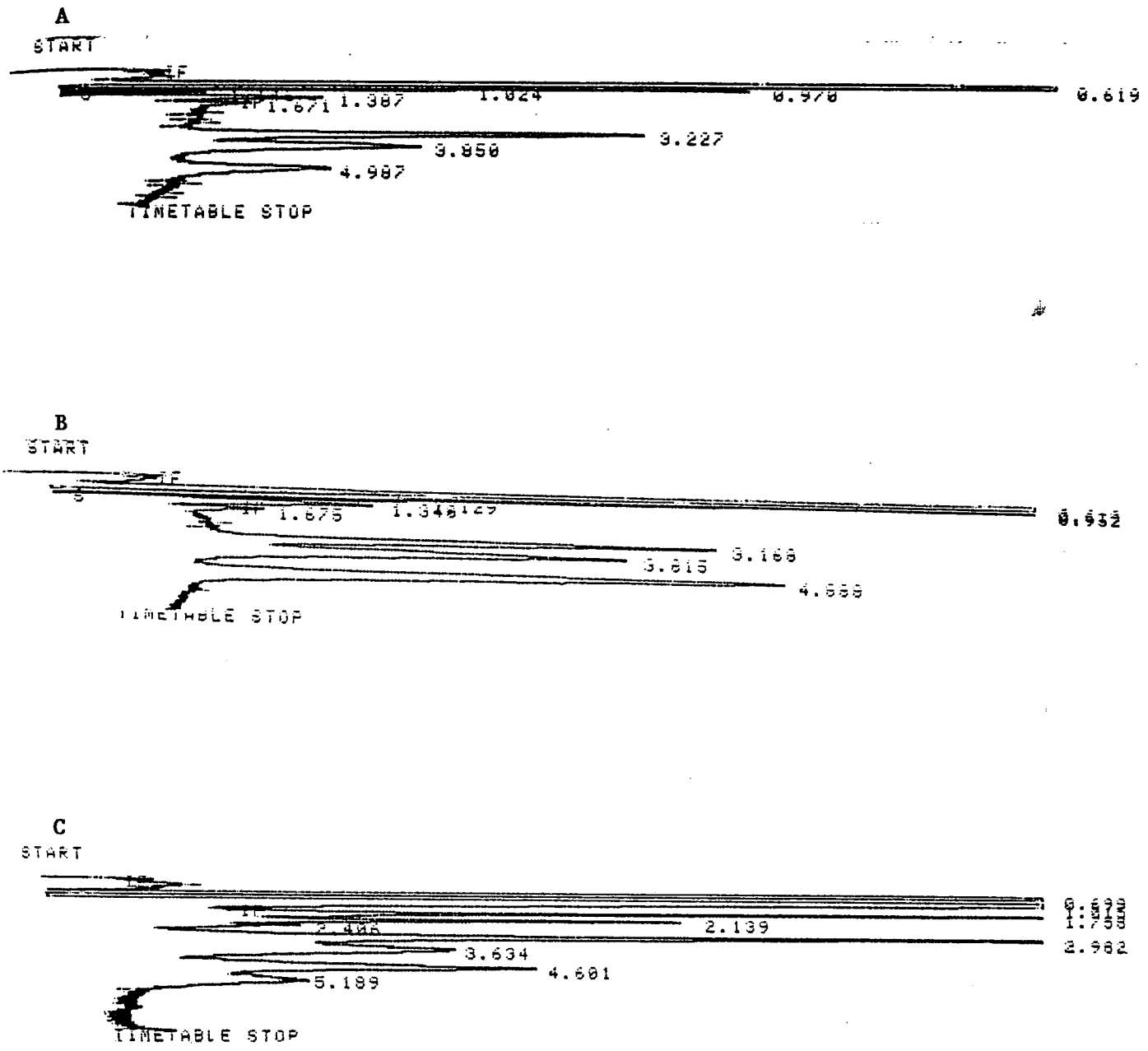


Fig. 2.13. Chromatographic analysis of different sample types.



2.5.3. Release Studies.

Cells maintained in 35mm diameter culture dishes were used to investigate the release of endogenous DA. On the day of experimentation, the cells were washed (2 x 1ml) in sEBSS containing pargyline at a concentration of 10^{-5}M and underwent a 15 min preincubation period. They were then incubated in sEBSS (0.5ml) containing basal (5.6mM) or elevated (56mM) levels of K^+ for periods of 1 hour (or times and conditions specified by the experimental design). The samples were then collected onto 10µl of 5.0 M perchloric acid (PCA), to give a final concentration of 0.1M PCA and stored at -80°C until the time of amine determination using reverse phase, high performance liquid chromatography with electrochemical detection (HPLC-ECD). To investigate the effect of E_2 on endogenous DA, release cells were grown from the time of plating, in a defined medium containing one of a range of concentrations of E_2 from 10^{-14} to 10^{-6}M. They were subjected to release studies as outlined above and the DA output under different steroidal environments was again measured by HPLC - ECD.

2.5.4. Sample Preparation for HPLC-ECD.

2.5.4.1. Adjustment of pH. On the day of assay, 0.5ml samples were thawed on ice and spiked with 3,4, dihydroxybenzylamine (DHBA, Sigma) at a concentration of 2.5ng/ml, which acted as an internal standard (see Section 2.5.2.3). The samples were originally stored in 0.1M PCA which lowered the pH to approximately 2.5. However it is reported that alumina absorbs amines more efficiently when the sample is adjusted to an alkaline pH (Bouloux et al 1985). The pH of the samples was therefore raised to approximately 8.4 by adding 0.5ml of 0.5M Tris - EDTA (pH 8.6) buffer prior to extraction, (sample pH was typically in the range 8.40 - 8.42, mean ± s.e.m. = 8.41 ± 0.004, n=5).

2.5.4.2. Extraction. DA was removed from the samples using commercially produced alumina cartridges, in conjunction with a vacuum system (Speed-mate 30) which allowed accurate regulation of the rate at which the samples passed through the alumina. Prior to extraction the cartridges were "primed" by rinsing through 0.5ml aliquots of the following solutions:

i. 3 x 50% methanol.
ii. 2 x 50% acetonitrile
iii. 3 x HPLC grade H_2O
iv. 3 x Tris- EDTA buffer (pH 8.5).

This procedure significantly enhanced the recovery of the amines. Samples were loaded onto the primed cartridges in 1ml volumes and passed through the alumina at a rate of 1ml per min which was determined to be the optimal speed (Table 13.0). The cartridges were then washed with 2 x 0.5ml aliquots of HPLC grade H_2O before elution of the amines in 200µl of 0.1M PCA. Recovery was typically in the region of 60% although there was batch-to-batch variation in the efficiency of the alumina cartridges. The percentage recovery was established for each batch prior to use and the variation in recovery was subsequently accounted for in the calculation of the results. As an example, for one batch of cartridges employed, the mean percentage recovery ± s.e.m. was 66.4% ± 6.0, n=6.

2.5.4.3. Optimization of the Extraction Procedure for DA. The procedure for the extraction of the DA and the other catecholamines was optimized by modification of both samples and cartridges as described above. Other modifications included altering the rate at which the sample was passed through the cartridge and changing the volume of 0.1M PCA used to elute the amines. Details of the optimization of the extraction procedure may be found in Table 13.0. Row 1 outlines the initial conditions and row 11 gives the optimal conditions which were subsequently used in the present study.

2.5.5. Content Studies.

In certain experiments the amount of DA held in intracellular storage sites within the hypothalamic cultures was also measured. The cells were scraped from the base of the culture dish in 0.5mls of 0.1MPCA and homogenized using mild sonication. The resulting "cell solution" was stored at -80°C until the time of assay. Sample preparation and measurement followed the procedures as detailed in Sections 2.5.2 and 2.5.4.

Table. 13.0. Optimization of the Procedure for DA extraction.

Modification	Approx. pH of sample	Volume of H_2O used to wash cartridges (ml)	Volume of MeOH used to wash cartridges (ml)	Volume of acetonitrile used to wash cartridges (ml)	Volume of Tris/EDTA buffer used to wash cartridges (ml)	Flow rate for sample through cartridge (ml/min)	Volume of 0.1M PCA used to elute the amines (µl)	Improvement of % recovery + = slight ++ = moderate +++ = good
1	2.5	1.0	----	----	----	5.0	1 x 200	Recovery < 15%
2	8.4	1.0	----	----	----	5.0	1 x 200	+++
3	8.4	1.5	----	----	----	5.0	1 x 200	++
4	8.4	1.5	0.5	----	----	5.0	1 x 200	+
5	8.4	1.5	1.5	----	----	5.0	1 x 200	++
6	8.4	1.5	1.5	0.5	----	5.0	1 x 200	++
7	8.4	1.5	1.5	1.0	----	5.0	1 x 200	++
8	8.4	1.5	1.5	1.0	1.5	5.0	1 x 200	+
9	8.4	1.5	1.5	1.0	1.5	1.0	1 x 200	+++
10	8.4	1.5	1.5	1.0	1.5	1.0	2 x 100	++
11	8.4	1.5	1.5	1.0	1.5	1.0	4 x 50	+++

2.6. Statistical Analysis.

The data obtained are presented as the mean ± s.e.m. (n=4). Significant differences compared with each control group were determined by use of Student's t - and Mann Whitney - U- tests.

CHAPTER THREE: RESULTS

3.1. Immunocytochemistry.

3.1.1. Tyrosine Hydroxylase ICC.

Using immunocytochemistry (ICC), it was possible to identify the presence of tyrosine hydroxylase immunopositive (THIP) neurones within the cell cultures.

3.1.1.1. Optimization. The technique of ICC involves a number of stages, each of which must be individually optimized in order to achieve specific and successful staining. It was important to arrive at optimal concentrations for both the primary and secondary antibodies and this was achieved by titration of the two antibodies, while keeping all other components of the procedure i.e. fixative, fixation time, incubation periods etc constant (Table 14.0). This allowed us to arrive at a satisfactory stain intensity whilst minimising non-specific binding of the antibodies to sites within the cultures. Low dilutions of the primary AB resulted in positive staining although background staining was significant and generally obscured the clarity of the neurones as shown in Plate. 1.0.c, as did low dilutions of the secondary antibody (Plate 1.0.b). When optimal concentrations of the two antibodies were employed background staining was minimal and did not significantly interfere with THIP neurone detection (Plate 1.0.a). Results of the optimization study are described in Table 14.0 and the primary and secondary antibodies were used subsequently at dilutions of 1:10 and 1: 100 respectively. When the DAB/PAP visualization method was used the PAP complex (tertiary layer) was employed at a dilution of 1:100.

Background staining was further reduced by an incubation stage after the primary antibody stage with NGoS, used at a dilution of 1:10. The serum saturated any sites to which the secondary antibody might bind non-specifically as it was raised in a goat and directed against mouse IgG. Higher concentrations had no further beneficial effect in reducing background staining, although the background signal was lower if the incubation period was 30 min as opposed to 10 min.

3.1.1.2. Peroxidase - antiperoxidase vs. Fluorescence ICC.

Initially the peroxidase - antiperoxidase/diaminobenzidine (PAP/DAB) method was employed to visualize the THIP neurones. The reaction product, due to its crystalline appearance provided a good degree of resolution and the processes (axons and dendrites) of THIP cells were easily distinguishable, bifurcating and extending over large distances of the culture plates. (Plate 2.0. A - D).

Table. 14.0. Optimization of TH ICC. Titration of the primary and secondary antibodies.

DILUTION		COMMENTS
PRIMARY ANTIBODY	SECONDARY ANTIBODY	
1:5	1:50	High background, obscuring any positive staining which may be present.
1:5	1:100	Good staining, obvious THIP neurones seen, but still high background
1:5	1:200	Good staining, low levels of background signal
1:10	1:50	More prominent staining, obvious large bipolar and multipolar neurones seen, but still high background signal
1:10	1:100	Good staining, backgound signal markedly reduced
1.10	1:200	No obvious THIP neurones seen
1:20	1:50	Excessive background with no THIP neurones observed
1:20	1:100	Faint staining, fluorescent signal only just detectable above background
1:20	1:200	No THIP neurones observed

As the primary antibody was expensive and in limited supply it was subsequently used at the highest dilution, which gave acceptable visualization (1:10), and the second antibody which was more readily available was used at a higher concentration (1:100), to further enhance the fluorescent signal.

However, although the PAP/DAB method was effective and allowed clear detection of the TH containing cells, its use was confined to single label studies and the technique employing the use of a fluorescent labelled second antibody was used to perform dual label studies allowing the detection of multiple antigens for co-localization studies.

Nevertheless, regardless of the visualization technique employed, it was possible to visualize THIP neurones with distinctly different morphologies.

3.1.1.3. Morphological Description.

Immunocytochemical studies revealed the presence of three morphologically distinct populations of THIP neurones. These were best characterised by the shape of their cell bodies and the number of processes. A unipolar neuronal type with an oval shaped cell body (Plate 3.0.a), was visualised as early as DIV 3, which was the earliest day studied. This neuronal cell type was however seen only during the initial stages of *in vitro* development and was considered either to mature into bipolar and/or multipolar neurones (see below) or to die as the culture aged.

The second type of neuronal morphology was characterised by an oval cell body and two processes extending from either pole of the cell body and hence were considered to be bipolar neurones. This cell type was prevalent during the early stages of development i.e. DIV3 (Plate. 3.0.b) to DIV7 (Plate 3.0.d) and increased in size (both in terms of perikarya size and neurite length) throughout the period of investigation, up to DIV 14 (Plate 3.0.f). It was frequently possible to observe a large opaque nucleus, surrounded by immunoreactive perikarya (Plate 3.0.d).

The third THIP neuronal cell type had characteristic fusiform or pyramidal shaped cell bodies with at least three processes (Plate 3.0.c, e and g for DIV 3, 7 and 14 respectively). As with the bipolar neurones, this sub-type increased in size as a function of culture age. By DIV 14 this sub-population of THIP neurones became the most prevalent of the three sub-types observed within this experimental model. Whether this depicts an increase in the number of multipolar cells which could possibly develop from the other morphological types, or the poor survival of the uni- and bipolar neuronal types is yet to be established. Varicosities were present on all three THIP neuronal populations and were more prominent during the initial stages of the investigation.

3.1.2. GABA ICC.

Immunocytochemical localization of GABA-ergic neurones within the hypothalamus was also performed using an antibody raised against the neurotransmitter itself. The majority of the GABA - immunopositive (GABA-IP) neurones had large, bipolar cell bodies with processes which underwent extensive bifurcation resulting in a complex dendritic/axonal network. Varicosities were clearly visible along the processes and growth cones were frequently observed at the nerve terminals.

3.2.3. Co-localization of TH and GABA Immunoreactivity.

Certain GABA-IP neurones were also found to contain TH immunoreactivity and the processes of this neuronal population could be traced over a greater distance when viewing the GABA fluorescence as compared with TH (Fig Plate 4.0. A - F).

Description of Plate. 1.0. Immunocytochemical visualization of tyrosine hydroxylase immunopostive (THIP) neurones in primary culture. The technique was optimized by titration of the primary and secondary antibodies as outlined in Section 3.1.1.1. Optimal staining, with minimal non-specific signal, was achieved using the primary and secondary antibodies at dilutions of 1:10 and 1:100 respectively (A). Higher concentrations of the antibodies produced marked background signal which obscured the visualization of the THIP neurones as shown in Figs. C and B for the primary and secondary antibodies respectively.

PLATE 1.0

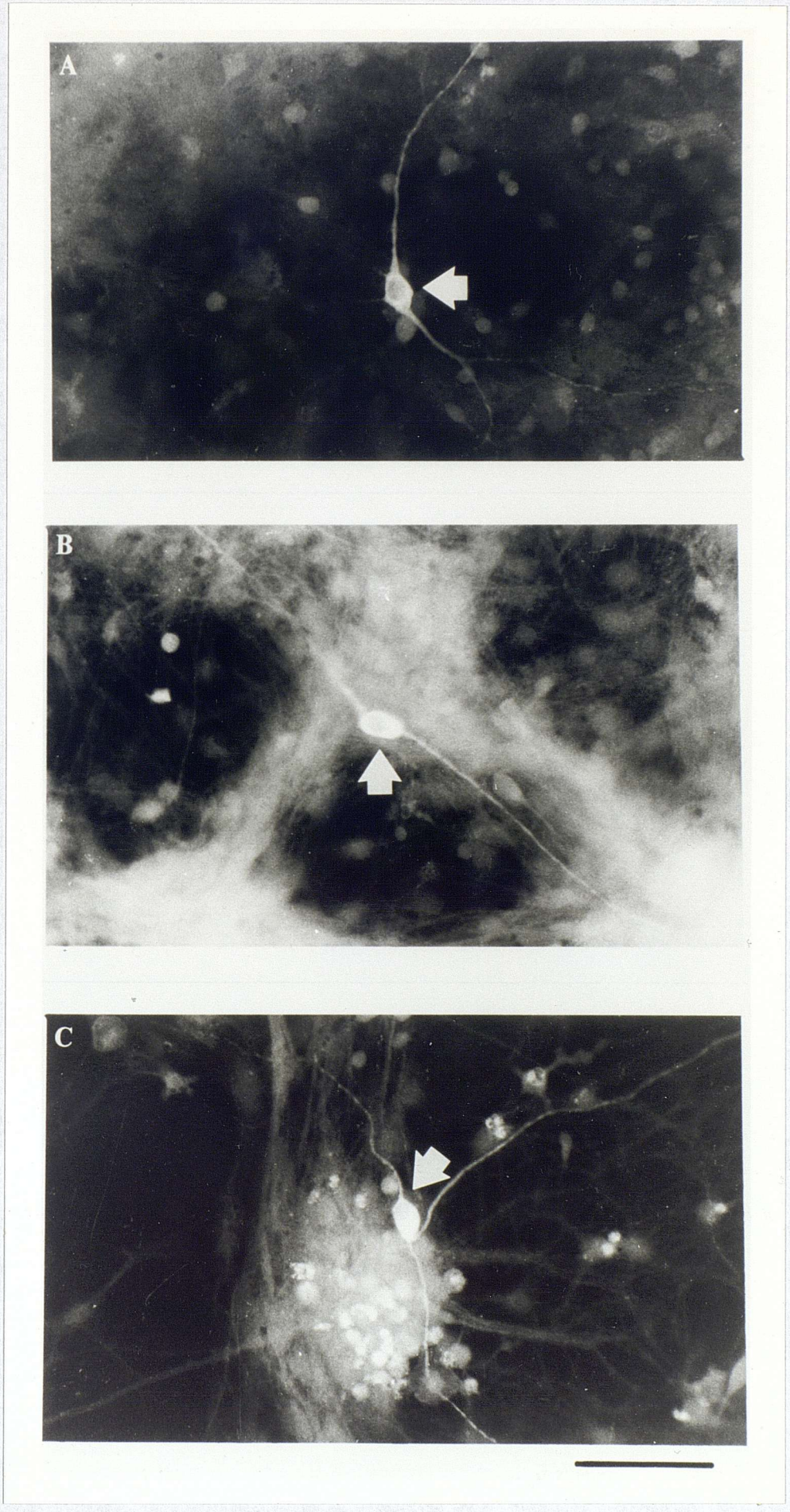
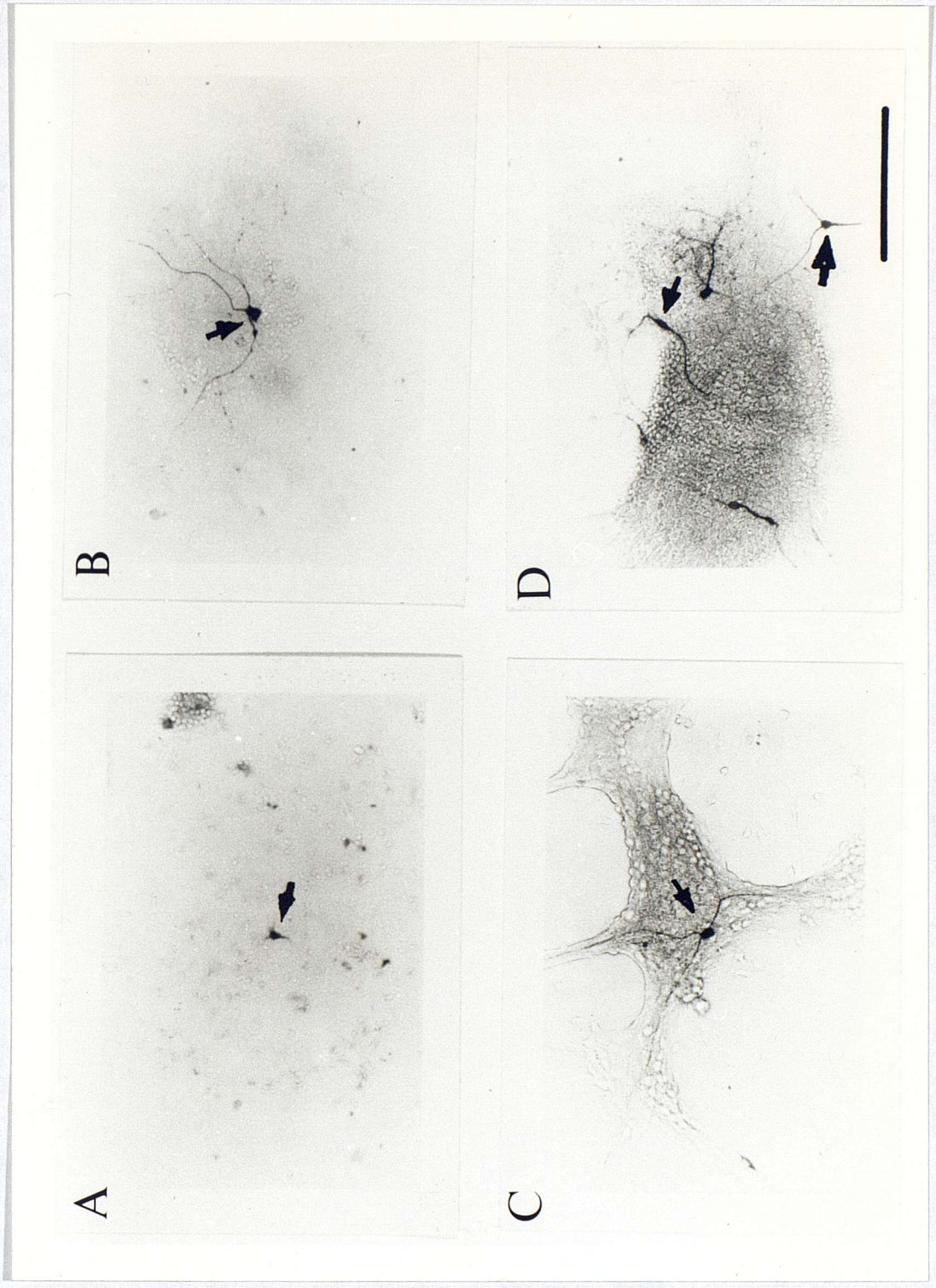
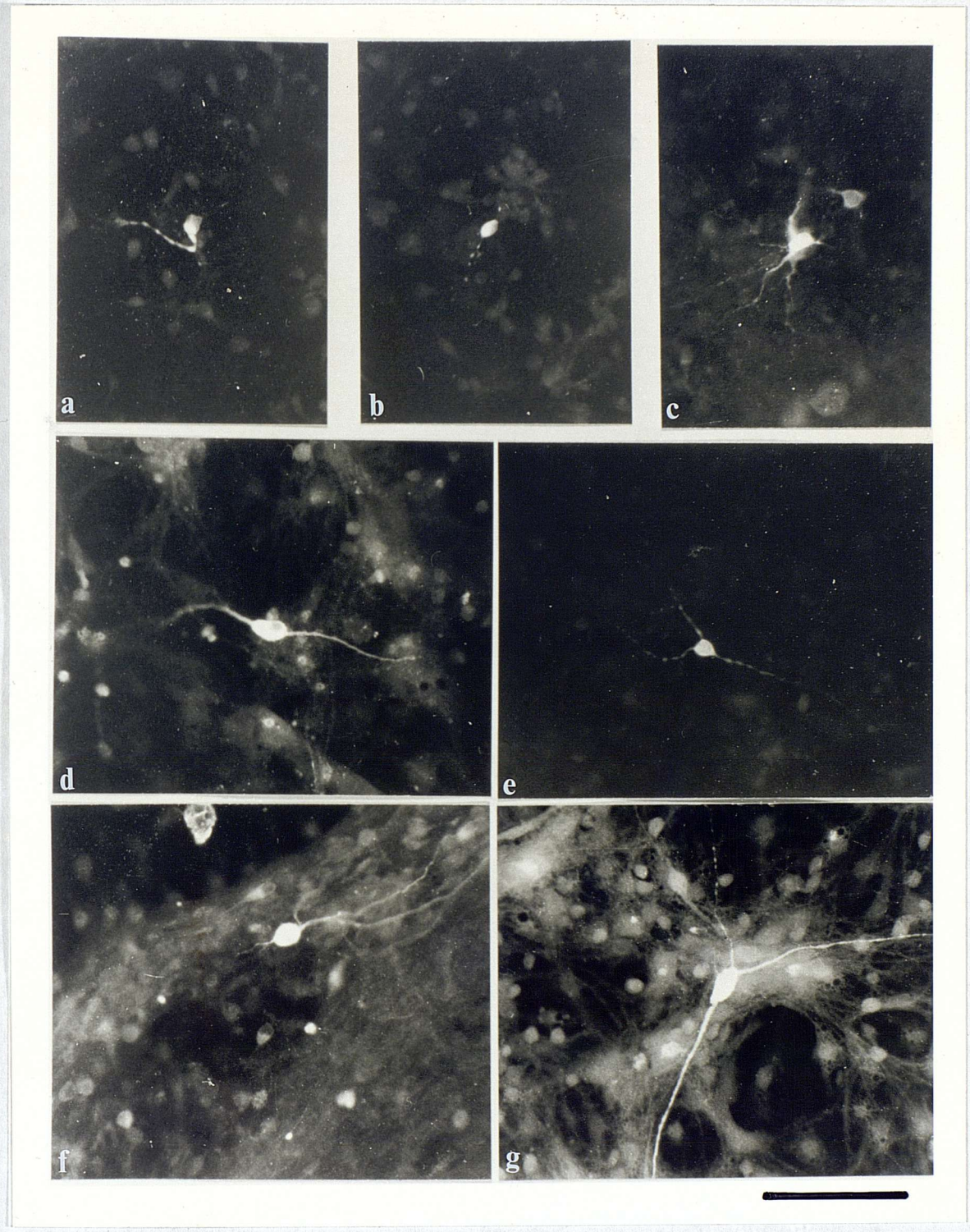


PLATE 2.0



Description of Plate. 2.0. Peroxidase-antiperoxidase immunocytochemical visualization of THIP neurones in primary cultures of foetal rat hypothalamic cells. Bar = 10μm.

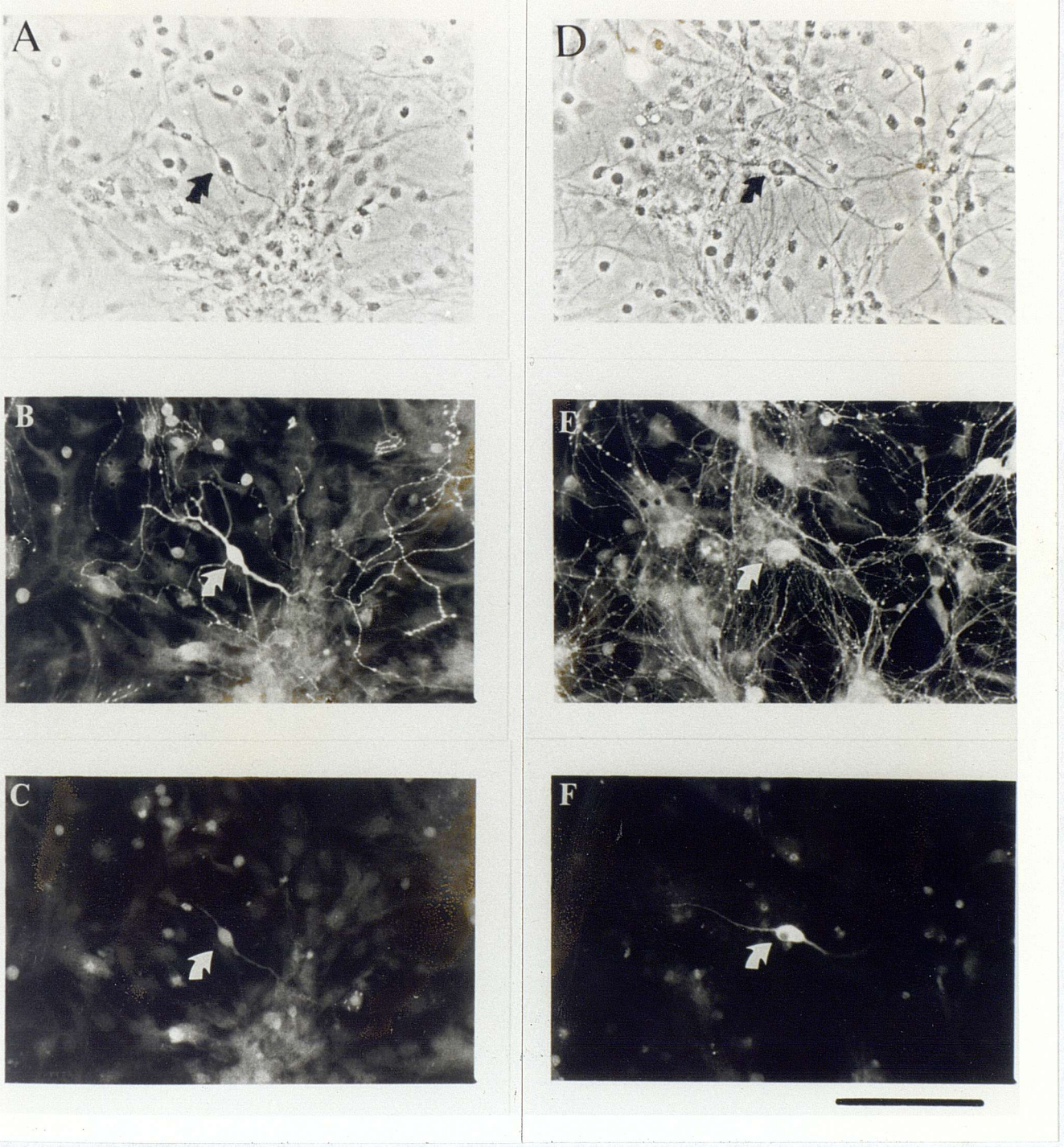
PLATE 3.0.



Description of Plate. 3.0. Immunocytochemical localization of THIP neurones in primary culture of foetal rat hypothalamic cells. Three distinct sub-types were seen on DIV 3, namely (a) a unipolar neurone seen only on the initial days in culture, (b) a bipolar type and (c) a multipolar type. The latter two sub-types persisted to later stages in culture. The bipolar type are shown for DIV 7 (d) and DIV 14 (f) and the multipolar type shown on DIV 7 (e) and DIV 14 (g). Bar = 20μm.

Description of Plate. 4.0. Co-localization of TH and GABA immunoreactivity in a single neurone. Figs A and D show two distinct neurones under light microscopy. Figs B and E show the same neurones displaying GABA immunoreactivity. Fig C shows the same neurone as shown in Figs A and B which was also stained for TH and fluorescence detected following this procedure was not markedly greater than the background signal and the neurones were thus considered to be TH immunonegative. Fig F shows the same neurone as shown in Figs D and E and when this neurone was co-stained for TH the neurone was found to be immunopositive. Bar = 10μm.

PLATE 4.0.



3.1.4. Discussion.

Using indirect ICC, it was possible to identify both TH and GABA immunopositive neurones within the hypothalamic cell culture preparation. Furthermore, neurones within each of these transmitter populations could be further subdivided on the basis of their morphology.

Regardless of the visualization system employed, we were able to locate three morphologically distinct subtypes of THIP neurones, which are known to have correlates *in vivo*. The uni- and bi- polar neuronal types reported in this *in vitro* investigation are similar to those observed under *in vivo* conditions (Daikoku et al 1986; Ugramov et al 1989a and b). A detailed study of the *in vivo* development of the dopaminergic systems of the hypothalamus, reported the presence of uni- and bi-polar THIP cells, detectable as early as E13, situated in the vicinity of the arcuate nucleus. The processes of these cells projected to the presumptive median eminence at a later gestational age (Ugramov et al 1989a). The authors concluded that the uni- and bi- polar neurones which they observed, constitute the TIDA neuronal system of the hypothalamus and on the basis of the morphological similarity, we propose that these subtypes as identified *in vitro* may belong to the same neuronal pathway.

In addition, the multipolar subtype is similar in morphology to a subset of THIP neurones reported by the same workers developing from as early as E15 in the area of the hypothalamus which would later form the zona incerta. It is thus reasonable to suggest that the multipolar THIP cells that we have identified belong to the rostral component of the ICHDA tract *in vivo*.

A strong correlation with *in vivo* observations also exists in relation to the morphological maturation of the THIP neurones. We noted a distinct increase in perikarya size and neurite length of TH containing cells over the 11 day period of investigation. On DIV 3, all three subtypes identified were "immature" looking, in terms of cell body size, and the short, varicose nature of their processes. By DIV 7, a striking increase in perikarya size was observed, in addition to extensive elongation and bifurcation of the neurites, a trend which continued up to DIV 14. A similar pattern for morphological development of dopaminergic neurones has been reported for the hypothalamus (Daikoku et al 1986; Ugramov et al 1989a)as well as for other dopaminergic systems of the CNS (Specht et al 1981). Within the hypothalamus, the *in vivo* development of THIP neurones is associated with an increase in TH expression, perikarya size and neurite length. Cell body growth is reported to begin

as early as E13, with neurite formation and elongation occuring slightly later (E15 - 16, Daikoku et al 1986).

For mesencephalic dopaminergic systems a similar pattern has been reported, although it is initiated at earlier gestational ages (E12.5 - 13.5) (Hemendinger 1980, Specht et al 1981). At this gestational age, TH - containing cells within the mesencephalon already possess at least one and frequently two processes, one of which may have undergone bifurcation. In contrast, by E12 hypothalamic dopaminergic neurones, if visible at all, possess very short weakly stained neurites and this suggests that the morphological development of hypothalamic dopaminergic systems "lag" behind that of mesencephalic dopaminergic pathways. We cannot, with our present findings, provide conclusive evidence for this, but in terms of functional development, evidence suggests that the diencephalic dopaminergic systems are retarded in relation to those of the mesencephalon (Engele et al 1989). A direct long-term comparison of the morphological development of mesencephalic and diencephalic dopaminergic systems *in vitro* remains to be performed.

ICC visualization of hypothalamic GABA- ergic systems highlighted a difference in the pattern of morphological maturation between these two neurotransmitter systems. GABA -IP neurones as visualized by fluoresence ICC appeared to develop rapidly and as early as DIV 3, GABA -IP cells were large with neurites which, although generally short, bifurcated extensively forming complex neuronal networks. We failed to see any obvious increase in cell size over the period of the investigation. It is interesting to speculate that, unlike the hypothalamic dopaminergic systems, the hypothalamic GABA-ergic systems within this model undergo the major part of their morphological development during earlier stages and that once re-established in culture conditions these cells initially grow at a much more rapid rate than their dopaminergic counterparts. This may constitute an important developmental difference between hypothalamic DA and GABA systems and it is possible that such a phenomenon may influence both the activity of these neurotransmitter systems and the role they play at later stages of postnatal development and in the regulation of neuroendocrine function. Furthermore, these observations suggest that certain aspects of pre-programmed developmental patterns are conserved under *in vitro* conditions.

Neuro-neuronal interactions are widely believed to influence cell development. This concept, however, does not exclude the possible influence of glial cells on neuronal development and a number of studies have associated glial cells with the morphological maturation of DA containing cells. A recent investigation reported that the type of glial cells with which foetal dopaminergic neurones were co-cultured,

influenced the final morphology of the cells (Denis Donini et al 1984). If cells from the mesencephalon were cultured on glial cells derived from striatal tissue, the neurones exhibited a bipolar morphology projecting long, linear processes, with occasionally shorter processes around the cell body. In contrast, those cells grown on glial cells from a mesencephalic origin demonstrated a markedly different morphology with larger THIP neurones possessing numerous long and extensively bifurcating neurites. From these observations the authors suggested that the DA neurones, when allowed to grow on top of glial cell monolayers, exhibited a morphology dictated by the glial cell type underneath. A molecular basis for such an influence is suggested by the work of Lieth et al (1989) who proposed that different glial cell populations exhibit differential cell surface characteristics with respect to the composition of proteins and glycoproteins. Thus, the influence of cell -to- cell contacts is likely to operate in addition to the effect of soluble CSF borne factors. To our knowledge, an investigation of glial influences on the development of hypothalamic dopaminergic systems has not been performed but, as most of them project locally i.e. they innervate other regions within the hypothalamus, it is likely that if glial cells are associated with their development, they will be present within the culture preparation used and will be able to exert their regulatory effect. Also, as the hypothalamus contains a heterogeneous population of dopaminergic neurones, it is reasonable to suggests that different glia types may differentially regulate the development of each distinct dopamine containing neuronal population.

The co-existence of DA and GABA in a single neurone has been reported *in vivo* for several brain regions including the hypothalamus. Wulle and Wagner (1990) reported localisation of GABA and tyrosine hydroxylase in a "simple" neurone model, i.e. the vertebrate retina. In addition, co-localisation of the two neurotransmitters has also been reported within cells occupying the region of the arcuate nucleus (Leranth et al 1985). Our dual staining ICC studies highlighted the fact that TH and GABA were localized in the same cell although they were not co-localized in all the processes, i.e. some processes of the same cell were immunopositive for both TH and GABA while others were positive only for GABA (Plate. 4.0). This may reflect differential storage or release of the transmitters within the same neuronal pathway and may provide a means by which DA released from a cell which produces both DA and GABA, may feed back on the same cell either to augment or inhibit GABA release and vice-versa. Alternatively, it is interesting to speculate that during these early stages of development, differentiation is incomplete and that these double labelled neurones represent precursor cells which have not fully adopted a single, distinct phenotype. We cannot of course ignore the most obvious possibility that the sensitivity of the

ICC technique used to detect TH is not as great as that of GABA and hence fails to highlight the enzyme located in the more distal segments of the processes.

The functional significance of these findings is not clearly understood, but as DA and GABA are considered to be intimately associated with neuroendocrine regulation, their localisation within a single neurone/neuronal population may go some way to explaining the interrelationship shared by these two neurotransmitter subtypes. The present results emphasise the need for further investigation of certain aspects of neuronal interaction, including the extra - and intra- hypothalamic factors involved in the regulation of the morphological development of the neurotransmitter systems involved in neuroendocrine function.

3.2. Investigations with [^{3}H]DA.

[^{3}H]DA was used to investigate parameters of uptake and release in the dopaminergic neurones in our experimental preparation. Similar studies were performed with [^{3}H]GABA to monitor the activity of the GABA-ergic systems intrinsic to the hypothalamus the results of which are detailed in Section 3.3. The results of these studies will be discussed together in section 3.3.3.

3.2.1. Uptake Studies.

Fig 3.1 demonstrates that the hypothalamic cultures accumulated [^{3}H]DA in a time and temperature dependent manner. Uptake increased with incubation time from 10 to 60 min, after which time it began to plateau. The cells, when incubated at 4°C, failed to take up the radiolabelled neurotransmitter to any appreciable extent (less than 5% of uptake at 37°C) and may represent non - specific accumulation. Subsequently, the value for uptake at 4°C was subtracted from the values for uptake at 37°C to give a measure of active uptake processes. As uptake was at a high yet submaximal level at 60 min, this incubation period was subsequently chosen for loading the DA neurones with radiolabelled neurotransmitter.

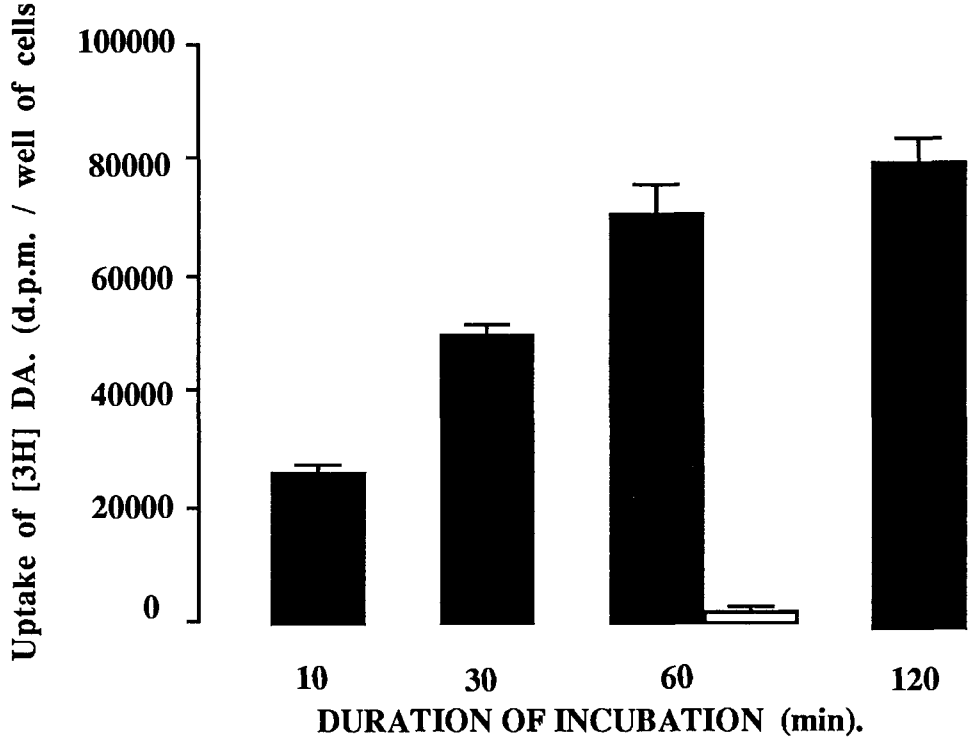


Fig . 3.1. Time course for the accumulation of [^{3}H]DA into foetal rat hypothalamic cells maintained in primary culture at 37°C (■) and 4°C (□). Cells were exposed to [^{3}H]DA (2μCi/well of cells 3.3 x 10^{-7}M), for increasing periods of time before terminating the reaction and measuring the amount of label taken up as described in Section 2.3.2.

Uptake studies were performed at different time points in culture from DIV 3 to DIV 14 (Fig 3.2). On early days in culture, viz. DIV 3, specific uptake was relatively low. A slight but significant increase in uptake occurred by DIV 5. Thereafter, uptake increased dramatically up to DIV 11 after which time it began to plateau.

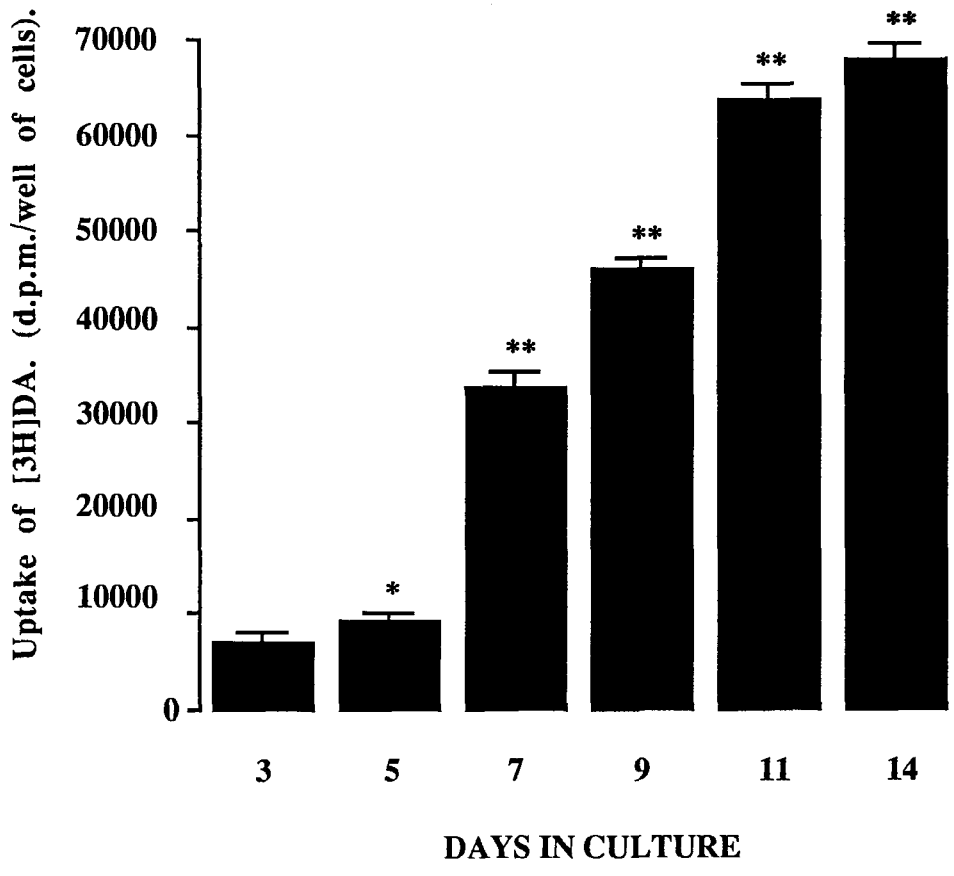


Fig. 3.2. Ontogenesis of [^{3}H]DA uptake into foetal rat hypothalamic cells in primary culture. Results represent the mean ± s.e.m., n=4. * $p < 0.005$, ** $p < 0.002$ compared with DIV3.

Kinetic analysis of the uptake of [^{3}H]DA revealed marked changes as the cultures matured (Table 15.0.). The value of K_m decreased progressively on DIV 3 and 7 and 14 ($p< 0.005$) and there was a conco mitant increase in the value of V_{max}.

Table 15.0. Apparent kinetic parameters for the uptake of [^{3}H]DA into hypothalamic cells in primary culture on different days in culture.

DIV	K_m (μM)	V_{max} (pmoles/min/well of cells)
3	56.23± 3.01	3.2 ± 0.075
7	7.0 ± 1.3	4.27 ± 0.09
14	3.98 ± 0.87	6.6 ± 0.54

The specificity of uptake i.e. whether the [^{3}H]DA was being taken up into dopaminergic neurones, was investigated using two DA uptake inhibitors, benztropine and nomifensine (Fig. 3.3). Both drugs reduced uptake in a dose-dependent fashion, with the highest concentration tested (10^{-4}M), significantly attenuating uptake by 58.6% ± 6.7 and 69.5% ± 0.5, for nomifensine and benztropine, respectively. We were not able, however, to abolish uptake totally with the range of concentrations used in the present study.

The activity of the uptake inhibitor nomifensine (10^{-6}M), increased with advancing culture age (Fig. 3.4). On DIV 3 and DIV 5, the inhibitor was relatively ineffective in blocking uptake but by DIV 7, nomifensine blocked uptake by 16.5 ± 1.1% and percentage inhibition rose to 44.5 ± 2.4 % by DIV 11.

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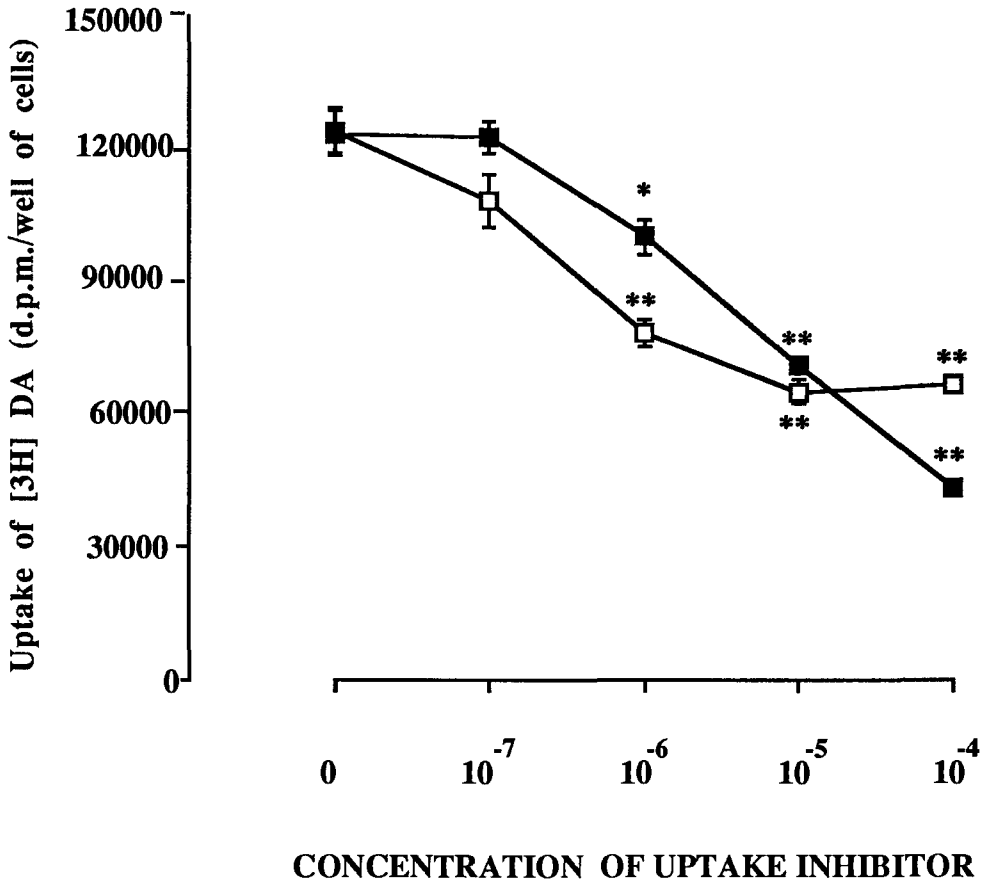


Fig. 3.3. Dose-dependent relationships for nomifensine (—□—) and benztropine (—■—) in inhibiting uptake of [^{3}H]DA into foetal rat hypothalamic cells in primary culture (DIV 10). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p < 0.002$ compared with the control value.

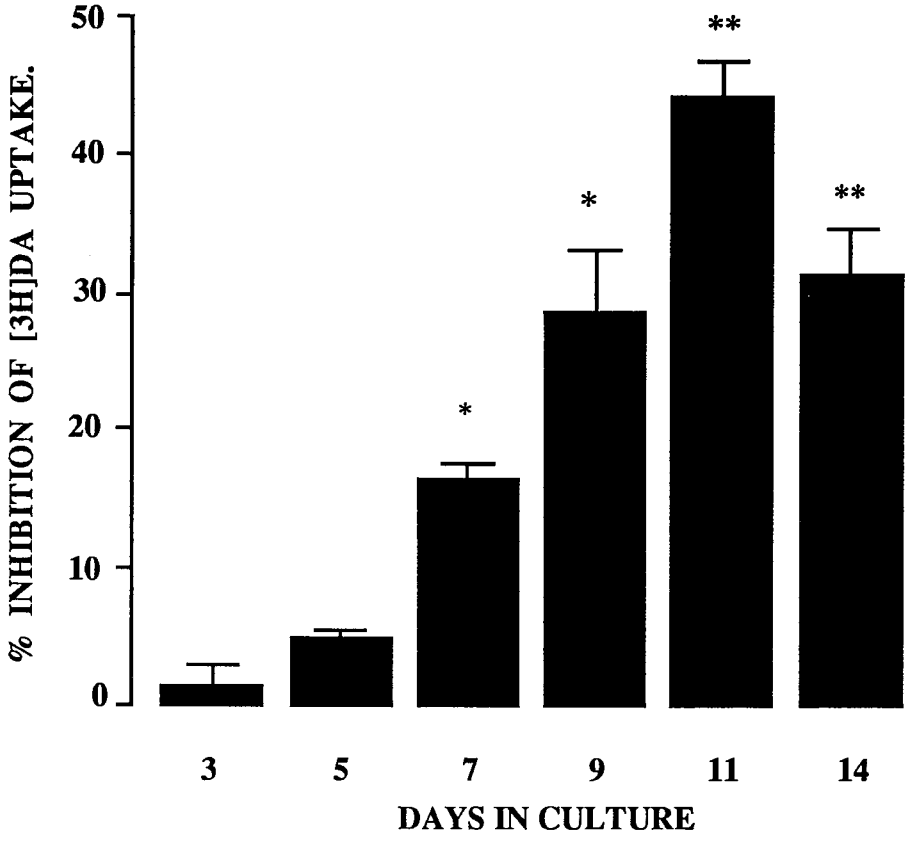


Fig. 3.4. Ontogenetic pattern for inhibition of uptake of [^{3}H]DA into cultured foetal rat hypothalamic cells. Uptake experiments were performed on different days in culture as detailed in Section 2.3.2, both in the absence or presence of the specific uptake inhibitor nomifensine at a concentration of 10^{-6}M. Results represent the mean ± s.e.m., n=4. * $p < 0.05$, ** $p < 0.02$, compared with the percentage inhibition on DIV3.

The ability of the natural ligand of the uptake process to block [^{3}H]DA uptake was also investigated. Thus graded concentrations of unlabelled DA were added to the labelled DA (3.3×10^{-7}M) during the 60 min loading period and produced a dose - dependent inhibition of uptake, with the highest concentration of "cold" DA (10^{-4}M) inhibiting uptake by 45% ($p < 0.005$, Fig. 3.5., solid bars).

This observation was expected if the unlabelled DA was in competition with [^{3}H]DA for the uptake sites, but the degree of inhibition was not as great as with the uptake inhibitors. A number of reports have suggested that the uptake of DA is regulated via a DA autoreceptor pathway whereby high levels of DA activate receptors (probably D_2), in order to promote its own removal from the synaptic cleft (Demarest & Moore, 1979a). To investigate this possibility, we studied the ability of a single concentration (10^{-9} M) of the D_2 receptor antagonist, sulpiride, to influence uptake in the presence and absence of "cold" DA (Fig. 3.5). Whereas sulpiride alone and DA alone at a

concentration of 10^{-8}M had no overall effect on [^{3}H]DA uptake, in combination uptake was markedly reduced by approximately 60% ($p < 0.001$). This inhibition was reversed in a dose - dependent manner by increasing the concentration of the "cold" DA. These results suggest that DA promotes [^{3}H]DA accumulation via a DA - receptor mediated mechanism which is reversed by sulpiride.

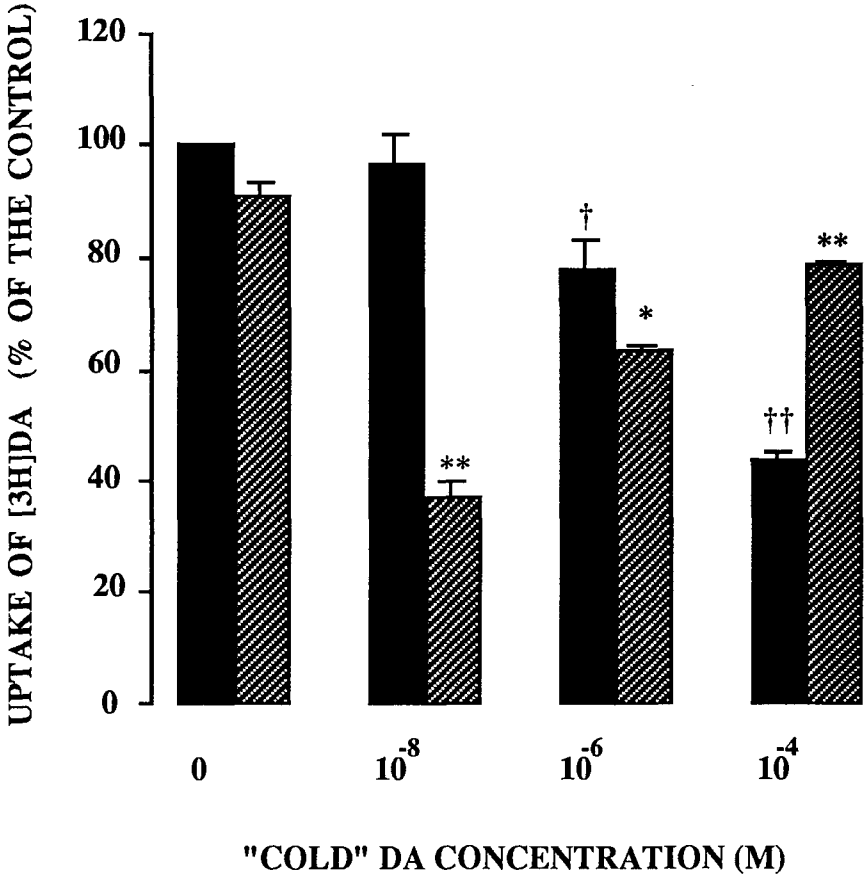


Fig 3.5. Effect on the uptake of [^{3}H]DA into foetal rat hypothalamic cells in primary culture by co - incubation with "cold" DA alone (■), or "cold" DA in the presence of the D_2 antagonist, sulpiride, at a concentration of 10^{-9} M, (▨). Results represent the mean ± sem. n = 4. † $p < 0.05$, †† < 0.005 for [^{3}H]DA uptake in the presence of "cold" DA compared with [^{3}H]DA uptake in the absence of "cold DA". * $p < 0.05$, ** p , 0.001, for [^{3}H]DA uptake in the presence of sulpiride compared with in its absence.

Fig. 3.6 shows that increasing concentrations of "cold" DA may also reverse the inhibitory effects of nomifensine (10^{-4}M) on [^{3}H]DA accumulation, suggesting that DA itself regulates the uptake process and may overcome the effect of DA transporter blockade.

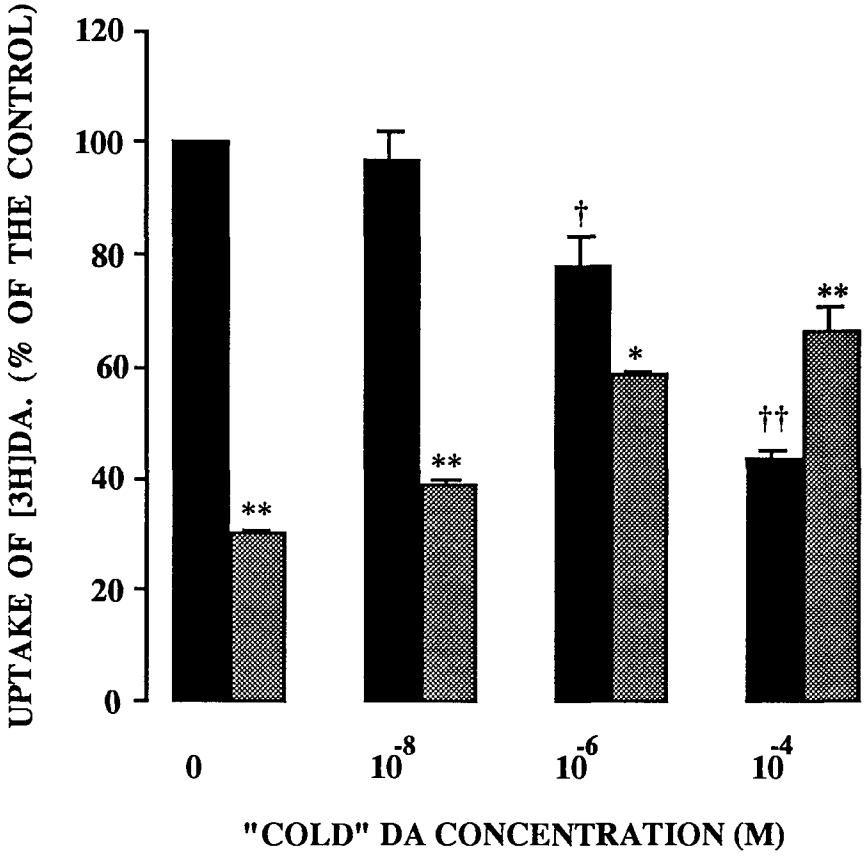


Fig. 3.6. Effect on the uptake of [^{3}H]DA into foetal rat hypothalamic cells in primary culture by co-incubation with "cold" DA alone (■), or "cold" DA in the presence of the uptake inhibitor, nomifensine at a concentration of 10^{-4}M (▩). Results represent the mean ± sem. n = 4. † $p < 0.05$, †† < 0.005 for [^{3}H]DA uptake in the presence of "cold" DA compared with [^{3}H]DA uptake in the absence of "cold DA". * $p < 0.05$, ** $p < 0.001$, for [^{3}H]DA uptake in the presence of nomifensine compared with uptake in its absence.

3.2.2. Release Studies.

The release of accumulated [^{3}H]DA from the hypothalamic cell cultures was investigated under both basal and stimulated (56mM K^+), conditions (Fig. 3.8) and both were significantly reduced by the exclusion of Ca^{2+} from the release medium, (* $p < 0.01$, ** $p < 0.002$, Fig. 3.7). Over a 7 min incubation period depolarization produced a threefold increase in the amount of [^{3}H]DA released when compared with basal levels (** $p < 0.002$). On repeated stimulation [^{3}H]DA release declined rapidly ($p < 0.01$). After a third stimulation, extraction of the cells indicated the presence of an appreciable intracellular pool of [^{3}H]DA which was not releasable even by

depolarization. As levels of [^{3}H]DA tended to decline after the initial stimulation, subsequent experiments used a single 56mM K^+ stimulation only.

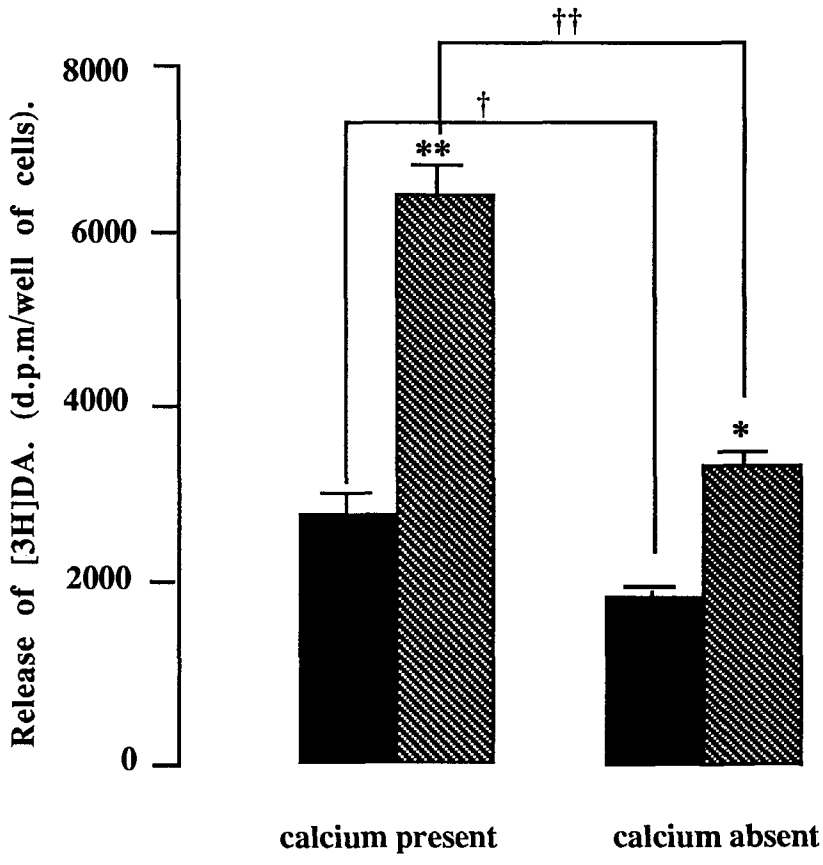


Fig. 3.7. The effect of exclusion of Ca^{2+} from the release medium on basal (■) and 56mM K^+ - stimulated (▨) release of [^{3}H]DA from foetal rat hypothalamic cells maintained in primary culture. (DIV 9). Results represent the mean ± s.e.m., n=4. * $p < 0.05$, ** $p < 0.002$ for basal vs K^+ induced release, † $p < 0.05$, †† $p < 0.002$, for basal and K^+ induced release in the presence vs absence of Ca^{2+}.

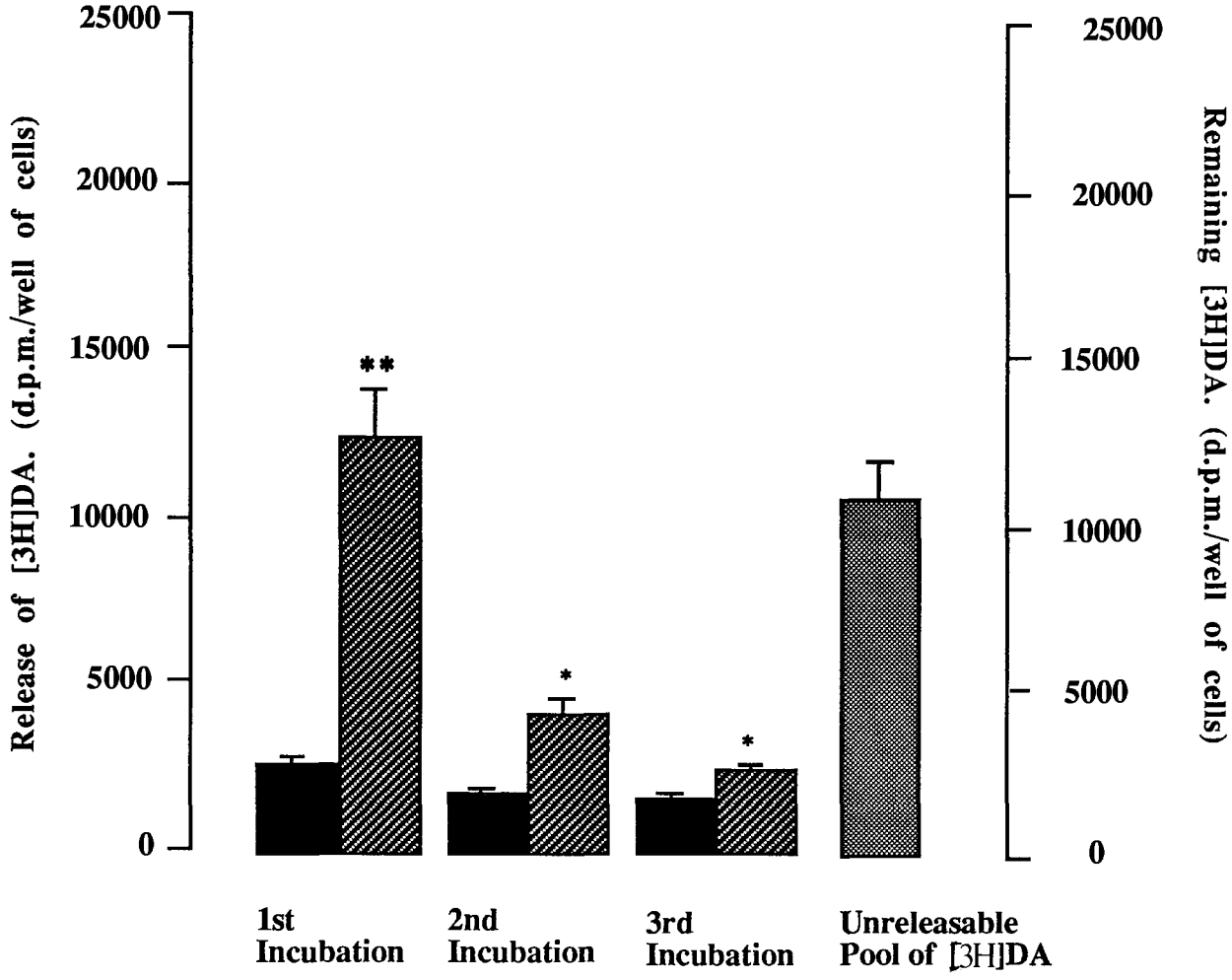


Fig. 3.8 The release profile for [^{3}H]DA on day 7 *in vitro*. The hypothalamic cell cultures were preincubated with [^{3}H]DA as outlined in 2.3.2 Once a steady state was attained three successive 7 min incubations were performed either under basal conditions (■) or in the presence of 56mM potassium (▨). The final column (▩) represents the non-releasable pool of [^{3}H]DA as indicated by the [^{3}H]DA content of the cells at the end of the release experiments. Results represent the mean ± s.e.m. n=4. *$p < 0.01$, ** $p < 0.002$ for basal v's 56mM K^+ - induced release.

When the release experiment was repeated, employing a 20 min incubation period, a different response was observed (Fig 3.9). Depolarization induced release was only 2 fold greater than basal release and the decline incurred by repeated stimulation occurred more rapidly. Indeed on the second and third incubation, 56mM K^+ - stimulated release was significantly lower than basal release and similar results were found with 60 min incubation periods (data not shown). As these observations suggested that longer incubation periods results in rapid depletion of available

radiolabelled neurotransmitter stores, we adopted a 7 min incubation period for all subsequent release studies.

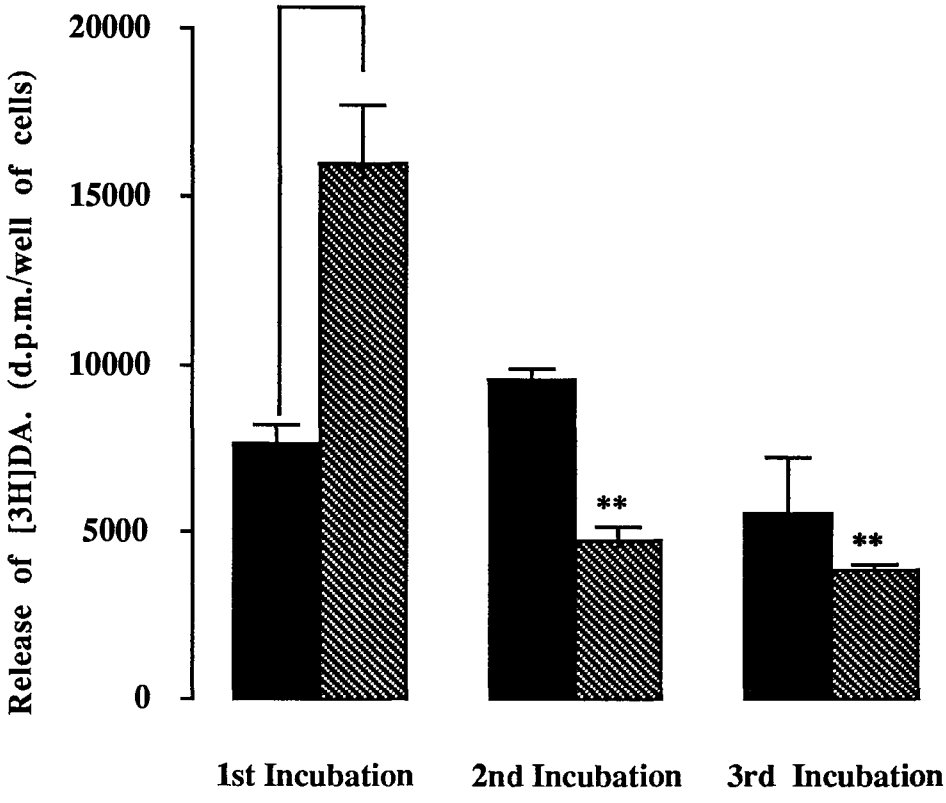


Fig. 3.9. The release of [^{3}H]DA during a 20 min incubation period. * $p < 0.05$ for basal vs K^+ induced release, ** $p < 0.01$ for K^+ induced release during the 1st incubation compared with that during the 2nd and 3rd incubation periods.

Both basal and 56mM K^+ - induced release of radiolabelled DA increased as a function of culture age. The greatest increase occurred between DIV 7 and DIV 9, (Fig. 3.10) with a further, less dramatic, increase at DIV 14. In the present investigation, release experiments were not performed on DIV 3 and DIV 5 as the uptake was low on these days and adequate label was not accumulated to make subsequent release experiments viable.

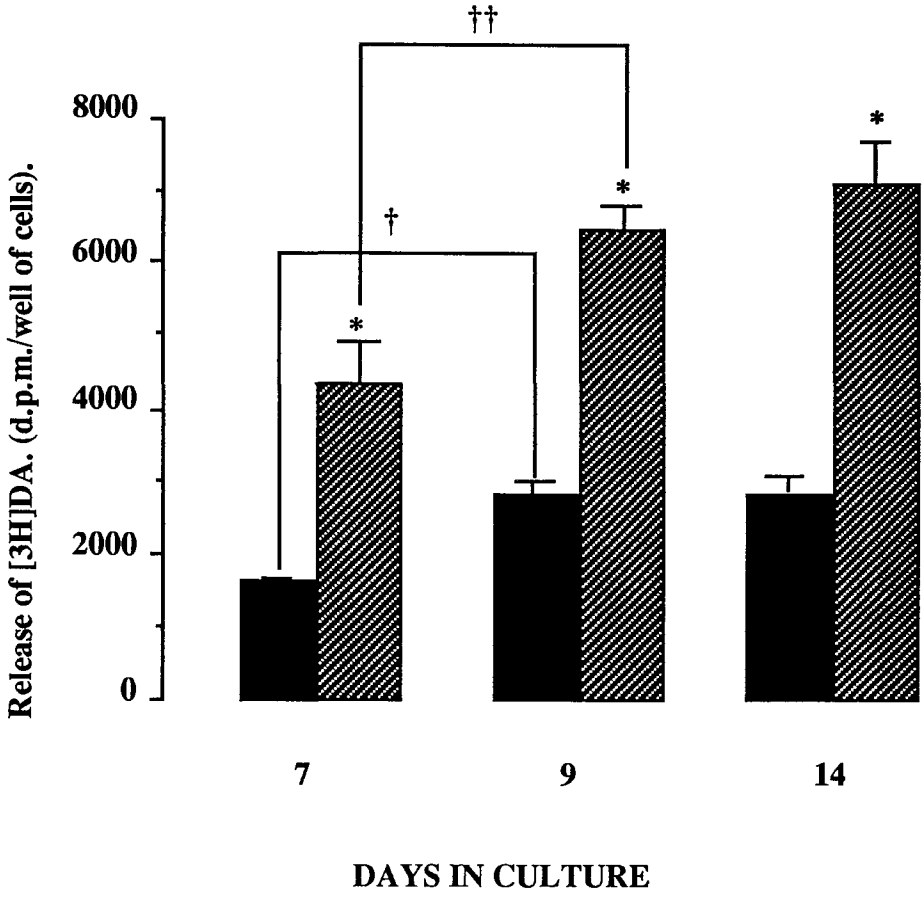


Fig 3.10. Basal ( ) and 56mM K^+ - stimulated ( ) release of [^{3}H]DA from a single batch of hypothalamic cells in primary culture during a 7 min incubation period. Release experiments were performed on different days in culture. Results represent the mean ± s.e.m. n=4. *$p < 0.005$, basal vs 56mM K^+ - induced release, † $p < 0.025$, †† $p < 0.01$ [^{3}H]DA release on DIV 7 vs DIV 9.

3.3. Investigations with [^{3}H]GABA.

3.3.1. Uptake Studies.

Uptake of [^{3}H]GABA was time - dependent and began to plateau after only a 10 min exposure to the radiolabelled neurotransmitter (Fig. 3.11). There was an overall increase in the uptake of [^{3}H]GABA over the investigational period, which followed a pattern distinctly different to that observed for [^{3}H]DA (Fig. 3.12). On DIV 3 uptake was relatively high and a significant increase in uptake occurred between DIV 3 and DIV 7 in culture with levels of radiolabel increasing from approximately 7, 000 to 14, 000 d.p.m./well of cells over a 4 day period. However between DIV 9 and DIV 15 there was no significant change in the level of uptake while on DIV 18 a further, significant rise in the accumulation of the [^{3}H]GABA was observed.

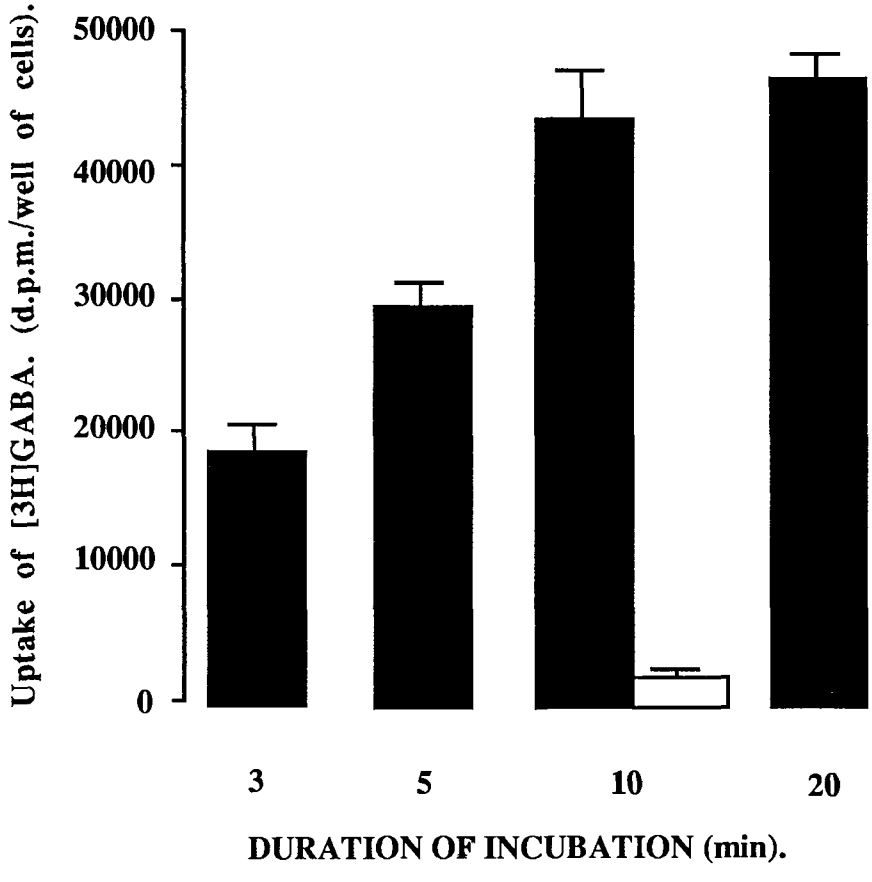


Fig. 3.11. Time course for the accumulation of [^{3}H]GABA by cultured hypothalamic cells at 37°C (■) and 4°C (□). Cells were exposed to [^{3}H]GABA (0.1μCi/well of cells - 2.5 x 10^{-8}M), for increasing periods of time before terminating the reaction and measuring the amount of label taken up as described in 2.3.4. Results represent the mean ± s.e.m. n = 4.

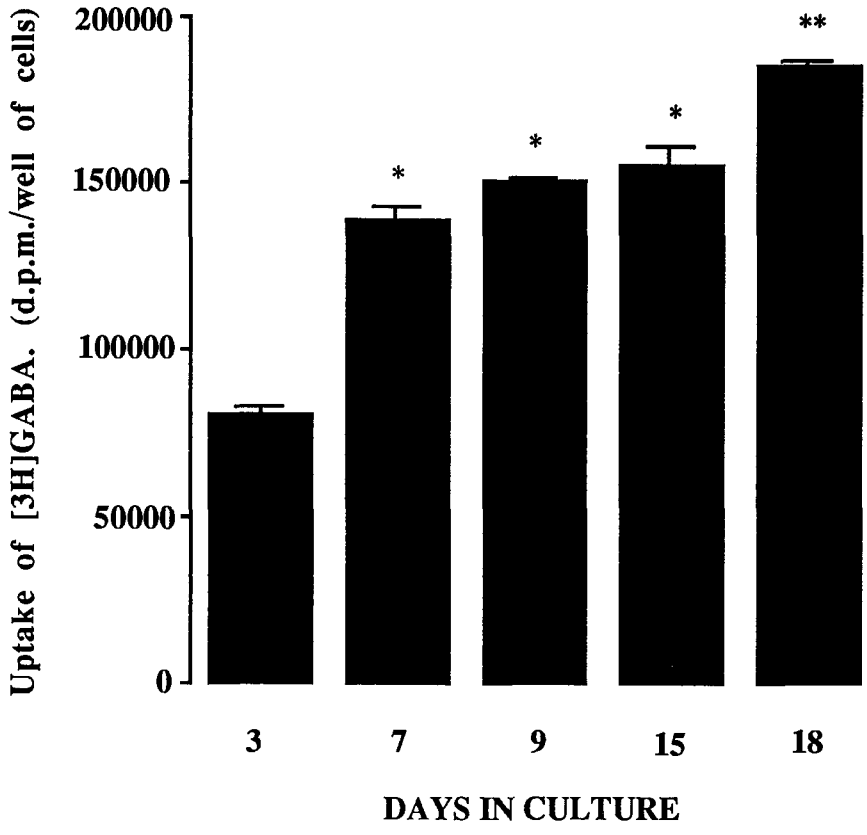


Fig. 3.12. Ontogenesis of the uptake of [^{3}H]GABA into foetal rat hypothalamic cells maintained in primary culture. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p.< 0.01$, compared with uptake on DIV 3.

Analysis of the kinetics of [^{3}H]GABA uptake revealed that there were significant changes in the values of both the K_m and V_{max}. The value for the Michaelis - Menten Constant decreased from 2.29 ± 0.4 to 1.0 ± 0.14 uM from DIV 3 to DIV 7 and remained constant between DIV 7 and DIV 14. Furthermore the value for V_{max} increased from 6.622 ± 0.213 to 10.85 ± 2.9 nmoles/min/well of cells between DIV 3 and DIV7 but there was no significant difference in the rate of uptake between DIV 7 and DIV 14 (Table 16.0).

Table. 16.0. Apparent kinetic parameters for the uptake of [^{3}H]GABA into hypothalamic cells in primary culture on different days in culture.

DIV	K_m (μM)	V_{max} (nmoles/min/well of cells)
3	2.3 ± 0.4	6.622 ± 0.213
7	1.0 ± 0.14	10.85± 2.9
14	0.9 ± 0.19	11.64± 2.1

The selective inhibitor of GABA uptake into neurones, SKF 89976 A inhibited uptake in a dose - dependent fashion with a 95% inhibition at 10^{-3}M (Fig. 3.13), suggesting the presence of a specific uptake process.

A single concentration of SKF 89976 A (10^{-5}M) was employed to investigate the ontogeny of the inhibition of the [^{3}H]GABA mechanism. On early days in culture (DIV 3) inhibition of uptake was as great as 52 ± 3.9%, but percentage inhibition decreased on DIV 8 to 38.5 ± 1.9% and declined further on DIV 15 to 21± 3.6% (Fig. 3.14).

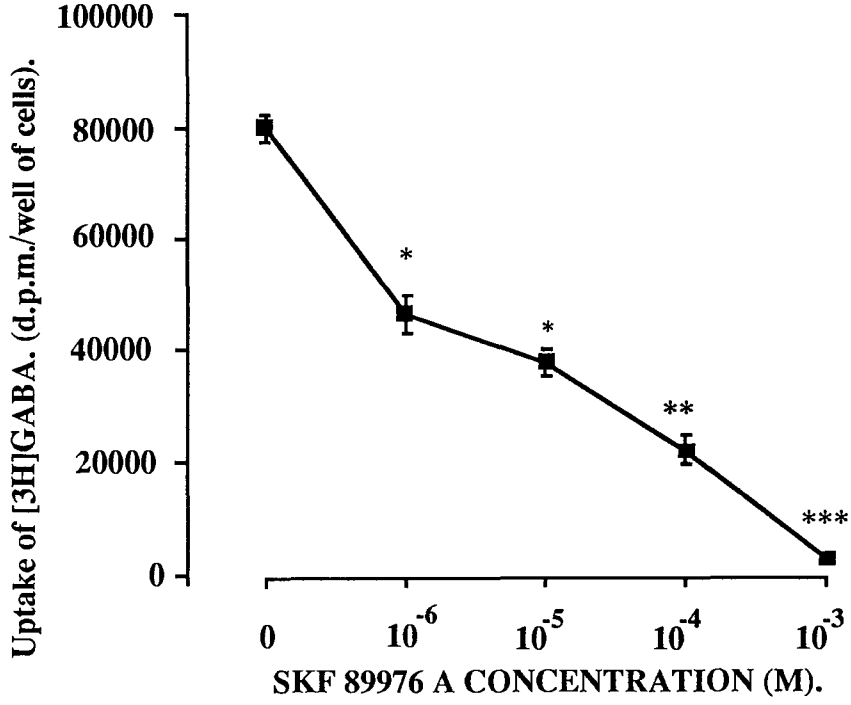


Fig. 3.13. Dose-dependent relationship for the specific inhibitor of GABA uptake into neuronal cells, SKF 89976 A in inhibiting uptake of [^{3}H]GABA into foetal rat hypothalamic cells in culture, (DIV 3). Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control.

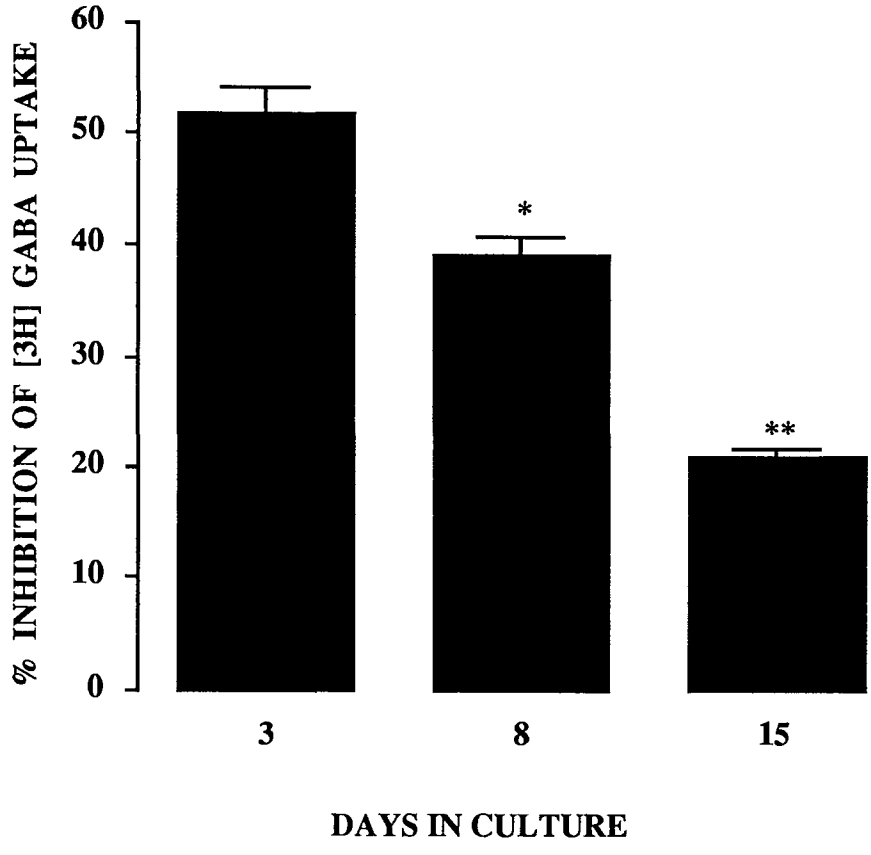


Fig. 3.14. Ontogenetic pattern for the inhibition of uptake of [^{3}H]GABA into foetal rat hypothalamic cells in primary culture. Uptake experiments were performed on DIV 3, 8 and 15 as detailed in Section 2.3.4., either in the absence or presence of the specific GABA uptake inhibitor, SKF 89976 A, at a concentration of 10^{-5}M. Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p.< 0.02$ compared with the inhibition observed on DIV 3.

Repeating the uptake experiments for [^{3}H]GABA in the absence of extracellular Na^+ revealed a transience in the Na^+ dependency of the uptake mechanism. The uptake mechanism displayed only slight Na^+ - dependency during initial stages of cultivation but the degree of dependence increased with extending periods *in vitro* as shown by the changes in the percentage uptake in the absence of Na^+ (Fig 3.15).

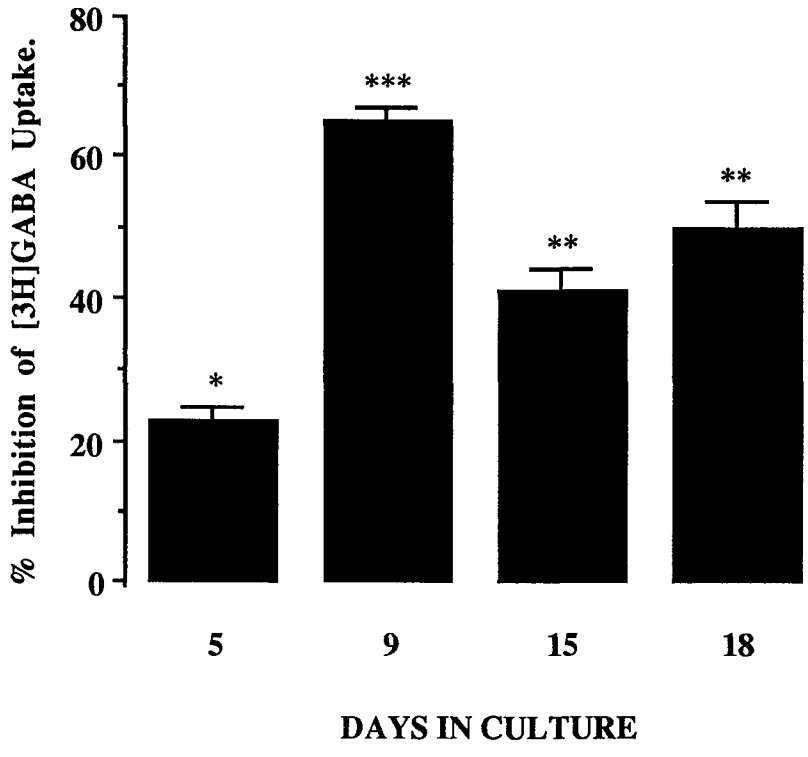


Fig. 3.15. The effect of omitting Na^+ from the loading medium on the uptake of [^{3}H]GABA into hypothalamic cells maintained in primary culture. Results represent the mean ± s.e.m. n = 4. *p < 0.05, ** p < 0.02 *** p < 0.001, compared with uptake in the presence of Na^+.

3.3.2. Release Studies.

The 56mM K^+ induced release of [^{3}H]GABA was 5 to 6 fold greater than that induced under basal conditions and was significantly reduced when Ca^{2+} was omitted from the release medium, (Fig. 3.16., * p < 0.05, **p < 0.002), although this reduction was not as profound as that observed for [^{3}H]DA.

Fig 3.17 illustrates the effect of repetitive stimulation on the release of [^{3}H]GABA. In contrast to the release of [^{3}H]DA, the amount of [^{3}H]GABA released during the second and third stimulation was significantly greater than during the first stimulation although there was no significant difference between the two.

[^{3}H]GABA release experiments did not demonstrate an increase in the amount of neurotransmitter released with increasing culture age at any time point studied during the period of investigation from DIV 5 to DIV 18 (data not shown).

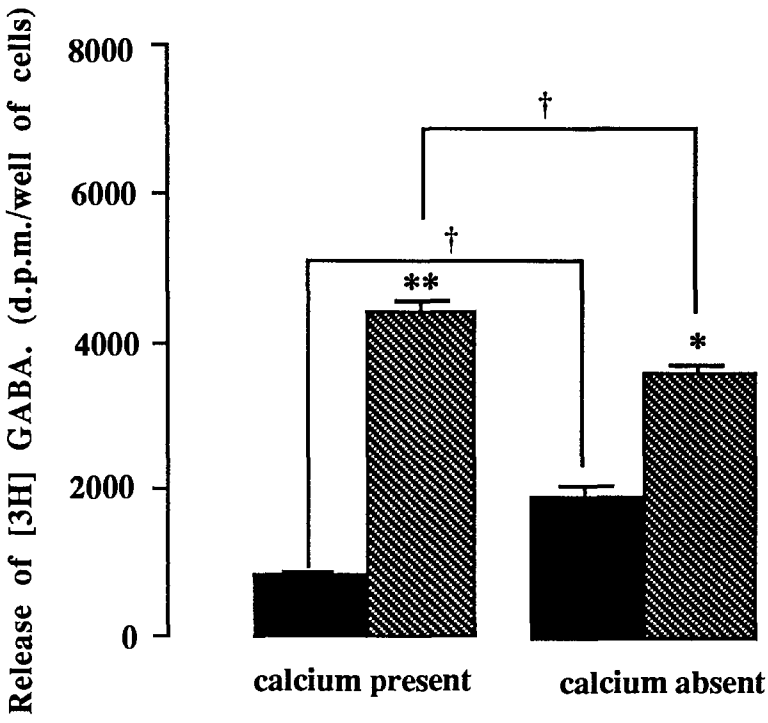


Fig 3.16. The effect of exclusion of Ca^{2+} from the release medium on the basal (■) and 56mM K^+ induced (▨) release of [^{3}H]GABA from foetal rat hypothalamic cells in primary culture. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.002$ for basal vs depolarization induced release, † $p < 0.05$ for release in the presence vs the absence of Ca^{2+}.

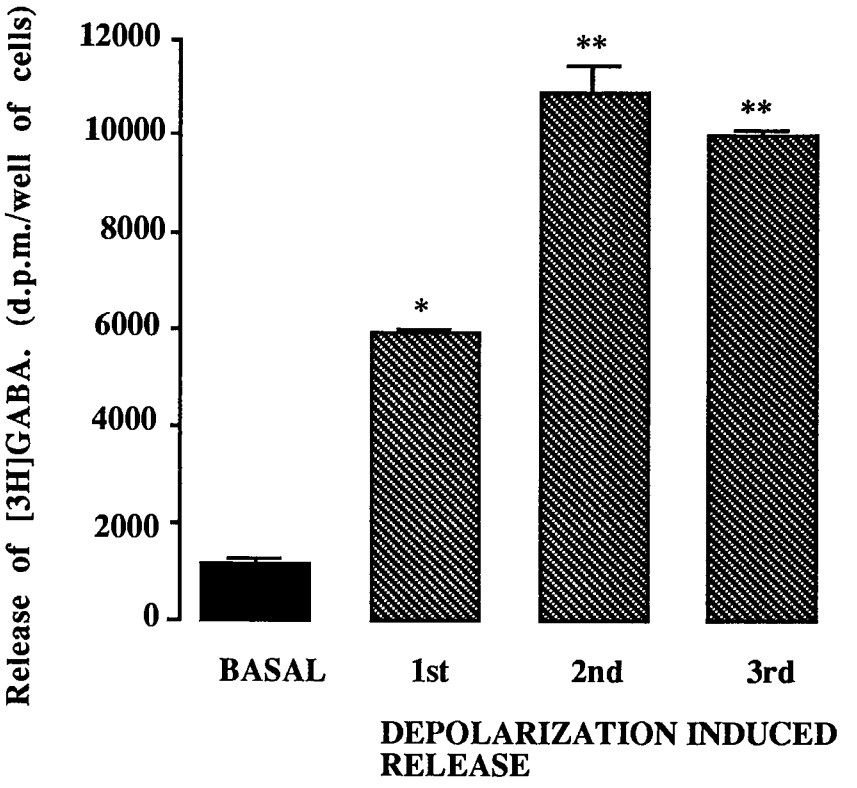


Fig. 3.17. Release profile for [^{3}H]GABA from foetal hypothalamic cells in primary culture. The cells were pre-incubated with [^{3}H]GABA before being subjected to three consecutive 7 min incubations either under basal (■) or 56mM K^+ induced (▨) conditions. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.005$ for depolarization induced release compared with basal.

3.3.3. Discussion.

These results show that hypothalamic cells in primary culture retain certain neuronal functions, the maturation of which may be investigated within this experimental model. The uptake of [^{3}H]DA into hypothalamic cells in this culture system displayed both time and temperature dependency and a similar finding was made for [^{3}H]GABA uptake. However, the [^{3}H]GABA uptake mechanism appeared to work with greater avidity than the mechanism for the uptake of [^{3}H]DA, with saturation of the mechanism occuring after only 10 min, whereas the DA mechanism took 6 times longer to attain the same state (Figs. 3.1 and 3.11). Furthermore, analysis of the kinetic parameters of uptake revealed that the value for K_m was more than one order of magnitude lower for [^{3}H]GABA than [^{3}H]DA uptake on all experimental days investigated, suggesting that the transporter protein involved in the removal of GABA

from the pre-synaptic cleft has a greater affinity and avidity for its ligand than the transporter protein involved in DA uptake. In addition, the value for V_{max} was consistently higher for GABA than DA, suggesting that there are a greater number of uptake sites for GABA than DA within our culture preparation. Thus, it is possible that uptake plays a greater role in GABA-ergic function than in dopaminergic function within the hypothalamus. The anatomical arrangement of certain intrahypothalamic dopaminergic tracts, especially the TIDA pathway, with its nerve terminals impinging upon the precapillary spaces of the hypophyseal portal vessels makes any requirement for rapid DA re-uptake less important as it may be "flushed away" to the anterior pituitary gland. However, it is generally accepted that termination of GABA-ergic transmission depends almost solely on re-uptake. This could represent a functional basis for the profound differences in the uptake parameters as exhibited for the two transmitters within our culture model. Alternatively, the highly active uptake for [^{3}H]GABA may reflect the greater maturity of these cells at this developmental stage relative to the dopaminergic neuronal populations. This view is in accord with the conclusions of our ICC studies and other results discussed below.

The characteristics of the uptake process for both [^{3}H]DA and GABA changed with age of the cultured cells suggesting that a pre-programmed maturation proceeds over extending periods *in vitro*. For [^{3}H]DA, the value of the Michaelis - Menten Constant (K_m; expressed as μM) decreased dramatically during *in vitro* development (viz. 56.2 ± 3.01, 7.0 ± 1.3 and 3.98 ± 0.87, on DIV 3, 7 and 14 respectively), suggesting an increase in the affinity of the transporter protein for DA. In addition, the value of V_{max} increased significantly with cultivation time, which is indicative of an increase in the expression of the transporter protein on the nerve terminals and co-incided with the increase in [^{3}H]DA uptake, which was greatest between DIV 3 and 7 (Fig 3.2). We propose that the observed increase is partly due to neurite growth, as demonstrated in Plate 3.0, which would allow for an increase in the number of DA uptake sites being expressed and similar results have been reported for mesencephalic dopaminergic neurones in culture (di Porzio 1980). In essence, these results suggest that in the perinatal period the apparatus for termination of DA neurotransmission in the hypothalamus is not fully developed but that their maturation continues *in vitro*.

Our finding of a change in the value of the K_m contrasts with the observation made in the *in vivo* study of Coyle & Henry (1973), using hypothalamic fragments at different

stages of development, who reported that the apparent K_m value remained constant during the postnatal period, although the studies are in agreement over the increase in V_{max}. However, as the hypothalamic tissue used in our study is taken at an earlier stage in neuronal development, the transporter complex may require a longer time interval to attain a maximal functional level. If the kinetic analysis were extended beyond DIV 14 it is likely that the value of K_m would have reached a steady level, as after DIV 14 the actual magnitude of uptake did in fact begin to plateau (Fig. 3.2). It is therefore possible that when hypothalamic dopaminergic neurones are removed from their *in vivo* environment and re-established in culture their rate of development, as measured by the ontogeny of certain biochemical parameters, is slower than *in vivo*.

The K_m values for [^{3}H]DA uptake in the present investigation are, in general, greater than those reported in studies using other hypothalamic preparations. Demarest & Moore (1979a) reported that the uptake of [^{3}H]DA into synaptosomal preparations of median eminence tissue had an apparent K_m value of 1.5μM, whereas Sarker et al (1983), using median eminence fragments, reported a K_m value of 0.56μM. However, as those values were derived from experiments on adult hypothalamic tissue and may exclude DA perikarya it is likely that the transporter mechanism in play functions differently to that in intact foetal neurones. It is known that changes in the DA transporter protein occur with aging (Bannon et al 1992) and our results support the hypothesis that similar changes in the transporter complex occur prior to birth and up to adulthood.

The uptake of [^{3}H]GABA also increased with advancing culture age, although the pattern of this increase was markedly different from that exhibited by the [^{3}H]DA uptake mechanism. Thus, while there was a rapid rise in the uptake of the radiolabelled GABA between DIV 3 and 7, similar to that displayed for [^{3}H]DA uptake, the amount of [^{3}H]GABA being accumulated (in terms of absolute levels of transmitter) was much greater even at the earliest time-point tested. Thereafter, [^{3}H]GABA uptake did not change again until DIV 15, after which time there was a second sharp rise at DIV 18. In agreement with these observations, the kinetic data showed an increase in the apparent V_{max} for [^{3}H]GABA uptake over a 14 day period, although we were unable to continue these studies beyond this time-point. There was also a significant decline in the apparent K_m value for GABA uptake between DIV 3 and 7 (2.3 ± 0.4 , 1.0 ± 0.14, respectively), reflecting an increase in the affinity of the transporter protein with advancing culture age. Both of these observations may account for the ready increase in [^{3}H]GABA uptake on initial experimental days. Between DIV 8 and DIV 14 there was no significant increase in the uptake of

[^{3}H]GABA and the maximal rate of uptake and the K_m value remained constant suggesting no change in either the expression or the affinity of the transporter protein.

The values for K_m observed for [^{3}H]GABA in this investigation, are similar to those reported in the literature for [^{3}H]GABA uptake into foetal/neonatal cerebral cortex tissue both in primary culture (Borg et al 1980) and slice preparations (Johnson & Davies 1973) where the value for the Michaelis Menten Constant ranged from 2.0 to 5.0μM. Furthermore the K_m value for [^{3}H]GABA uptake into adult hypothalamic preparations is also within this range (Anderson & Mitchell 1986) suggesting that unlike the uptake mechanism for [^{3}H]DA, the [^{3}H]GABA uptake system does not undergo a great degree of maturation postnatally. To our knowledge, no kinetic data has been published for foetal hypothalamic GABA-ergic systems in culture.

The specificity of uptake of both [^{3}H]DA and [^{3}H]GABA was investigated by use of their respective uptake inhibitors. In the case of [^{3}H]DA, uptake was significantly attenuated in a dose - dependent fashion by benztropine and nomifensine which at the highest concentrations tested (10^{-4}M), produced a maximal inhibition of [^{3}H]DA uptake of 60 and 70% respectively. These drugs are reported to be highly specific inhibitors of neuronal DA uptake mechanisms by interacting with the DA transporter protein (Dubochovitch & Zahniser 1985). We cannot eliminate the possiblity that the degree of inhibition which we have observed was not maximal as Sarkar et al (1983) using fragments of the median eminence and Puymirat et al (1987), employing mouse hypothalamic cells in culture have reported an inhibition of 70 and 90% respectively using the same inhibitors and it is likely that higher concentrations of the inhibitor would have resulted in a greater degree of inhibition. However, the ability of a single, sub-maximal dose of nomifensine to attenuate [^{3}H]DA uptake into the hypothalamic neurones increased approximately three-fold with advancing culture age (Fig. 3.4). This may reflect a maturation of the DA transporter molecule and possibly an increase in the number of functional [^{3}H]DA uptake sites in accord with the kinetic analysis data and reports by Engele et al (1989) and Fizman (1991). This distinct ontogenetic pattern for the ability of these compounds to inhibit [^{3}H]DA accumulation may thus account for the sub-maximal level of inhibition demonstrated in this study and we propose that the uptake mechanism had not reached maturity at the time of these experiments. It is possible that performing the experiments at later stages of *in vitro* development may have produced a greater maximum degree of inhibition.

Alternatively, glial cells, known to be present in this culture model (Davidson & Gillies, 1993) may account for a proportion of [^{3}H]DA accumulation although it has been reported that glial cells possess a considerably reduced ability to take up DA compared with neurones of the same developmental age (Barachovsky & Bradford 1987). Therefore, we propose that the uptake of [^{3}H]DA observed in our study was principally neuronal. Further investigations would require the use of specific inhibitors of DA uptake into glial cells which to our knowledge were not available at the time of carrying out this series of experiments.

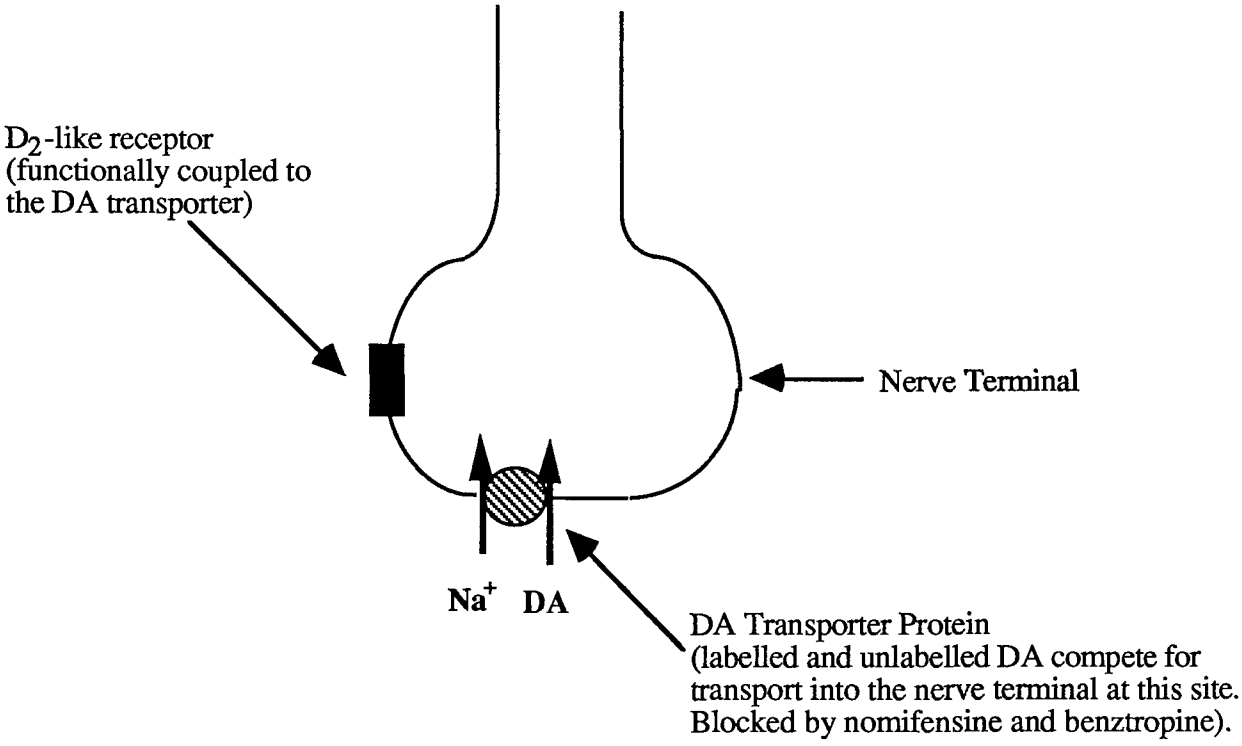
The uptake of [^{3}H]GABA was very effectively inhibited by the specific inhibitor of GABA uptake into neuronal cells, SKF 89976 A, with the block being virtually complete at 10^{-3}M. Furthermore, uptake of [^{3}H]GABA was not affected by ß-alanine which is considered to be a specific inhibitor of GABA uptake into glial cells. Therefore, we conclude that the uptake of [^{3}H]GABA is exclusively into neuronal cells within this hypothalamic culture preparation. SKF 89976 A, in contrast to the [^{3}H]DA uptake inhibitor nomifensine, became less effective in blocking [^{3}H]GABA uptake as the cultures matured. The loss of effectiveness of the uptake inhibitor, the change in the Na^+ - dependency of uptake (Fig. 3.15) and the second sharp rise in the amount of [^{3}H]GABA accumulated (Fig. 3.12) all suggest some fundamental change in the behaviour of the transporter protein although the connection between these phenomena and their physiological relevance in the ontogeny of hypothalamic GABA - ergic systems, are yet to be elucidated.

These results suggest that, in contrast to dopaminergic neurones, which display considerable functional and morphological immaturity at early *in vitro* ages up to DIV 7, the GABA-ergic neurones in primary culture gain functional competence at a very early stage in development. It is thus likely that once established *in vitro*, GABA-ergic neurones rapidly re-develop processes and begin to express uptake sites on their newly formed axons and dendrites. This proposal is further substantiated by our ICC studies, as during early stages of cultivation GABA-ergic neurones were already exhibiting well developed processes which do not appear to undergo further growth at later time-points in culture. We conclude that the apparatus for transport is in place early on in the developmental process of GABA-ergic cells and that this degree of maturity is retained and continued under *in vitro* conditions.

Attempts to totally abolish [^{3}H]DA uptake by direct competition with unlabelled DA failed, producing no greater than a 45% inhibition at the highest concentration of "cold" DA employed. This contrasts with some studies on murine hypothalamic cell cultures where 10^{-5}M DA produced a 75% inhibition of [^{3}H]DA uptake (Puymirat et

al 1987). We therefore considered the possibility that the uptake of [^{3}H]DA within our culture model was under some form of receptor mediated regulation by DA itself, as suggested by (Ross 1991). The D_2 receptor antagonist sulpiride, displayed little inherent activity in affecting the magnitude of [^{3}H]DA uptake but was highly effective in reducing [^{3}H]DA accumulation in the presence of "cold" DA. Thus we have constructed a theoretical model for the regulation of DA uptake at the site of the nerve terminal (Fig. 3.18).

Fig. 3.18. Theoretical model for the autoregulation of DA uptake into dopaminergic nerve terminals in the hypothalamic culture preparation.



In this model, it is proposed that presynaptically located DA transporters are functionally coupled to a DA receptor which exhibits pharmacological similarities to the D_2-like receptor sub-type. At equilibrium, the net uptake of DA is determined by the amount of the neurotransmitter in the synaptic cleft, uptake occuring only when the concentration of DA approximates the Michaelis - Menten Constant for the uptake mechanism in play. D_2-like receptor activation by high concentrations of DA results in facilitation of uptake, ensuring that excessive levels of DA are not allowed to flood the synaptic cleft. Thus, [^{3}H]DA is readily taken up into the nerve terminal via the transporter molecule as it bears a structural resemblance to the endogenous ligand.

However, as sulpiride alone had a negligible effect on [^{3}H]DA upake, it follows that the tritium labelled DA has minimal activity at the D_2-like receptor proposed to be involved in regulating its uptake. Introduction of unlabelled and therefore pharmacologically active DA to the system, serves to disrupt the equilibrium and we propose that "cold" DA at a concentration of 10^{-8}M DA has two potential actions. Firstly, there is direct competition between labelled and unlabelled ligand at the DA transporter complex, which would normally result in the reduction of [^{3}H]DA accumulation. This is, however, counteracted by the second action of the exogenous DA at the D_2-like receptor, to facilitate [^{3}H]DA uptake, the net result being no apparent change in uptake. When the secondary action is blocked by the introduction of the D_2 antagonist sulpiride, [^{3}H]DA uptake was inhibited by approximately 50%. As the concentration of "cold" DA is increased it would compete more effectively with [^{3}H]DA at the transporter molecule and thus reduce accumulation. Simultaneously, however, it would compete with sulpiride at the D_2-like receptor which would have the opposite effect of facilitating uptake. This interpretation merits further study as it implies that the net effect of raising DA levels depends critically on the prevailing DA concentration which could be influenced by the efficacy of a number of DA agonists which are used clinically.

An alternative explanation for these resultsought also to be considered. Our uptake studies were performed with a 60min incubation period for [^{3}H]DA accumulation. Net accumulation of [^{3}H]DA will therefore depend on a balance between uptake and basal release processes over that hour. Thus, differences in net accumulation in the presence of varying concentrations of "cold" DA could also be influenced by a D_2-receptor mediated inhibition of [^{3}H]DA release (Sarkar et al 1983). In view of the low levels of basal release, this latter explanation seems less likely. The correct interpretation of these results would be aided by molecular biological methods for measuring expression of the DA transporter protein and by a series of K_m estimations, employing shorter incubation periods for [^{3}H]DA accumulation, under different prevailing levels of "cold" DA and in the presence and absence of specific uptake inhibitors.

Whatever the explanation, however, these findings suggest that the concentration of DA within the synaptic cleft influences DA accumulation. By inference, autoregulation of DA uptake has been reported previously, especially for the TIDA neuronal population (Demarest & Moore 1979a) where the uptake mechanism for DA operates at a lower affinity than in other populations (K_m for dopaminergic neurones in the corpus striatum being 0.6μM compared with 1.5μM in the TIDA neurones

Annunziato 1979). The uptake mechanism in the TIDA pathway is not however dysfunctional but is activated only when DA concentrations in the hypophyseal portal blood perfusing the nerve terminals reach a particular level. This in itself suggests some form of DA (receptor?) mediated modulation of uptake, as the presence of DA at the site of the nerve terminal is vital to its activation.

The magnitude of the release of both [^{3}H]DA and [^{3}H]GABA in response to elevated K^+ was Ca^{2+} - dependent and typically 2-5 times greater than that achieved under basal conditions, which suggests that the release mechanism displayed in this culture model possess a degree of functional integrity. However, [^{3}H]GABA release appeared to be less dependent on extracellular Ca^{2+} and it has been reported that certain GABA-ergic systems within the CNS, like cholinergic systems may release their transmitter via a Ca^{2+} - independent mechanism (Arias & Tapia 1986). Such a mechanism may therefore account for a portion of release observed here.

The ability of the hypothalamic cells to release pre-accumulated [^{3}H]DA increased with age in culture, the most marked increase occuring between DIV 7 and DIV 9. Whether the increase in the total release of [^{3}H]DA represents a maturation of the release mechanism displayed within this experimental model or whether it is partially a consequence of maturation of the uptake system is difficult to determine from this data, but several arguments suggest the former. Firstly, in a number of situations we have found that changes in uptake are not closely paralleled by changes in release (Section 3.5). Secondly, we have observed a similar increase in the release of endogenous DA over the same culture period (following section). Finally, synaptogenesis, which is thought to co-incide with the development of Ca^{2+}-dependent neurotransmitter release (Zurgil (1981), occurs extensively at DIV 7 in whole brain cultures (Bradford & Barachovsky 1987) which is the stage at which [^{3}H]DA release increased most dramatically in our study (Fig 3.10). Furthermore, the increase in release of neurotransmitter with advancing cultivation time may be due to the development of growth cones and varicosities as demonstrated by our ICC studies which results in increased transmitter output.

In contrast to the situation with dopaminergic neurones, the release of [^{3}H]GABA remained unchanged over the period of investigation and was high even at DIV 5, when [^{3}H]DA release studies were not viable. This difference suggests a degree of plasticity for the [^{3}H]DA release mechanism *in vitro* which is absent within the GABA-ergic population and may indicate that GABA-ergic systems mature more rapidly.

The significant decline in [^{3}H]DA release observed when the cells were subjected to a rapid train of depolarizing stimuli was considered to be indicative of a depletion of pre-accumulated [^{3}H]DA stores. However, the significant amount of [^{3}H]DA remaining in the cells following extraction shows that this is not so and suggests additional storage sites for the radiolabelled neurotransmitter, from which it cannot be readily released. This phenomenon has been suggested by other authors (Barachovsky & Bradford 1987). In contrast, a rapid train of depolarizing stimuli elicited an increase in [^{3}H]GABA release, with the second and third responses being significantly greater than the first. These observations point to marked differences in the dynamics of storage and/or release of [^{3}H]DA and [^{3}H]GABA in our culture model, and may reflect fundamental differences in the dynamics of secretion of the two neurotransmitters *in vivo*.

In conclusion we have demonstrated that foetal rat hypothalamic cells maintained in primary culture retain certain parameters of neuronal function, including the ability to accumulate and release exogenous radiolabelled neurotransmitters and that these markers of neuronal activity undergo a degree of maturation with advancing time in culture. Differences in the patterns of expression and development of these parameters between the dopaminergic and GABA-ergic systems indicate that different hypothalamic neuronal populations carry through distinct pre-programmed developmental profiles *in vitro*. Furthermore, the differences suggest that in late foetal life hypothalamic GABA - ergic neuronal systems have reached a greater level of maturity than their dopaminergic counterparts and that the advanced developmental potential of the former is preserved and continued under *in vitro* conditions.

3.4. Studies with Endogenous DA.

Using HPLC -EC for the measurement of endogenous DA we wished to determine to what extent the release of pre-accumulated [^{3}H]DA was a measure of the behaviour of endogenously synthesised DA.

3.4.1. Time dependency of DA release.

Fig 3.19 illustrates a time course study for the release of endogenous DA. During a 7-min period DA output was barely above our limits of detection (Section 2.5.2.3). Increasing the incubation period led to a significant increase in the amount of DA released under both basal and 56mM K^+ - stimulated conditions. In view of these results it was decided that the release of endogenous DA would be investigated over incubation periods of 60 min, so that the levels of the amine could be detected with greater accuracy.

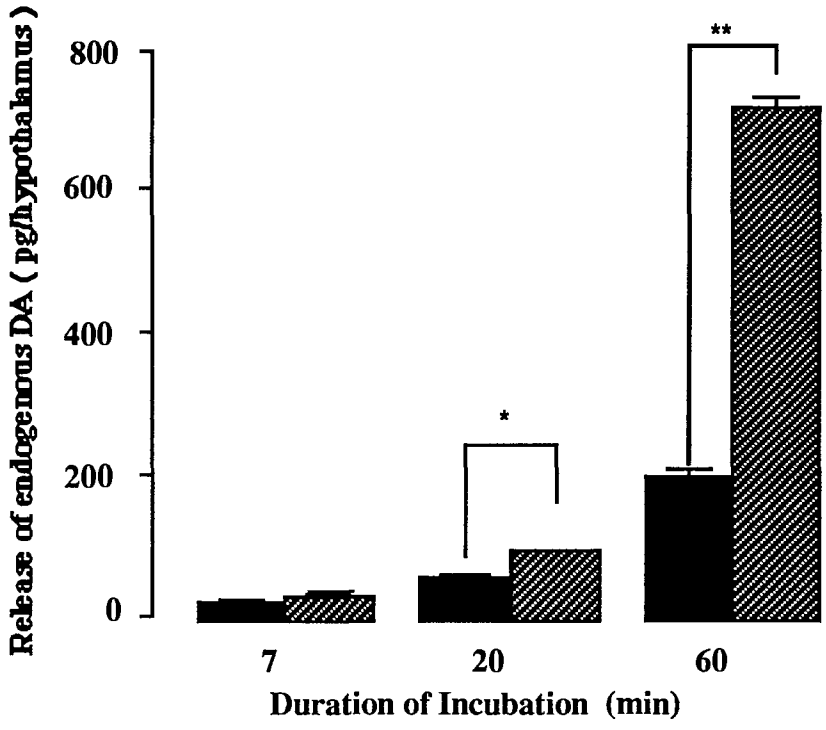


Fig. 3.19. The effect of incubation time on the basal (■) and 56mM K^+ induced (▨) release of endogenous DA from cultures of hypothalamic cells on DIV 7 as measured by HPLC-EC. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.001$ for 56 mM K^+ induced release compared with basal release. For basal and 56mM K^+ - induced release $p < 0.05$ and $p < 0.02$ respectively for 7 min compared with 20 min incubations. For basal and 56mMK^+ induced release, $p < 0.02$ and $p < 0.001$ respectively for 20 min compared with 60 min incubations.

3.4.2. Maturation of DA release from the hypothalamic cultures.

Basal and K^+ - stimulated release of DA from naive cultures (i.e. which had not been previously treated) was studied between days 3 and 21 *in vitro* (Fig. 3.20). On DIV 3 release was low but detectable under basal conditions. There was a slight, but significant increase in the basal release of DA on DIV 7 followed by a slight fall on DIV 11. Thereafter basal release increased with culture age throughout the period of the investigation. Potassium - induced release increased throughout the time of the study not only in terms of absolute output but also when expressed as the percentage above basal. As none of these cells had received any prior stimulus these responses represent an inherent maturation of the dopaminergic neurones within our culture preparation.

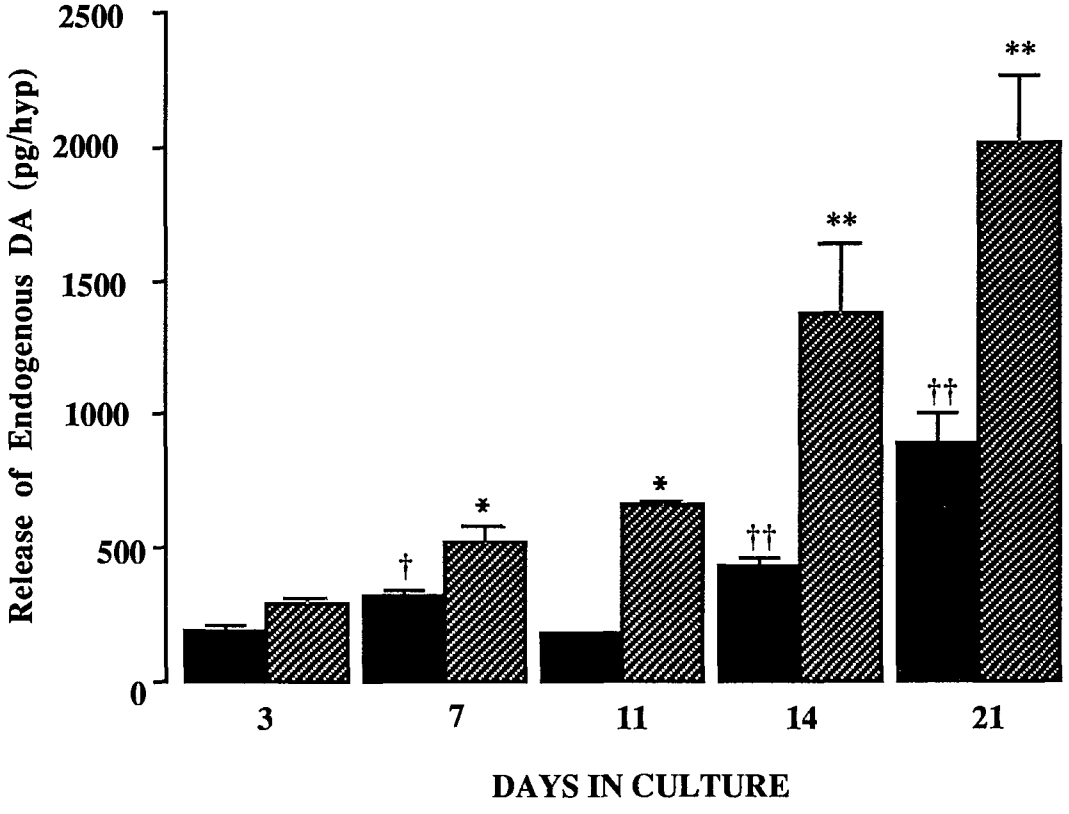


Fig. 3.20. Release of endogenous DA from foetal rat hypothalamic cells maintained in primary culture during 1 hour incubations under either basal (■) or 56mM K^+ - stimulated (▨) conditions. Experiments were performed on DIV 3, 7, 11, 14, and 21. Results represent the mean ± s.e.m. n=4. † $p < 0.05$, †† $p < 0.025$ for basal release as compared with the release on DIV 3, * $p < 0.05$, ** $p < 0.025$ for 56mM K^+ induced release as compared with the release on DIV 3 .

3.4.3. The Ontogeny of DA content in hypothalamic cultures.

At certain time-points equivalent to those in Fig 3.20 the DA content was measured in naive cultures and in cultures which had just received a 1 hour depolarizing stimulus. There was no significant increase in DA content between DIV 7 and 14. Furthermore, on DIV 7, there was no significant difference in DA content between those cells which experienced a single 1 hour incubation with 56mM K^+ prior to extraction and those which had not been pre-stimulated. However, on DIV 11 and 14 DA content in those cells which were subjected to a depolarizing stimulus, was significantly higher (by approximately 30%) than that in naive cells.

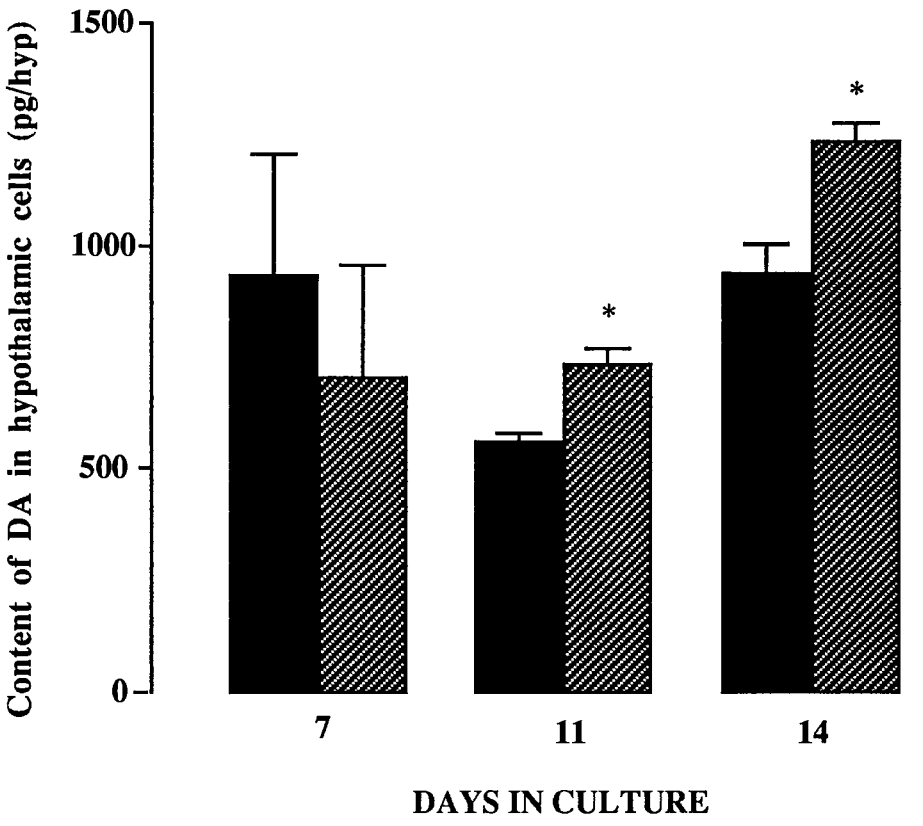


Fig. 3.21. DA content in hypothalamic cells in primary culture immediately following a single 1 hour incubation in the presence of 56mM K^+ (▨) or without pre - stimulation (■), on DIV 7, 11 and 14. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$ for unstimulated vs pre-stimulated cultures.

3.4.4. Depolarization induced maturation of DA release and content.

Previous work demonstrated that prior exposure of the cultures to elevated levels of potassium enhanced the response of peptidergic neuronal cells to depolarizing stimuli during later stages in culture (Gillies; 1989). We therefore investigated whether this phenomenon occurred for hypothalamic dopaminergic neurones. A single batch of

cells was cultured as outlined in Section 1.1, and divided into five groups (n=4). Group 1 was exposed to 56mM K^+ for 1 hour on DIV 3 and continued to receive a depolarizing stimulus on DIV 7, 11, 14 and 21. Group 2 was first exposed to elevated levels of K^+ on DIV 7 and received similar treatment to group 1 on subsequent experimental days. Groups 3, 4 and 5 did not receive their first depolarizing stimulus until DIV 11, 14 or 21, respectively. Thus in total group 1 received five potassium stimulations and groups 2, 3, 4 and 5 received four, three, two or one stimulation(s) respectively.

Basal levels of DA release were not influenced by a prior depolarizing stimulus (data not shown) and Fig. 3.22 illustrates the response to K^+ depolarization. The inherent maturation described earlier (Fig 3.20) is once again apparent in the increasing magnitude of the first response of successive groups. In addition, a depolarization - dependent maturation of stimulated release is evident. Thus, on DIV 7 and 11 those cells which had been exposed to 56mM K^+ on DIV 3 (group 1), released greater amounts of dopamine than those cells which were stimulated only once previously or not at all (groups 2 and 3). By DIV 14 in culture, fewer prior exposures to elevated levels of K^+ were required to produce a response of a magnitude similar to that of group 1 which had received the greatest number of K^+ depolarizations. By DIV 21 the responses of groups 1 - 4 to K^+ - depolarization were not significantly different and therefore the number of prior depolarizations required for each group to "catch up" with group 1 decreased as the inherent maturation proceeded.

The cultures from the experiment shown in Fig. 3.22 were also extracted on DIV 24, under basal conditions and the DA content in these cells was measured (Fig. 3.23). There was no significant difference in the DA content of the cells in groups 1, 2, 3 or 4 which had received five, four, three or two stimulations respectively, over the investigational period. Only those cells which had been stimulated for the first time on DIV 21 (group 5), contained significantly higher levels of DA than groups 1 - 4.

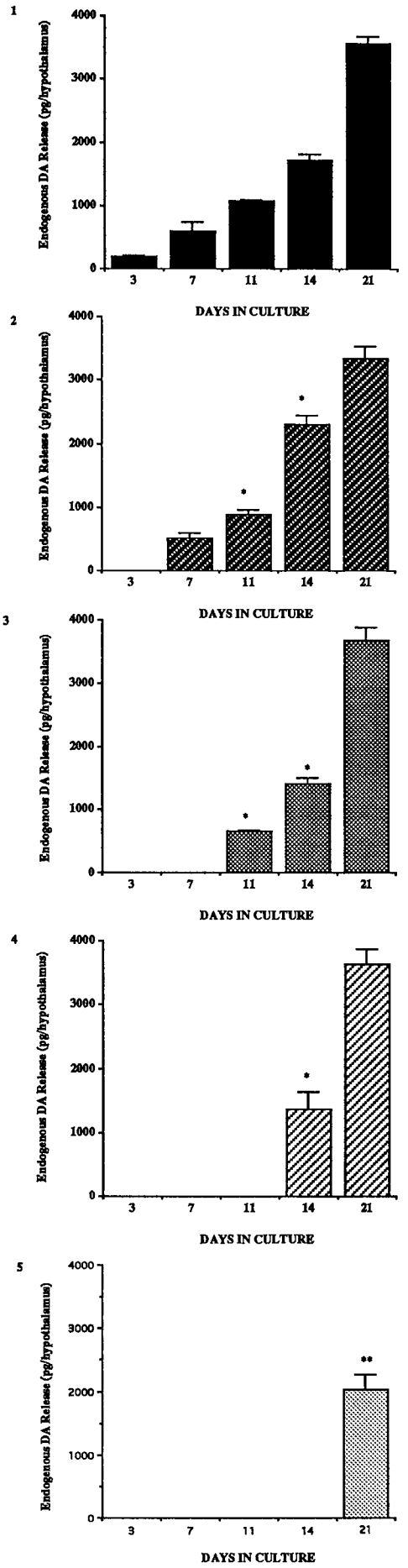


Fig. 3.22. Depolarization - induced release of endogenous DA from hypothalamic dopaminergic neurones in primary culture. Release studies were performed on a single batch of cells divided into 5 groups. Group 1 (■) received a 1 hour 56mM K^+ - stimulation on DIV 3 and 7, 11, 14, and 21. Group 2 (▨) was first stimulated on DIV 7 and each of the remaining experimental days. Group 3 (▩) received an initial stimulation on DIV 11, Group 4 (▨) on DIV 14 and Group 5 (▩) on DIV 21. Results represents the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.005$ compared with the corresponding value for DA release in Group 1 cells.

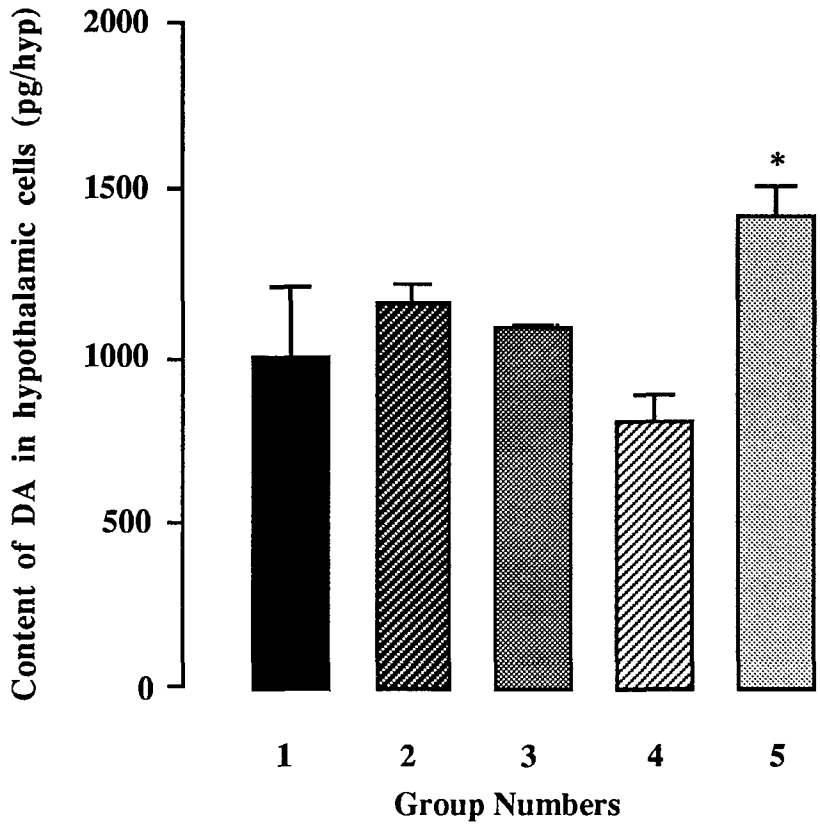


Fig. 3.23. The effect of prior potassium depolarization on the content of DA in hypothalamic cells in primary culture. Group 1 (■) received five stimulations prior to extraction whereas groups 2 (▨), 3 (▩), 4 (▨), and 5 (□) received four, three, two or one stimulation(s) respectively. There was no significant difference between groups 1, 2, 3 and 4. The DA content in group 5 was significantly higher than the other four groups. Results represent the mean ± s.e.m. n = 4. * $p < 0.05$ for group 5 compared with groups 1, 2, 3 and 4.

3.4.5. DA release vs Conceptional Age.

Using an alternative approach to investigate the perinatal development of hypothalamic DA neuronal systems, we initiated cultures from hypothalami collected on embryonic days 18 (E18) and 21 (E21), and postnatal day 1 (P1). Release experiments were performed between DIV 7 and DIV 21 (Fig. 3.24.a and b).

As with our previous developmental studies, the DA output increased with increasing culture age throughout the investigational period. Under basal conditions, cultures initiated from E18 tissue released markedly less DA than those cultures initiated from E21 and P1 tissue on all experimental days except DIV 7, when the levels were

slightly higher (Fig 3.24.a). DA output induced by a depolarizing stimuli was also lower from E18 than E21 and P1 cultures at all time points tested. Depolarization - induced release of DA from the postnatal cultures was dramatically greater on DIV 21 than from the other two culture types (Fig 3.24.b).

When the same data were expressed as release v's age relative to conception (CA), the pattern for basal release was similar to that seen in the previous figure, with E18 cultures releasing less DA than E21 and P1 cultures (3.25.a). Depolarization induced DA release differed slightly, where the rise in output of DA exhibited by the P1 cultures appeared initially to lag behind the E21 and P1 cultures, followed by a sharp rise in output by CA +45 (Fig 3.25.b). DA release from the E18 and E21 cultures increased steadily over the period of study from CA +25 to +39 and CA +28 to +42, respectively.

When the E18 and P1 cultures were observed under light microscope, some differences were apparent (Plate 5). Under low power the cells in the E18 cultures appeared to form "tight" aggregations and individual neurones were almost indistinguishable as the culture proceeded (Plate 5A). In contrast, the typical neuronal - type cells within P1 cultures remained separated one from another (Plate 5B). Similar observations were seen under high power and in addition those cells from the P1 cultures appeared to be larger than their E18 culture counterparts (Plate. 5C -F). In addition, the P1 cultures remained intact for a much longer time period than the E18 (and E21) cultures and the monolayer failed to "peel" even after a five week period.

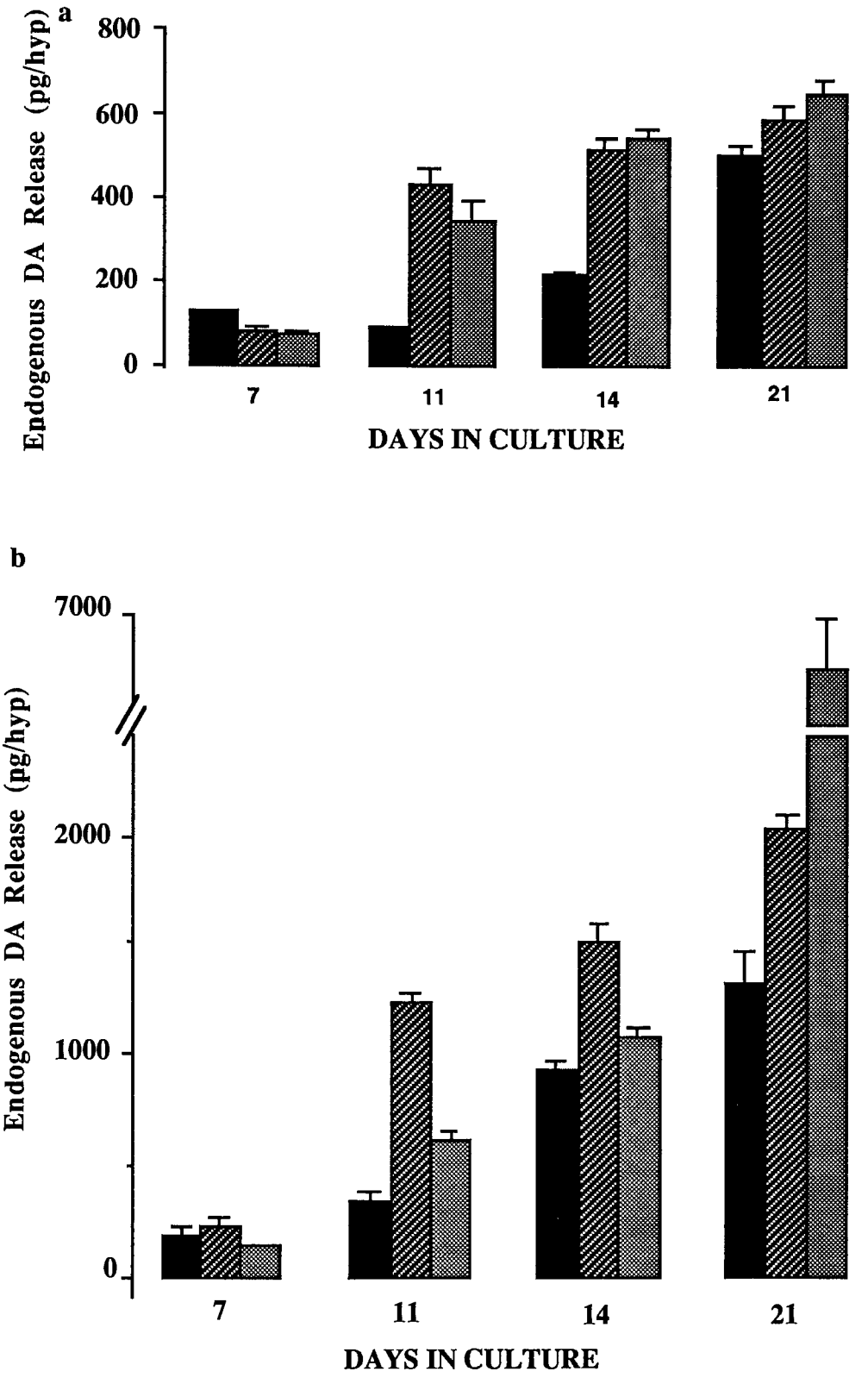


Fig. 3.24. Release of endogenous DA from hypothalamic dopaminergic neurones in primary culture. Cultures were initiated from foetal hypothalamic tissue removed on embryonic days (E) 18 ( ) and 21 ( ) and postnatal day (P) 1 ( ). Basal release (a) and 56mM K^+ - stimulated release (b) of DA was measured on different days in culture.

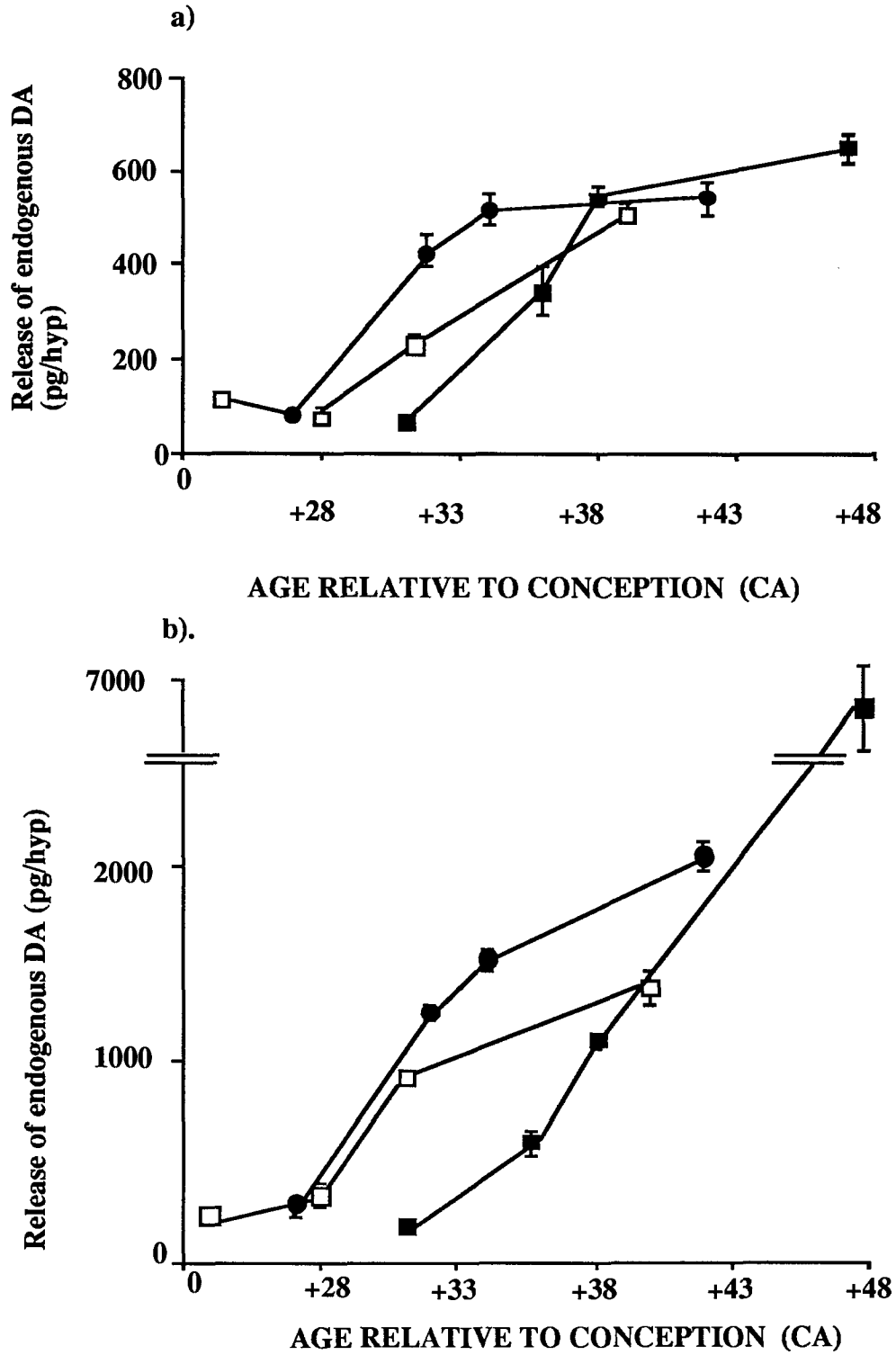
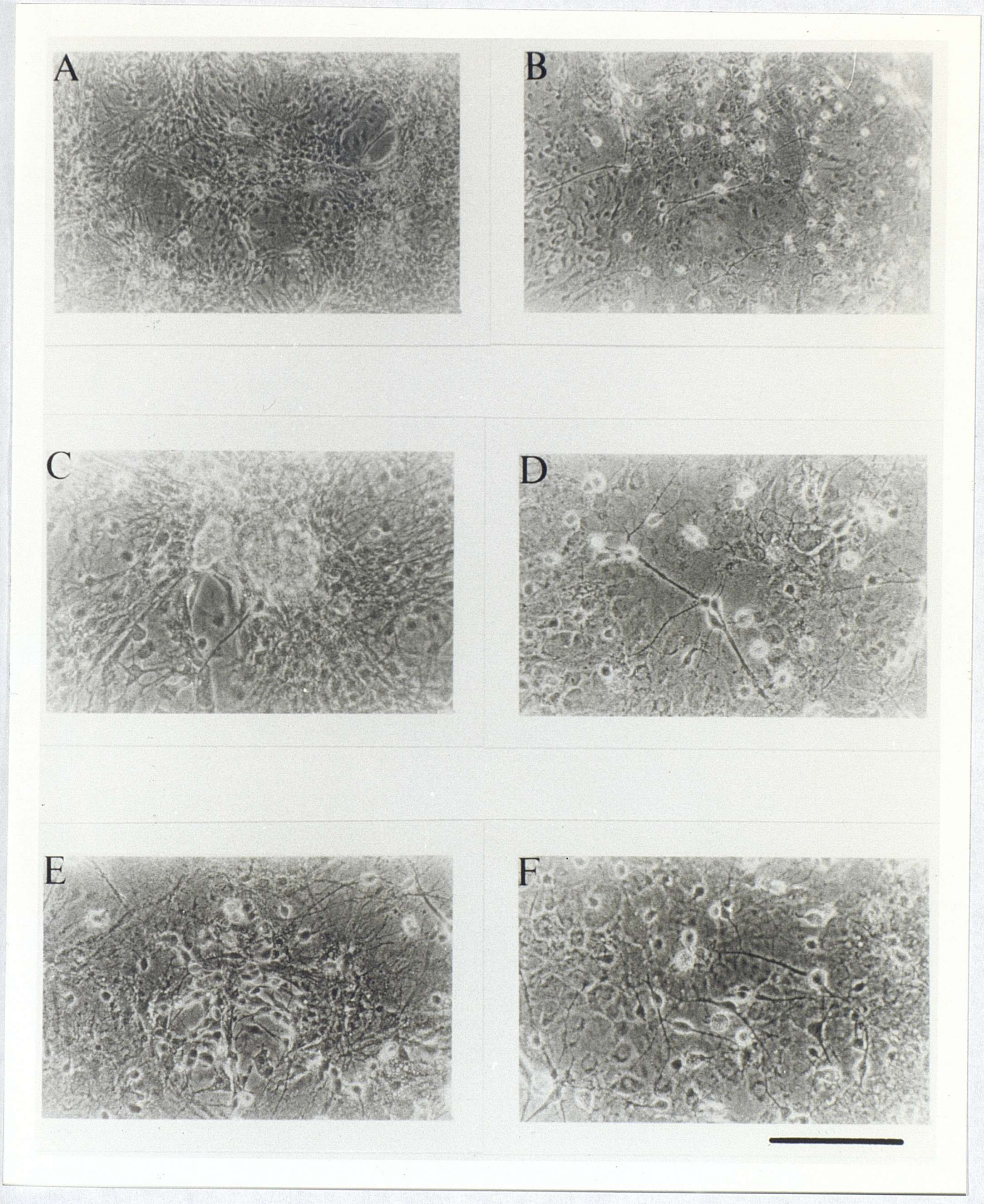


Fig. 3.25. Release of endogenous DA from foetal rat hypothalamic cells maintained in primary culture. Cultures were initiated from foetal hypothalamic tissue removed on embryonic (E) days 18 (—□—) and 21 (—✱—) and postnatal day 1, (—■—). Basal release (a) and 56mM K+ - stimulated release (b) of DA was measured on different days relative to conception. Results represent mean ± s.e.m, n = 4.

Description of Plate 5.0. When the E18 and P1 cultures were observed under light microscope, some differences were apparent Under low power the cells in the E18 cultures appeared to form "tight" aggregations and individual neurones were almost indistinguishable as the cultures proceeded (Fig. A). In contrast, the typical neuronal-type cells within P1 cultures remained separated one from another (Fig. B). Similar observations were seen under high power, and in addition those cells from the P1 cultures appeared to be larger thantheir E18 culture counterparts (Figs. C-F). In addition, the P1 cultures remained intact for a much longer time period than the E18 (and E21) cultures and the monolayer failed to "peel" even after a five week period. Bar = 5μm for Figs A and B, Bar = 10μm for Figs C-F.

PLATE 5.0.



3.4.6. Discussion.

Both basal and K^+ - induced release of DA from our hypothalamic cultures was demonstrable and increased with **increasing incubation time.** Thus after a 7 min incubation in the presence of 56mM K^+, DA output was only 20% above that released under basal conditions and this percentage difference increased to 50% and 250% over a 20 and 60 min incubation period respectively (Fig 3.19). The apparent reduced ability of the cells to respond to potassium depolarization over short time periods cannot be due to lack of stored DA as DA content is over 500 times greater than that released over a 7 min incubation period (Fig 3.21). These results could suggest therefore, that the DA held in long term storage sites within the nerve terminal is not readily releasable and that a more prolonged stimulus, which activates DA synthesis and facilitates the release of the newly sythesized amine, is required before a large response to a depolarizing stimulus can be seen. Alternatively, during a short time period it is possible that the uptake mechanism for DA is highly effective in maintaining low levels of DA around the synaptic cleft, while with prolonged stimulation the nerve terminal becomes "swamped" and re-uptake is not sufficient to recapture the large amount of DA released, thus accounting for the high levels within the release medium. The latter possibility might be investigated by measuring the release of endogenous DA in the presence of uptake blockers.

As the cultures matured, the response to elevated K^+ increased. Thus during a 1 hour incubation period the increase in magnitude of DA output in response to depolarization on DIV 3 was 40% above basal but rose to greater than 165% by DIV 21 (Fig. 3.20). This enhanced responsiveness of the cultures to stimulation with advancing culture age suggests a degree of maturation of the synthetic and/or release machinery for DA. Much evidence supports a role for synaptogenesis in neuronal development *in vitro* and *in vivo* using whole brain tissue (Barachovsky & Bradford 1987). Certain indicators of synapse formation including Ca^{2+} influx, the phosphorylation of the proteins involved in neurotransmitter release and Ca^{2+} - dependent release of DA, increase with increased cultivation time in parallel to an enhancement of neuronal activity (Zurgil & Zisapel 1984; Barachovsky & Bradford 1987). Thus, the *in vitro* development of specific cell -to- cell connectivity may account for both the increase in absolute amounts of DA secreted and the increase in magnitude of response of these dopaminergic systems to elevated K^+. Therefore we propose that the development of synaptic contacts may play a role in the maturation of hypothalamic dopaminergic neurones in this experimental model, in a manner analogous to that proposed for the development of other dopaminergic systems *in vitro* (Barbin 1985) and *in vivo* (Santana et al 1992).

In addition to the inherent maturation of the synthesis/release mechanisms discussed above, we were also able to induce a depolarization (56mM K^+) - dependent acceleration of functional maturation as demonstrated in experiment 3.22. During early stages of *in vitro* development, repeated depolarization enhanced DA output, above the inherent pattern of development. At later stages of cultivation this depolarization - induced maturation was superseded by the pre-programmed development and all cultures, with the exception of those which had not received any prior stimulation, behaved in a similar manner by DIV 21. The release of certain neuropeptides from our culture model may be similarly modulated (Gillies 1989; Hannah et al 1993) and it is likely that the action of exogenous depolarizing stimuli in addition to those being generated endogenously, may serve to enhance the activity of either the synthesis or secretory apparatus.

Repeated depolarizations may also change the dynamics of the storage and release of endogenous DA at the nerve terminal. A component of neurotransmitter release is thought to be mediated via a carrier-dependent process involving the DA transporter complex (Raiteri et al 1979). Thus K^+ stimulation, like amphetamine, is believed to elicit the release of cytoplasmic stores of DA and repeated exposure of the cells to such stimuli causes a switch in the route of newly synthesized DA away from cytoplasmic degradation by MAO, to the synapse with subsequent release. This is exemplified by the observation that repetitive, prolonged exposure of dopaminergic neurones to elevated K^+ reduces the amount of DOPAC in the extracellular compartment, which is generally accepted to be derived from the breakdown of cytoplasmic stores of DA (Zetterstrom et al, 1983, 1986).

While there was no significant change in content of naive cells with increasing cultivation time, those cultures which were subjected to a single 1 hour incubation in the presence of elevated K^+ prior to extraction contained significantly higher levels of DA than did their untreated counterparts (Fig 3.21). Although we were unable to investigate the likely intracellular effects leading to an increase in neurotransmitter sy nthesis, it has been reported that membrane depolarization stimulates the production of tyrosine hydroxylase in brain regions such as the substantia nigra (Knusel et al 1990). Any increase in DA content is likely to return rapidly to basal levels as frequent depolarising stimuli did not influence the resting levels of neurotransmitter in dopaminergic neurones (Fig 3.23).

When primary cultures were initiated from tissue at different stages of embryonic or postnatal development, the capacity of the DA - containing neurones to mature and survive differed markedly. Those cultures initiated from postnatal tissue ultimately displayed a greater maximal response in terms of stimulated DA output than cultures initiated from foetal tissue (Fig. 3.24). When expressing DA output in terms of age relative to conception, it appeared that the dopaminergic neurones within the P1 cultures required a longer period of re-adjustment under *in vitro* conditions before they "overtook" those in the E18 or 21 cultures (Fig. 3.25). These results are difficult to interpret as we are unable to assess the degree to which initiating hypothalamic tissue at different stages of development influences their *in vitro* behaviour. Yet it is believed that during embryogenesis certain aspects of neuronal development, including the formation of synaptic contacts, are inhibited (Santana et al 1992). Thus when embryonic cells are re-established in culture conditions it is possible that they are still exposed to these inhibitory influences and hence their rate of development appears to be relatively suppressed and retarded. Therefore we propose, that after birth these restraining influences on neuronal development are eventually "lifted" allowing processes such as synaptogenesis and other forms of cell -to- cell communication to occur. This may account for the dramatic increase in neuronal activity displayed by DIV 21 in the P1 cultures. The primary source for this inhibition is yet to be elucidated but it has been propsed that glial cells are intimately involved in neuronal development (Denis - Donini 1984), and if they exerted some restraining influence it is likely that they would modulate the rate of development of neuronal cells. If these cells and therefore the inhibitory influence they exert, decline in postnatal tissue this would account for the more rapid development and enhanced activity in the P1 culture subtype. It remains to be definitively established whether these results are reflecting genuine pre-programmed events in neuronal development which are being reflected *in vitro* , or whether simply taking the tissue at later stages of *in vivo* development is merely increasing the percentage survival of neurones once they are removed to an *in vitro* environment.

However, a parallel and simultaneous study investigating the activity of SRIH containing cells in these hypothalamic cultures yielded markedly different results, with the P1 cultures releasing the **lowest** levels of neuropeptide (Davidson & Gillies personal communication). Furthermore, in all three culture types (E18, E21, P1), the levels of SRIH output **decreased** as a function with time after DIV 10. The SRIH data is more in accord with the view that the survival rate of neurones cultured postnatally is significantly less than for those cultured during embryonic development (Barbin et al 1985). This is obviously not the case for hypothalamic dopaminergic neurones although it does appear to be true for mesencephalic dopaminergic systems

(Barbin 1985). These results therefore, further support our view that specific patterns of neuronal development *in vitro* do not solely reflect an artefact of the culture condition but may represent important developmental processes occuring in brain-specific regions in discrete neuronal populations at different developmental stages, many of which may be critical to the adequate functioning of the neuroendocrine system in adult life.

Our results for endogenous DA are similar in several respects to those for [^{3}H]DA. The release of both are time dependent and stimulated release increases as the cultures mature. The latter is especially significant in that it supports our argument that increased [^{3}H]DA output depends more on the development of some component of the release mechanism and is not solely a consequence of the increase in [^{3}H]DA uptake. It is likely that the maturation of the uptake and release mechanisms may proceed independently and at different rates.

Although the similarities in the release profiles of endogenous and [^{3}H]DA may justify our use of radiolabelled neurotransmitter to investigate neuronal activity, some apparent differences were also noted. Over a 7 min incubation period endogenous DA release was not significantly enhanced by membrane depolarization (Fig. 3.19), whereas the magnitude of response of [^{3}H]DA release to a depolarizing stimulus was markedly greater than under basal conditions (Fig. 3.8). It is possible that the storage site from which [^{3}H]DA is secreted is more responsive to a depolarizing stimulus over short intervals, than endogenous DA, with recently re-uptaken amines being positioned in intracellular locations which are more readily mobilized than the newly sythesized pools from which endogenous transmitter is released. However, as [^{3}H]DA release exhibits Ca^{2+} - dependency, we propose that it is released from genuine vesicular bound storage sites and thus mimics endogenous neurotransmitter secretion. Over longer durations of incubation endogenous DA release became highly responsive to membrane depolarization (Fig. 3.19) and it is therefore likely that increasing the incubation period allowed for both the mobilization of newly sythesised DA stores and also enabled a degree of neurotransmitter synthesis to occur as DA output in one hour under stimulated conditions was greater than the DA typically contained in intracellular storage sites (see Fig. 3.21).

The results from this series of experiments add further support to our proposal, that the dopaminergic neurones within this hypothalamic culture model exhibit a functional maturation, and show a remarkable capacity for survival under *in vitro* conditions. As perinatal levels (viz content) of DA are relatively high, it has been suggested that the neurotransmitter may play some modulatory role in the

development of the CNS. DA has been implicated in the adaptation of the newborn rat to extrauterine life (Lanier et al 1976). In addition, transient changes in the TH activity within the foetal CNS elicits permanent modifications in adult behaviour, suggesting that DA is involved in the "laying down" of certain behavioural patterns during early development, which are manifested in later life (Arevalo et al 1987). In addition, at a structural level, DA can modulate neuronal outgrowth and influence the morphological and architectural design of the developing brain (Matteson et al 1988). Most of the studies which support a role for DA in organizing CNS development have been performed on the brain as a whole. However, DA may play a similar role in the hypothalamus to regulate the development of neuroendocrine function in particular. Further studies would be required to investigate this proposal and this culture system may well aid in the elucidation of the neuronal mechanisms involved in such important developmental processes.

3.5. Regulation of DA Activity by Gonadal Steroids.

The role of steroids such as oestrogen and progesterone in the regulation of neuroendocrine function is well documented. However, many of the hypothalamic neuropeptide systems, notably GnRH neurones and the somatostatin neuronal populations of the PeVN appear not to be directly oestrogen responsive because they lack oestrogen receptors (Shivers et al 1983, Herbison et al 1993). Hypothalamic neurotransmitter systems have more recently been implicated as intermediaries in the steroidal regulation of hormone release both from the hypothalamus and the anterior pituitary gland (Lookingland & Moore 1984a; Flugge et al 1986; Herbison et al 1989) and evidence suggests that the TIDA system and a portion of the ICHDA neurones are directly responsive to oestrogens and possess oestrogen receptors (Sar 1984). We therefore investigated the effect of the gonadal steroids 17 ß-oestradiol (E_2) and progesterone (Prog) on the functional properties of the dopaminergic neurones, employing both measurement of the uptake and release of [^{3}H]DA within our experimental model. Further studies were performed to determine the influence of E_2 on endogenous DA release and [^{3}H]GABA uptake and release the results of which may be found in Sections 3.5.3 and 3.5.4 respectively.

3.5.1. 17ß - oestradiol.

Uptake experiments were performed on cultures which had been maintained in defined medium containing one of a range of concentrations of E_2 from 10^{-14} to 10^{-6} M. Investigations were carried out from DIV 8 to DIV 21 and optimal accumulation occurred at a concentration of 10^{-10}M E_2 at all time points tested. Fig. 3.26 shows the results of uptake studies performed on DIV 8 and DIV 15.

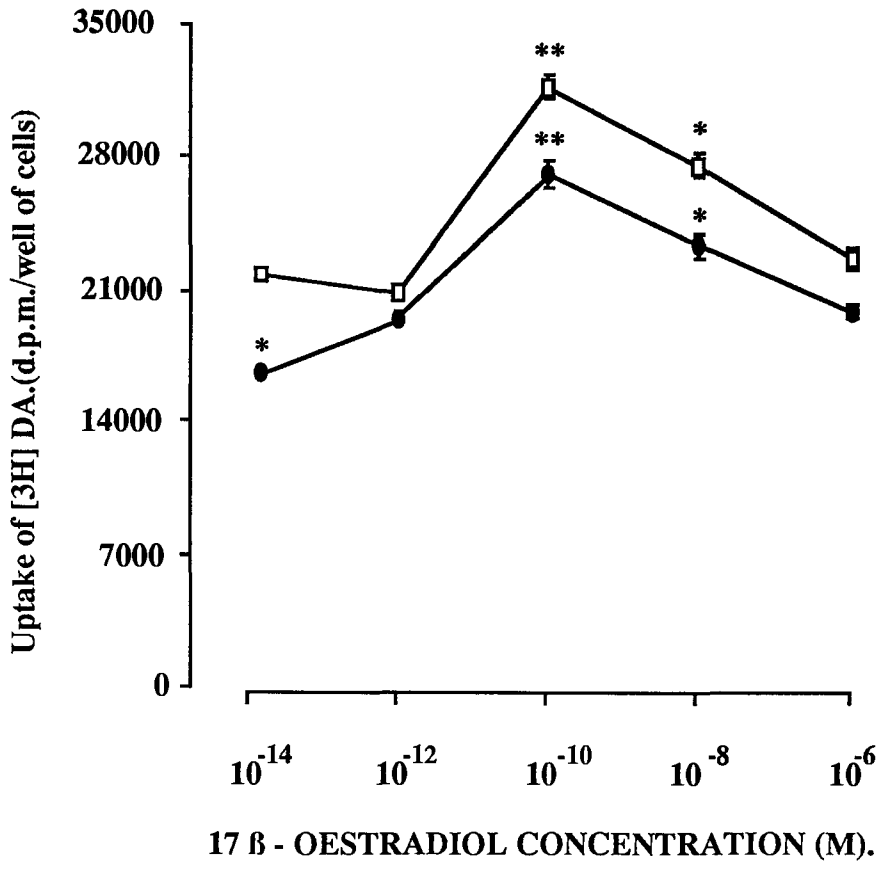


Fig. 3.26. Modulation of the uptake of [^{3}H]DA into hypothalamic dopaminergic neurones by E_2. Uptake experiments were performed as detailed in 2.3.2 on DIV 8 (—✱—) and DIV 15 (-□-). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p < 0.01$ compared with uptake at 10^{-12}M which is the concentration of E_2 routinely used in the defined medium.

The release of [^{3}H]DA,was also modulated by E_2. Maximal release of the tritiated neurotransmitter was observed in those cells maintained in defined medium containing 10^{-12}M E_2, while higher concentrations of the steroid reduced both basal and 56mM K^+ - stimulated release. Quantitatively similar influences of the steroid were observed at all time points tested. Optimal release was not purely a reflection of the increased level of uptake as the concentration of E_2 at which uptake was maximal, differed by two orders of magnitude from the steroid concentration which maximally stimulated [^{3}H]DA release. Fig. 3.27 illustrates the results of release experiments performed on DIV 12.

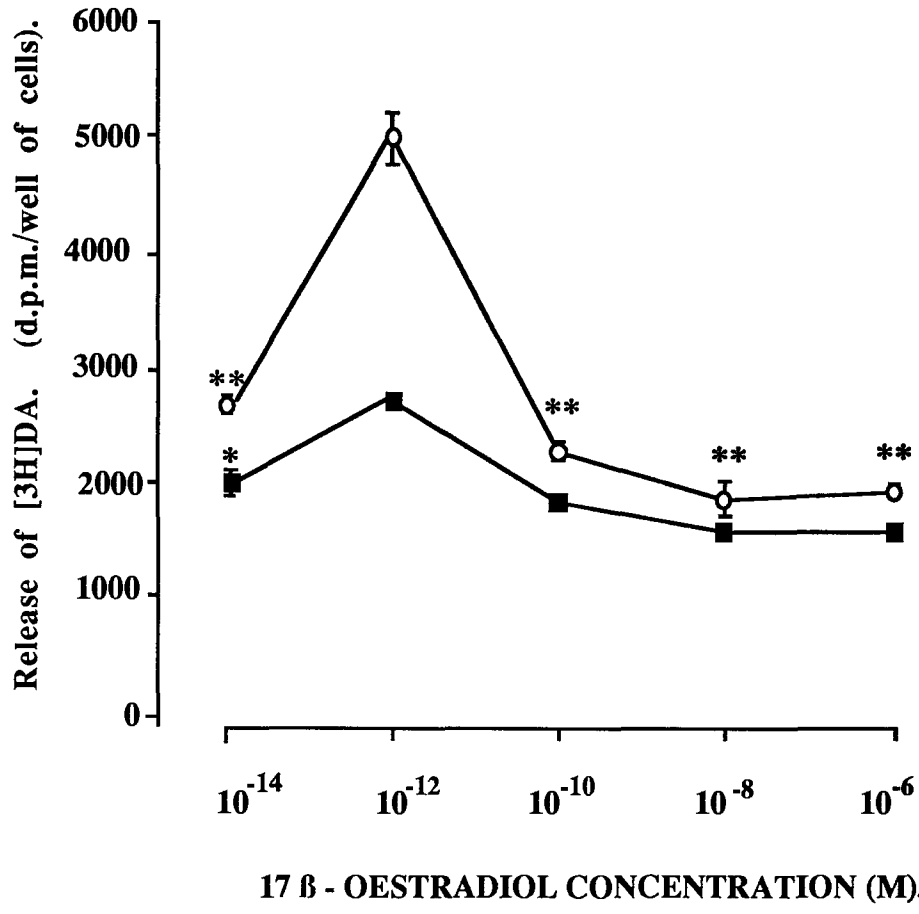


Fig. 3.27. Modulation of the basal (—■—) and 56 mM K^+ - stimulated (—o—) release of [^{3}H]DA from foetal rat hypothalamic cells maintained in primary culture, by E_2 (DIV 12). Results represent the mean ± s.e.m., n = 4. * $p < 0.05$, ** $p < 0.025$ compared with release at 10^{-12}M which is the concentration of E_2 routinely used in the defined medium.

3.5.2. Progesterone.

Cells which were grown and maintained in a range of concentrations of Prog from 10^{-12} to 10^{-8}M were subjected to uptake experiments. Fig. 3.28 demonstrates that those cells grown in the absence of Prog exhibited the greatest capacity for accumulating the radiolabelled neurotransmitter while the higher concentrations of the gonadal steroid significantly ($p < 0.001$) attenuated uptake.

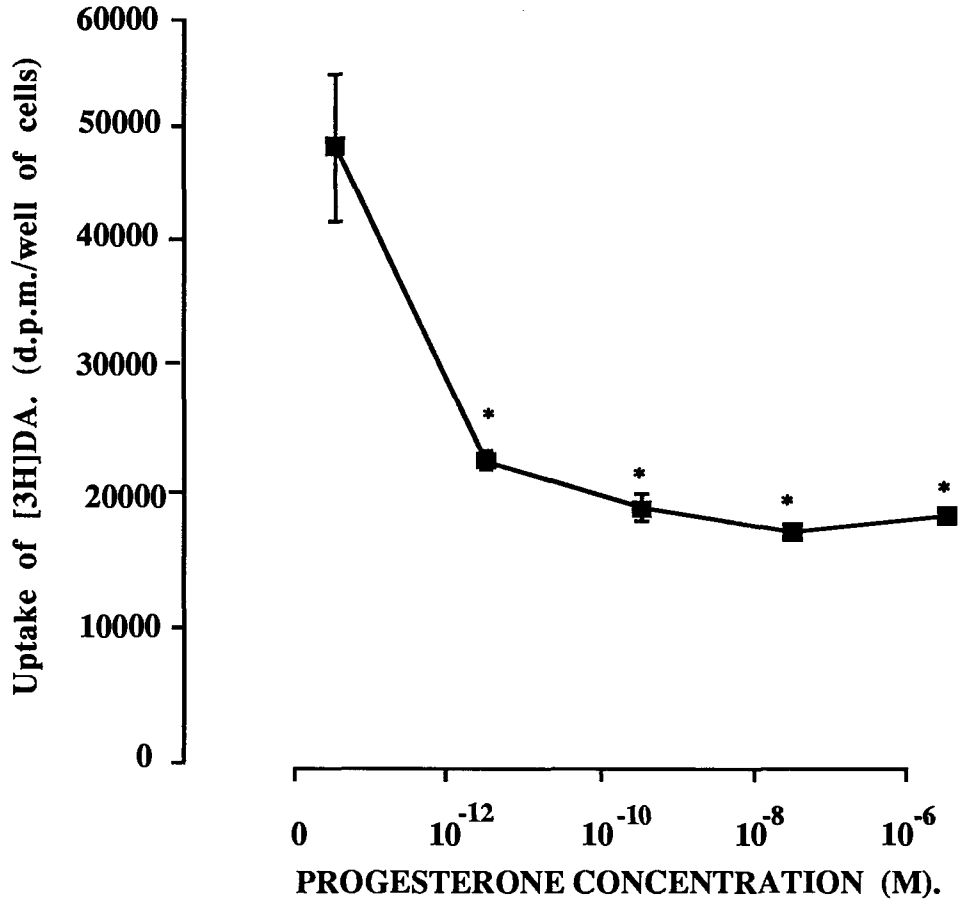


Fig. 3.28. Modulation of the uptake of [^{3}H]DA into foetal rat hypothalamic cells in culture by the gonadal steroid Prog (DIV 10). Results represent the mean ± s.e.m. n=4. * $p < 0.001$ compared with uptake in the absence of the steroid.

Release experiments performed on the same batch of cells showed that the release mechanism for [^{3}H]DA was also modulated by Prog (Fig 3.29). Both basal and 56mM K^+-stimulated release of radiolabelled DA was significantly reduced by concentrations of Prog from 10^{-12}M to 10^{-8}M when compared with release from cells grown in the absence of the steroid (* $p < 0.05$, ** $p < 0.005$). However, in those cells maintained in a high concentration of Prog (10^{-6}M), both basal and depolarization induced release of [^{3}H]DA were raised to levels of output higher than that of the control.

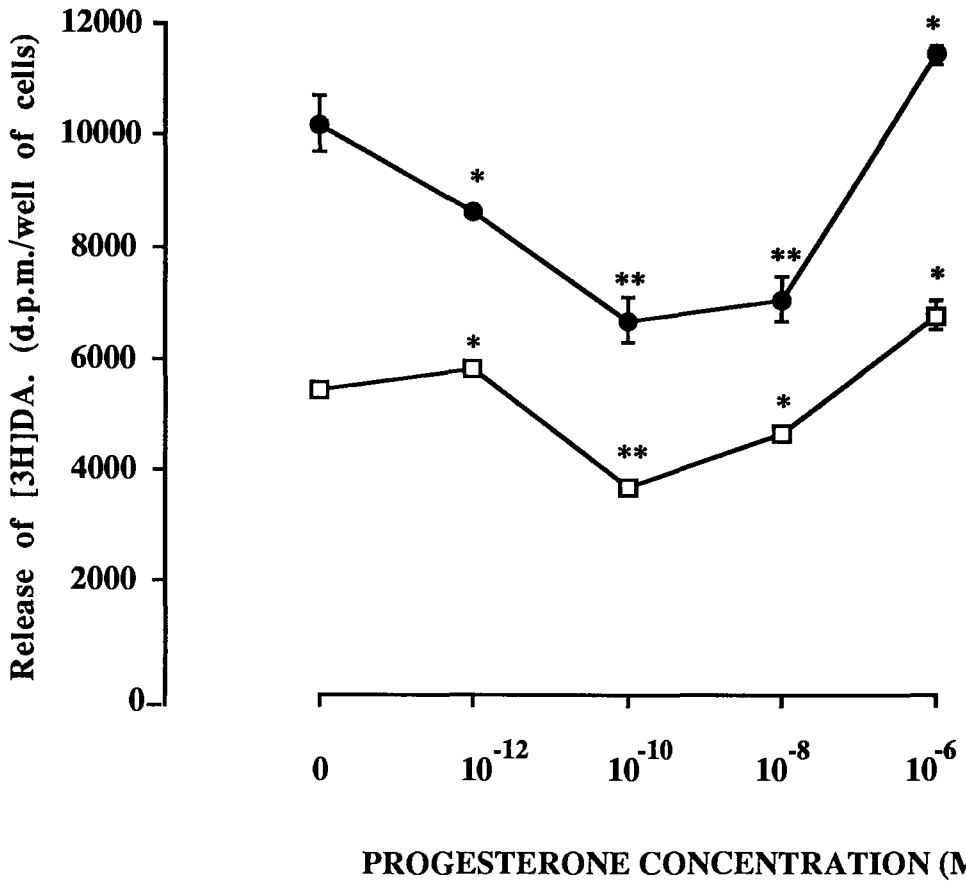


Fig. 3.29. Modulation of the basal (—□—) and 56 mM K^+ - stimulated (—●—) release of [^{3}H]DA from foetal rat hypothalamic cells in culture by Prog (DIV 10). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p < 0.005$ compared with release in the absence of the steroid.

3.5.3. Endogenous DA release and E_2.

The influence of E_2 on the release of endogenous DA was also investigated (Fig. 3.30). Cells were grown in a defined medium containing a range of concentrations of the gonadal steroid from 10^{-12} to 10^{-8}M and the effect on DA release was observed. On DIV 7, both basal and 56mM K^+ - stimulated release of DA were adversely affected when the cells were grown in the total absence of E_2. At a concentration of 10^{-12}M E_2 both basal and depolarization-induced release of DA were at a maximum, while higher concentrations of the steroid caused a significant attenuation of release and also reduced the magnitude of response to 56mM K^+, although the cells continued to respond significantly to the depolarizing stimuli.

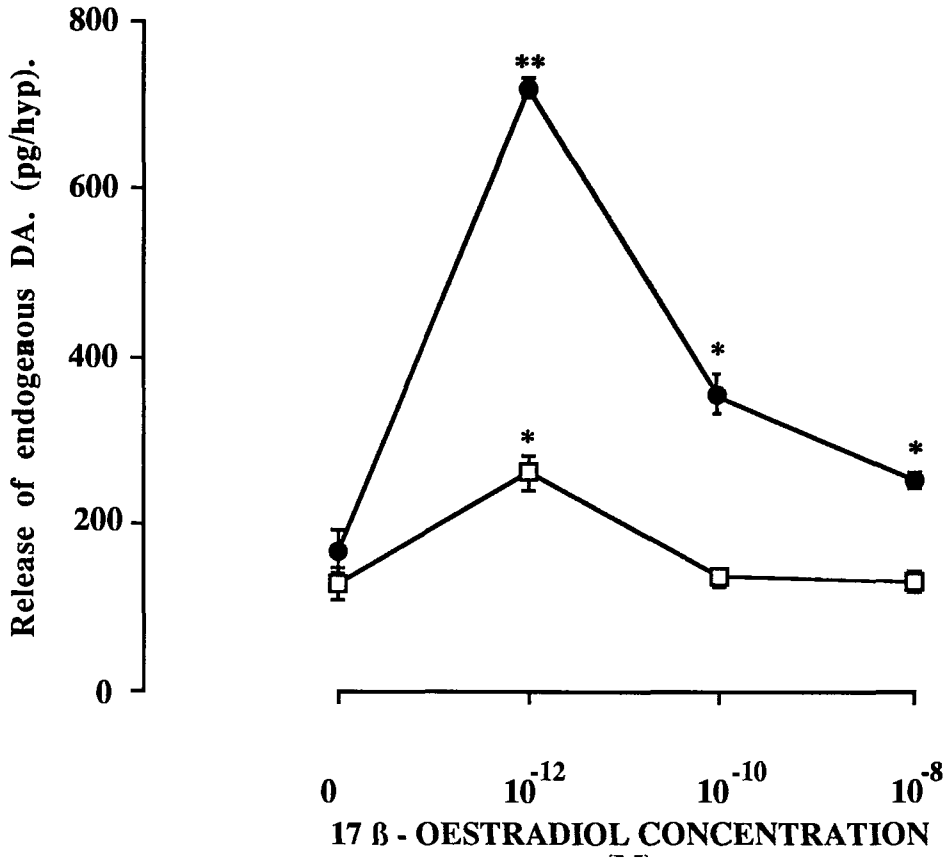


Fig. 3.30. Modulation of the basal (—□—) and 56mM K^+ induced (-●-) release of endogenous DA from foetal rat hypothalamic cells in primary culture by E_2 (DIV 7). Results represent the mean ± s.e.m. n = 4. * $p < 0.01$, ** $p < 0.005$ release in the presence of E_2 compared with release in the absence of the steroid.

On very late ages *in vitro* (DIV 28, Fig. 3.31) DA release was reduced and the pattern of responsiveness to E_2 changed. The output of DA from those cultures maintained in the absence of the steroid was not significantly different from that of cells grown in medium containing the steroid at a concentration of 10^{-12}M. Optimal depolarization - induced output of DA was observed from cells grown in 10^{-10}M E_2 and was again reduced at higher concentrations. Basal release appeared to be unresponsive to changes in E_2 levels at this stage in culture.

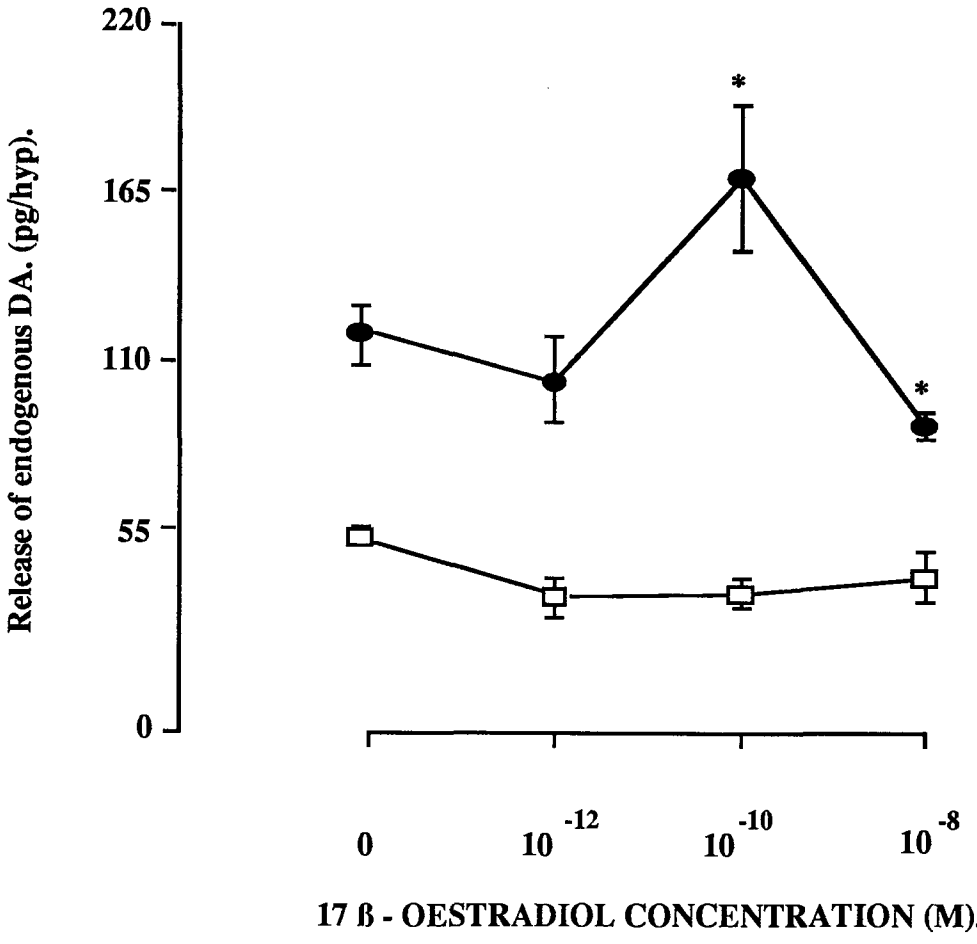


Fig. 3.31. Modulation of basal (—□—) and 56mM K^+ induced (-●-) release of endogenous DA from foetal rat hypothalamic cells in primary culture by E_2 (DIV 28). Results represent the mean ± s.e.m. n = 4. * $p < 0.05$ for depolarization induced release at 10^{-10}M E_2 compared with release in the absence of the steroid.

3.5.4. The Effect of E_2 on GABA - ergic Neurone Activity.

As the GABA -ergic systems within the hypothalamus are also considered to be linked to neuroendocrine regulation and have been reported to be oestrogen responsive (Herbison 1989), a similar experimental design was employed to monitor the effect of E_2 on the uptake and release of [^{3}H]GABA.

Unlike the uptake of [^{3}H]DA, [^{3}H]GABA uptake was totally unresponsive to steroidal modulation. E_2 failed to affect the degree of uptake on any of the days in culture investigated from DIV 7 to DIV 17 (3.32, shows the unresponsiveness of [^{3}H]GABA uptake to E_2 on DIV 7 and 17).

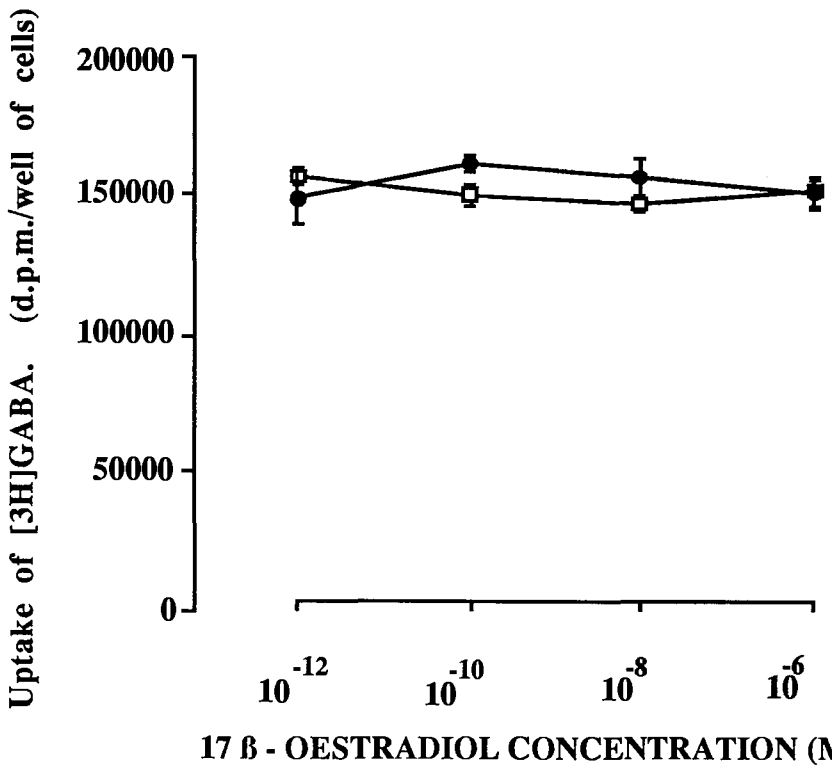


Fig. 3.32. Lack of modulation of the uptake of [^{3}H]GABA into foetal rat hypothalamic cells in primary culture by E_2, on DIV 7 (—□—) and DIV 17 (—●—). Results represent the mean ± s.e.m. n = 4.

Investigations to determine the influence of E_2 on the release of pre-accumulated [^{3}H]GABA showed a different pattern of modulation to that observed for [^{3}H]DA. The release of [^{3}H]GABA, like that of its uptake, was unresponsive to changes in E_2 concentration during early stages of *in vitro* development (viz prior to DIV 18). However as culture age increased, the release mechanism for tritiated GABA became steroid sensitive and 56 mM K^+ stimulated release of GABA was modulated by altered E_2 levels, although basal release remained unaffected. On DIV 18 depolarization induced release of [^{3}H]GABA was optimal at a concentration of 10^{-10}M E_2 (Fig. 3.33).

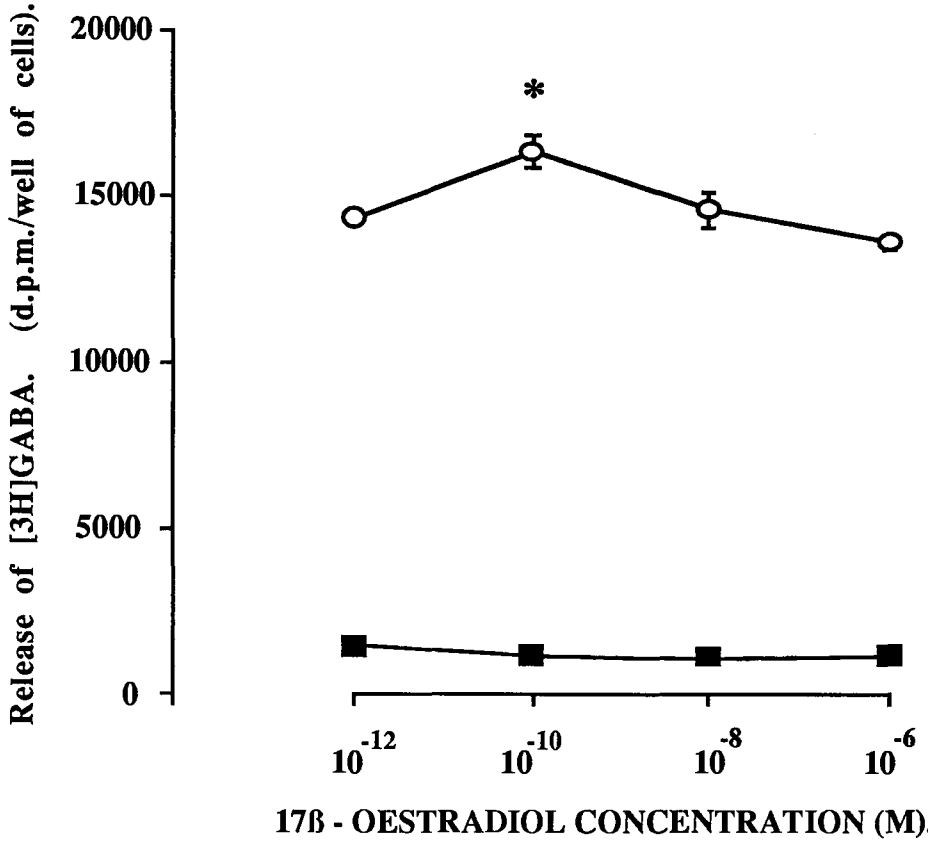


Fig. 3.33. Modulation of the basal (-■-) and 56mM K^+ induced (-○-) release of [^{3}H]GABA from foetal rat hypothalamic cells in primary culture, by E_2, (DIV 18). Results represent the mean ± s.e.m. n = 4. * $p < 0.05$ for 56mM K^+ induced release at 10^{-10}M steroid compared with release at 10^{-12}M which was the concentration of E_2 routinely used in the defined medium.

3.5.5. Discussion.

In this series of experiments we have demonstrated that E_2 and Prog significantly, but differentially, modulate hypothalamic dopaminergic neuronal function, even at very early stages of development.

In the presence of a constant background level of Prog (10^{-8}M), both basal and potassium - stimulated release, whether of endogenously synthesised (at early time points in culture) or pre - accumulated [^{3}H]DA, was optimal at a concentration of 10^{-12}M (Figs 3.27. and 3.30). At lower concentrations of E_2 (< 10^{-14}M), the magnitude of the depolarization - induced response (as judged by the percentage increase above basal), was reduced to less than one - seventh of that observed at the

optimal concentration, while higher concentrations of E_2 (viz. 10^{-10} - 10^{-6}M), virtually suppressed the response to elevated potassium. In contrast, Prog, in the presence of 10^{-12}M E_2, effectively reduced both basal and K^+ - stimulated release of [^{3}H]DA at all concentrations tested. However, the magnitude of response to depolarization, expressed as the percentage above basal, was only moderately reduced (by no greater than 40% compared with that observed in the absence of Prog) by any concentration of the steroid.

Prog also profoundly reduced [^{3}H]DA uptake and this effect was maximal even at the lowest concentration tested (10^{-12}M). In contrast, E_2 facilitated uptake maximally at a concentration of 10^{-10}M, although both lower and higher levels of E_2 attenuated uptake in a concentration - dependent fashion. We do not believe that the effect of E_2 on the release of [^{3}H]DA was purely a reflection of the increased level of uptake as the concentration of E_2, at which uptake was maximal differed by two orders of magnitude from the level of E_2 which maximally potentiated [^{3}H]DA release. Furthermore, other studies involving the investigation of uptake and release mechanisms indicate that the two systems do not alter in parallel as discussed in Sections 3.2 and 3.3).

To summarize the effects of these two gonadal steroids on both uptake and release, it appears that the actions of E_2 on dopaminergic transmission are biphasic while those of Prog are universally suppressive. Furthermore, whereas 10^{-14} and 10^{-10} - 10^{-6}M E_2 almost totally blunted the DA neurones' ability to respond to a depolarizing stimulus, Prog modulated this response only slightly. Thus, while both steroids influence dopaminergic transmission, they appear to do so via distinctly different mechanisms.

It is well established that E_2 and Prog act in concert in many tissues, including the hypothalamus. For example the stimulatory effects of E_2 on TH activity in the median eminence, as well as the effect of E_2 on DA uptake site expression, are enhanced in the presence of Prog (Wang & Porter 1986). Such interactions cannot be ruled out in our culture system and the actions of each steroid individually may differ significantly from their combined action. Further studies would therefore be required to ascertain any possible synergism between E_2 and Prog which may be occuring *in vitro*.

A responsiveness of hypothalamic dopaminergic pathways to gonadal steroids is highly relevant to neuroendocrine control, both perinatally and at later time - points in

development. During the perinatal period, gonadal steroids exert important "organizational" actions in the brain and especially in the hypothalamus, whereas in the adult the gonadal steroids influence hypothalamic function by actions which are known as "activational" processes. Such actions have profound effects on the HP - axis, at the onset of puberty and in adult life (Arnold & Gorski 1984). Thus, castration of the male rat immediately after birth, results in the emergence of female patterns of LH release, including pre - "ovulatory" LH - surges, as well as the characteristic female pattern of GH secretion. Although perinatally many of these influences are androgen - dependent, they are believed to be mediated via E_2 receptors after the conversion of testosterone to E_2 by aromatases which are widely distributed within the foetal CNS (Canick et al 1986). Surprisingly therefore, neither GnRH neurones (Shivers et al 1983) nor the SRIH containing cells of the PeVN (Herbison et al 1993) possess E_2 receptors which suggests that an intermediary neurotransmitter is involved. Our results provide evidence that intrahypothalamic dopaminergic systems may play a role during the organizational period as they display distinct responsiveness to E_2 and Prog at this critical time point in development. As yet we are unable to distinguish which of the several intrahypothalamic DA systems are involved.

The mechanisms via which gonadal steroids influence dopaminergic neurone function are not fully understood and most studies have been performed in the adult. The effect of E_2 alone in regulating TIDA activity is a subject of great controversy and it has been ascribed both a stimulatory (Gunnet et al 1986) and an inhibitory (Arita et al 1990; Tong & Pelletier, 1991; Pasqualini et al 1993) role. The literature largely favours the latter and evidence suggests that it is via an inhibitory action on the TIDA system that E_2 stimulates the release of prolactin (Reymond et al 1978; Ferland et al 1979). Levels of DA in the ME and hypophyseal portal blood are significantly reduced on the morning of pro - oestrous when E_2 levels are high and chronic exposure of TIDA to E_2 has an intense inhibitory action on TIDA activity. By contrast, ovariectomy results in a steroid reversible reduction of TIDA performance in the ME (Gunnet et al 1986), which suggests a stimulatory action of E_2 on TIDA neuronal function.

Reports suggest that E_2 may either attenuate (Michel et al 1987;) or enhance (Engele et al 1989 Morrisetti et al 1993) DA uptake. Such discrepancies in the literature may arise due to differences in the experimental preparation although, the biphasic nature of the actions of E_2 on [^{3}H]DA uptake demonstrated in our studies suggest that the concentration employed is critical. Both the inhibitory and stimulatory effects of E_2 on DA transport may be mediated via a direct action of the steroid with its

intracellular receptor leading to either up or down - regulation of the DA transporter protein and hence an increase in uptake site expression. It has also been suggested that E_2 may act directly in the neuronal membrane causing functional distortions which may or may not be physiologically relevant depending on the concentration. It has been suggested that significant degeneration of TIDA neurones is caused by exposure of the cells to high E_2 concentrations during experimentation (Sarkar et al 1982). It could therefore be argued, that the effect of the higher concentrations of E_2 in inhibiting the uptake and release of DA may be due to general cell death as a result of neurotoxicity. We do not however believe that this is the case, as observation of the cultures under the light microscope did not reveal any differences, in terms of their morphology and size, in the cells grown in high concentrations of the steroid compared with those maintained in lower, more physiological levels of the steroid.

The activity of the TIDA system is regulated by circulating levels of both E_2 and Prog. The effect is likely to be mediated by a direct action of these steroids at their respective receptors which are known to be present on the DA - containing cells of this pathway (Sar et al 1984, 1988). Furthermore, some of the TIDA neurones will posses both E_2 and Prog receptors (90% of them posses progesterone receptors). The physiological significance of this, in terms of the regulation of neurohormone release is yet to be fully established but evidence suggests that these two steroids have clearly distinct actions. Thus, in ovariectomized animals E_2 administration results in daily LH surges whereas Prog treatment advances and amplifies peak plasma gonadotrophin concentrations (De Paola & Barraclough 1979). These effects are strongly considered to be mediated via DA and we propose that the different actions of E_2 and Prog on DA uptake and release demonstrated in our culture model may indicate a mechanism whereby these two sex steroids may exert their differential effects on gonadotrophin release.

Endogenous DA was also affected by E_2 and it is possible that the steroid exerts its influence on DA synthesis as well as release. Chronic administration of E_2 has been reported to inhibit K^+ induced release of DA from the median eminenc via an action on neurotransmitter synthesis (Wang and Porter 1986). Thus, in our investigation, the inhibition of depolarization - induced release of endogenous DA by levels of E_2 from 10^{-10} to 10^{-6}M may be reflecting a disruption of its synthetic pathway, whereas the lower more physiological concentrations (10^{-12}M) may actually increase the expression of the synthetic enzymes.

The activity of the ICHDA neuronal system is also believed to be steroid responsive *in vivo* (Gunnet et al 1986) and *in vitro* (Sanghera et al 1991). A number of reports have documented a modulatory effect of ICHDA turnover by E_2 and these effect are considered to be direct actions mediated via E_2 receptors located on ICHDA neurones (Sar et al 1984). Furthermore, the rostral and caudal portions of the ICHDA tract have been shown to be differentially but directly modulated by E_2 (Gunnet et al 1986; Lookingland and Moore 1984b). Pharmacological studies have also shown that the ICHDA tract and more specifically the rostral component of this tract, serves to regulate the release of GnRH (MacKenzie et al 1984; 1988; James et al 1987; Leranth et al. 1988) and that catecholamine systems in this area may thus be steroid responsive (Wilson et al 1991). In support of this view, DA antagonists have been shown to abolish ovulation presumably by prevention of the preovulatory luteinizing hormone surge (MacKenzie et al 1986). Clearly, in its present form, this model does not allow for a differentiation of DA neuronal sub - populations, but microdissection of the hypothalamus at a later stage of development may provide additional information.

The physiological relevance of the steroid concentrations achieved in any experimental design are difficult to determine, although we would argue that those used in the present investigation may well be within the range reported for circulating levels found *in vivo*. For example, plasma concentrations of oestradiol of 2×10^{-9}M have been reported in the newborn rat (Pang et al 1979), while maternal levels of E_2 are in the order of 1×10^{-9}M at day 14 of gestation (Shiverick et al 1983) and are known to increase further at parturition. Furthermore, in the adult female rat, during the course of the oestrous cycle, levels of circulating E_2 range from 2×10^{-11} to 1.6×10^{-10}M (Smith et al 1975). Predictions of CNS steroid levels are however, complicated by the actions of aromatase enzymes converting circulating androgens to E_2 and also by sex hormone binding globulins which regulate circulating levels of oestrogens (Ekins 1990). Furthermore, the exact role played by sex steroids and their binding globulins in the developing CNS are not fully understood. Despite this, we suggest that the very low levels of E_2 and Prog (10^{-12} - 10^{-10}M), which had significant effects on dopaminergic neurone function in our culture model, are likely to be physiologically appropriate.

In contrast to the dopaminergic systems in our cultures, we found that both [^{3}H]GABA uptake and release demonstrated an insensitivity to changes in E_2 levels during early and mid - stages of *in vitro* development. While the lack of responsiveness persisted for uptake at later stages of the investigation (DIV 18), the K^+ - induced release of [^{3}H]GABA was enhanced at 10^{-10}M E_2. Studies observing

GABA release (Herbison et al 1989), turnover (Mansky 1982), and ICC co-localization of glutamic acid decarboxylase (GAD) with E_2 receptors (Tappaz 1983), suggest that GABA - ergic systems are E_2 responsive in the adult hypothalamus. Thus, the appearance of a responsiveness of the GABA - ergic neurones to E_2 in our culture model may indicate the emergence of a pre - programmed pattern of development which merits further investigation. The importance of Prog levels should also be investigated as our studies, titrating E_2 concentrations in the medium have, so far been performed only in the presence of 10^{-8}M Prog.

In conclusion, we have demonstrated that the activity of at least one sub - population of dopaminergic neurones within the foetal cell culture model is responsive to the actions of both E_2 and Prog but that these actions are different and most likely mediated via distinct mechanisms. These studies have also revealed that gonadal steroid influences on hypothalamic GABA - ergic neurones occur in a manner which is distinctly different from that exerted on dopaminergic neurones. We conclude therefore that the gonadal steroids are not merely influencing neuronal development in a non-specific manner (e.g. behaving as a neurotrophic factor) but they specifically and differentially influence the maturation and/or function of discrete populations of hypothalamic neurones involved in the control of endocrine function. We have also shown that the nature of response to each steroid depends critically on its concentration and it is therefore possible that the contradictory roles ascribed to E_2 concerning its influence on adult dopaminergic function as found in the literature, may be due to fundamental differences in the dose and regimen employed for individual experiments.

3.6. Regulation of DA Activity by GABA-ergic Agents.

Over 80% of neurones within the hypothalamus possess $GABA_A$ receptors (Purdy et al 1991) and there is evidence for an interaction between the dopaminergic and GABA-ergic systems of the hypothalamus in the regulation of neuroendocrine function. Therefore, we investigated the influence of drugs which influence GABA - ergic transmission on the release of endogenous DA from the cultures. Incubation periods of 60min in the presence of the drugs were employed and DA release was measured using HPLC -EC.

3.6.1. MUSCIMOL and Endogenous DA Release.

Fig. 3.34. shows that the release of endogenous DA from the hypothalamic neurones was significantly attenuated by the $GABA_A$ receptor agonist, muscimol, when used at a range of concentrations from 10^{-9} to 10^{-5}M (* $p < 0.04$, ** $p < 0.002$)

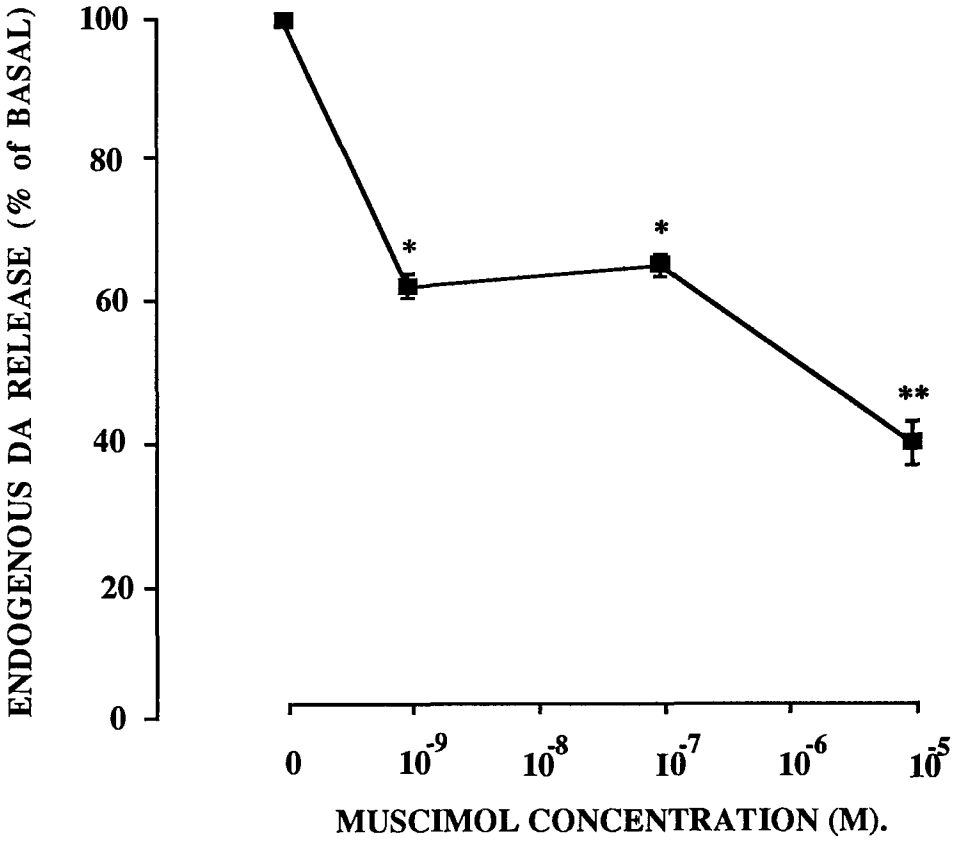


Fig. 3.34. The effect of muscimol on the release of endogenous DA from foetal rat hypothalamic cells maintained in primary culture during 1 hour incubations (DIV 8). Results represent the mean ± s.e.m. n = 4. * $p < 0.05$, ** $p < 0.002$ compared with basal release.

3.6.2. BICUCULLINE and Endogenous DA Release.

The $GABA_A$ receptor antagonist, bicuculline, was stimulatory on the release of endogenous DA at concentrations between 10^{-6} - 10^{-4}M as depicted in Fig. 3.35.

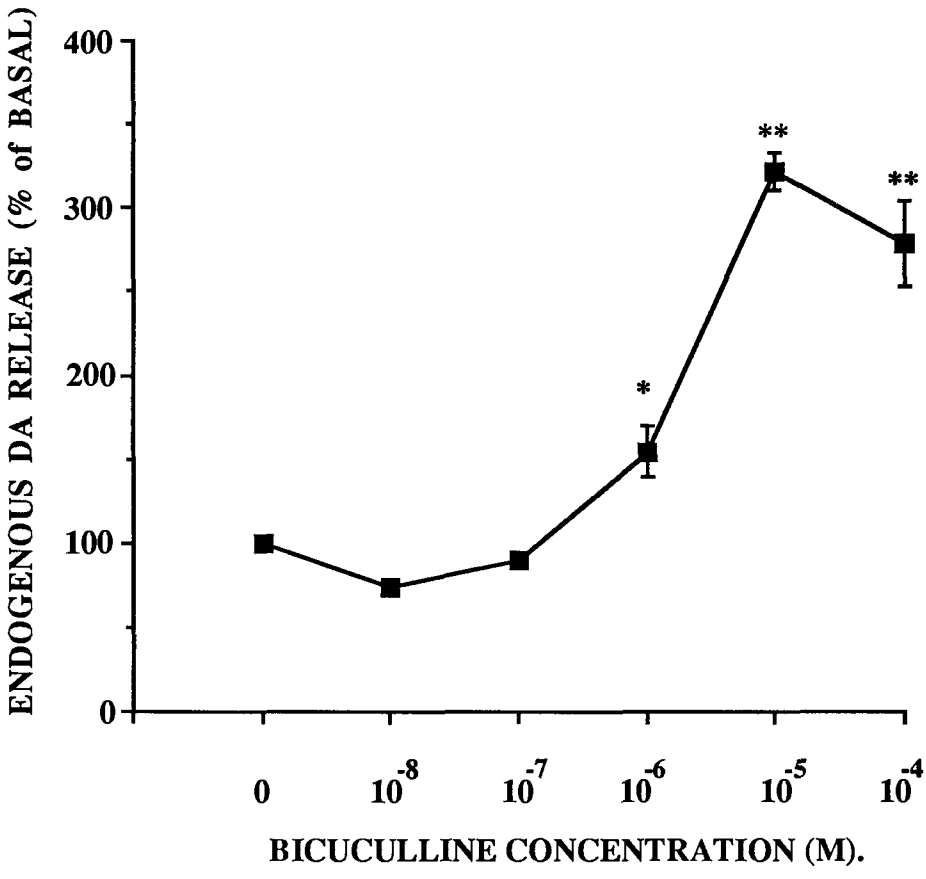


Fig. 3.35. The effect of bicuculline on the release of endogenous DA from hypothalamic dopaminergic neurones in primary culture during 1 hour incubations (DIV 7). Results represent the mean ± s.e.m. n=4. * $p < 0.005$, ** $p < 0.0001$ compared with basal release.

3.6.3. BACLOFEN and Endogenous DA Release.

The $GABA_B$ receptor agonist, baclofen, like the $GABA_A$ receptor agonist, muscimol, inhibited the release of endogenous DA (Fig. 3.36). At concentrations of 10^{-8}, 10^{-7} and 10^{-6}M, DA release was significantly lower than that of the control (* $p < 0.05$, ** $p < 0.02$) and at a concentration of 10^{-5}M the amount of DA released was below the limits of detection.

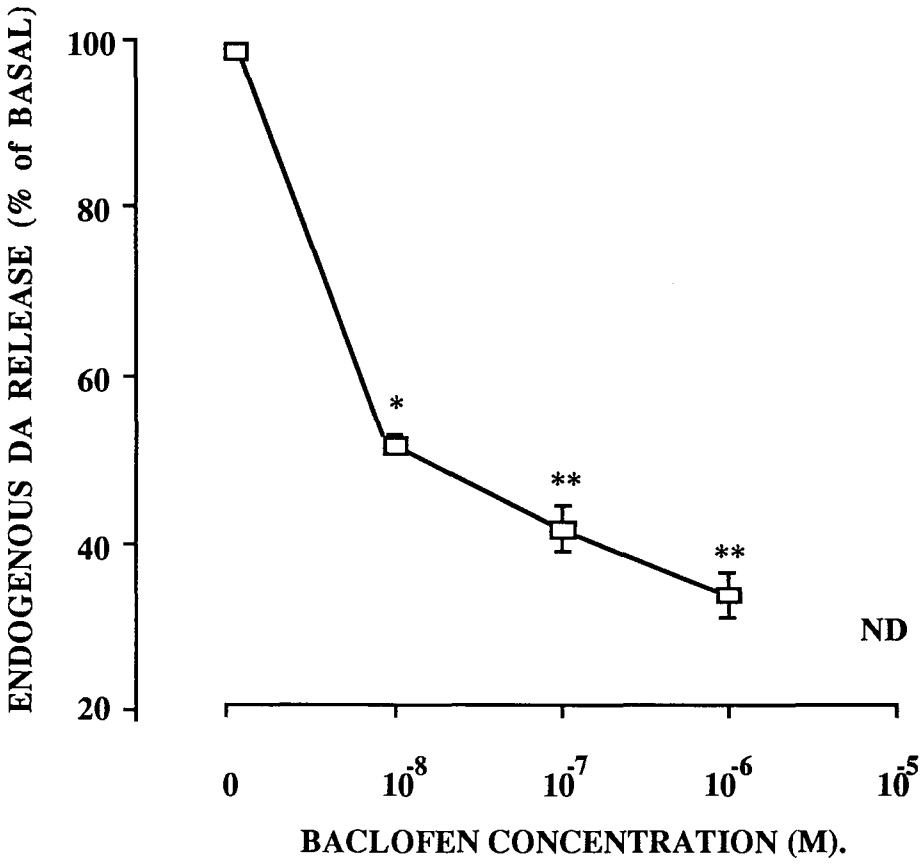


Fig. 3.36. The effect of baclofen on the release of endogenous DA from hypothalamic dopaminergic neurones in primary culture during 1 hour incubations (DIV 10). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $P < 0.02$ compared with basal release. ND = not detectable.

3.6.4. SKF 89976 A and Endogenous DA Release.

Fig. 3.37 illustrates the effect of the specific inhibitor of GABA uptake into neuronal cells, SKF 89976 A on endogenous DA release. An increase in the release of DA was observed in the presence of 10^{-7} and 10^{-6}M SKF 89976 A, but the higher concentrations of the inhibitor reversed this stimulatory effect.

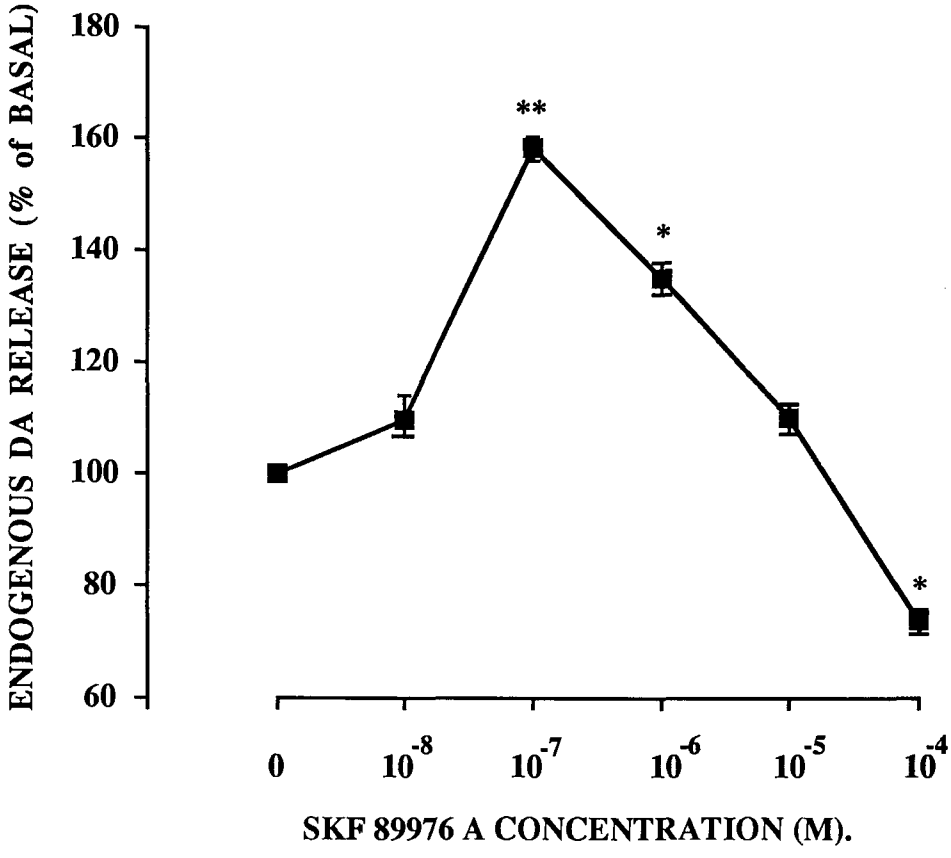


Fig. 3.37. The effect of SKF 899876 A on the release of endogenous DA from foetal rat hypothalamic cells maintained in primary culture during 1 hour incubations (DIV 9). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p.< 0.02$ compared with basal release.

These observations prompted the study of the effect of drugs influencing GABA-ergic transmission on the release of pre-accumulated [^{3}H]DA as such an approach required fewer hypothalamic cells, could be performed more quickly and could thus potentially provide a more economic model for the investigation of GABA-ergic influences on DA release. However results were not directly comparable as initial investigations showed that muscimol failed to modulate [^{3}H]DA release using our standard 7 min incubation period. Therefore, the experimental design was modified to observe the time - course of the effects of these drugs on [^{3}H]DA release during a 7 min incubation period followed by a 20 min incubation period.

3.6.5. MUSCIMOL and [^{3}H]DA Release.

Fig. 3.38 demonstrates the response of [^{3}H]DA release to the $GABA_A$ receptor agonist, muscimol (10^{-9} to 10^{-5}M). Over a 7 min incubation period the drug failed to alter the output of [^{3}H]DA with the exception of the highest concentration employed (10^{-5}M), when a slight yet significant stimulatory effect on release was observed

(*p < 0.05). In contrast, during the 20 min incubation, a significant (** p < 0.02) attenuation of release was achieved by concentrations of muscimol of 10^{-7} and 10^{-5}M.

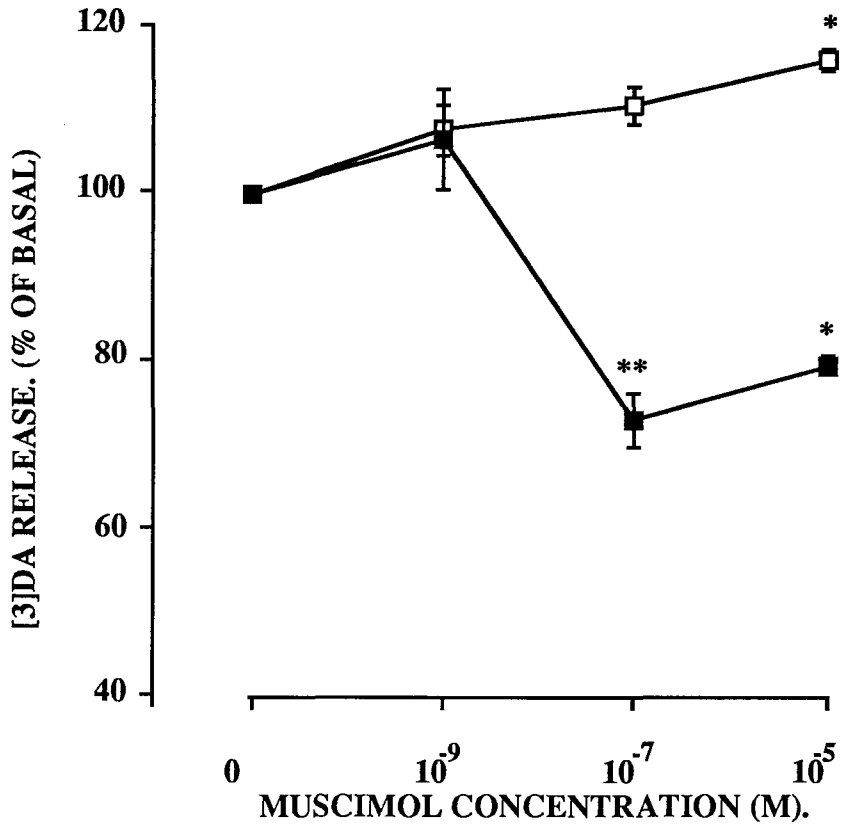


Fig. 3.38. The effect of muscimol on the release of [^{3}H]DA from hypothalamic dopaminergic neurones during 7 min (—□—) and 20 min (—■—) incubation periods (DIV 18). Results represent the mean ± s.e.m. n=4. * p < 0.05, ** p < 0.02 compared with the control.

3.6.6. BICUCULLINE and [^{3}H]DA Release.

The $GABA_A$ receptor antagonist, bicuculline was effective in modulating the release of [^{3}H]DA during both 7 min and 20 min incubation periods (Fig. 3.39). Low concentrations of the drug (10^{-9} and 10^{-7}M) significantly increased the magnitude of [^{3}H]DA release during the 7 min incubation and 10^{-7}M, bicuculline also significantly increased [^{3}H]DA release during the 20 min incubation period. In both instances, the higher concentrations of the antagonist reduced [^{3}H]DA release, compared with the release of [^{3}H]DA observed with 10^{-7}M. During the 7 min incubation, 10^{-5}M bicuculline was still higher than the control whereas with 20 min incubation, release at this concentration was significantly lower than that of the control.

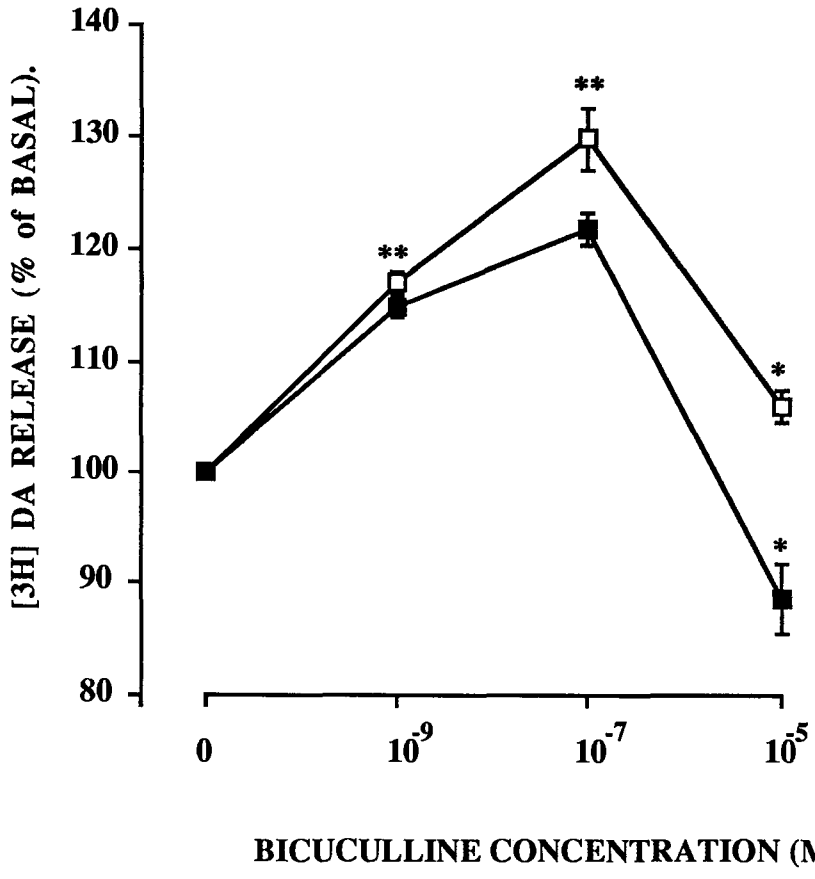


Fig. 3.39. The effect of bicuculline on the release of [^{3}H]DA from foetal rat hypothalamic cells in culture, during 7 min (—□—) and 20 min (—■—) incubation periods (DIV 18). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p < 0.02$ compared with the control.

3.6.7. BACLOFEN and [^{3}H]DA Release.

The $GABA_B$ agonist, baclofen, demonstrated a biphasic modulation of [^{3}H]DA release as illustrated in Fig. 3.40. During a 7 min incubation period lower concentrations of the drug (10^{-9}M), significantly (* $p < 0.05$), potentiated the release of pre-accumulated [^{3}H]DA. During both 7 and 20 min incubation periods, the release of [^{3}H]DA was slightly but significantly (** $p < 0.002$) attenuated by baclofen at a concentration at 10^{-7}M. In both instances, in the presence of the highest concentration of the drug, [^{3}H]DA release was not significantly different to that of the control.

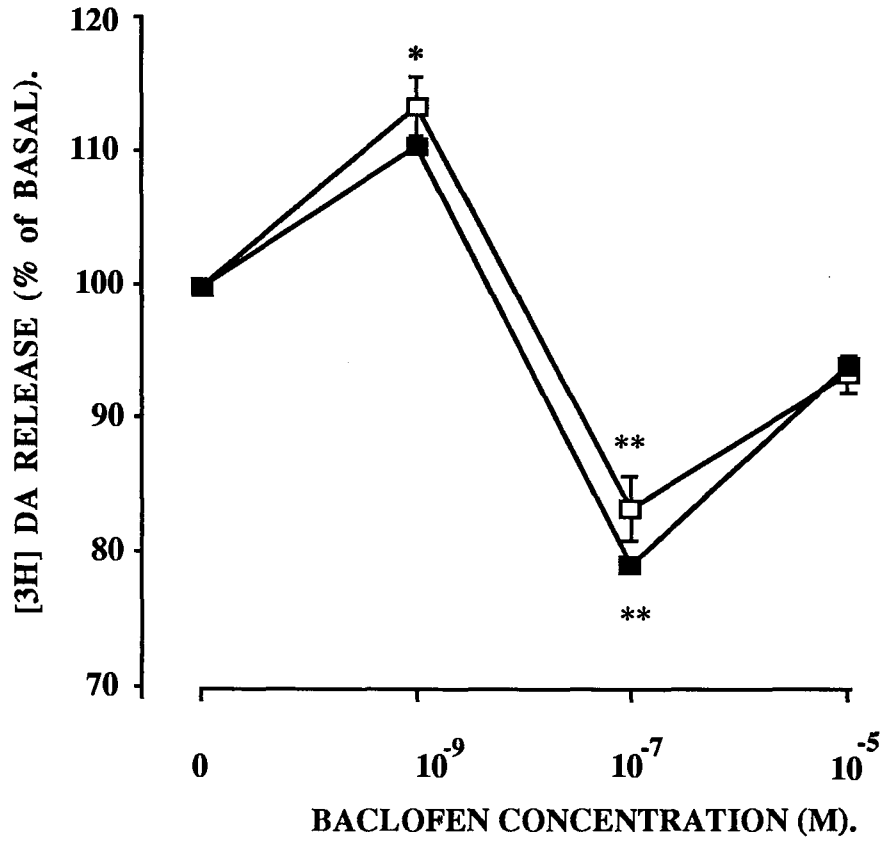


Fig. 3.40. The effect of baclofen on the release of [^{3}H]DA from foetal rat hypothalamic cells in primary culture, during 7 min (—□—) and 20 min incubation (—■—) periods (DIV 18). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p < 0.002$ compared with the control.

For ease of comparison, the data for this series of experiments is reproduced in tabulated form as presented below (Tables 17 and 18).

Table. 17.0. Summary of the effects of GABA-ergic agents on the release of endogenous DA from hypothalamic cells in primary culture.

AGENT	CONCENTRATION (M)	% RELEASE OF BASAL	STATISTICAL SIGNIFICANCE (p value)
MUSCIMOL	10^{-9}	61.9 ± 2.0	< 0.05
	10^{-7}	64.8 ± 1.6	<0.05
	10^{-5}	40 ± 1.57	< 0.002
BICUCULLINE	10^{-8}	80 ± 2.3	NS
	10^{-7}	92 ± 1.1	NS
	10^{-6}	155 ± 4.49	< 0.05
	10^{-5}	323 ± 3.12	< 0.001
	10^{-4}	279 ± 2.9	< 0.001
BACLOFEN	10^{-8}	51.6 ± 0.83	< 0.05
	10^{-7}	41.6 ± 4.29	< 0.02
	10^{-6}	33.5 ± 2.12	< 0.02
	10^{-5}	ND	---
SKF 89976 A	10^{-8}	110 ± 3.85	NS
	10^{-7}	158 ± 2.21	< 0.02
	10^{-6}	136 ± 1.36	< 0.05
	10^{-5}	110.3 ± 2.06	NS
	10^{-4}	74 ± 2.0	< 0.05

NS = not significantly different from basal release.

ND = not detectable.

Table. 18.0. Summary of the effects of $GABA_A$ and $_B$ receptors agonists and a $GABA_A$ antagonist on the release of [^{3}H]DA from foetal hypothalamic cells in primary culture.

AGENT	CONCENTRATION (M)	% RELEASE OF BASAL	
		7 min incubation	20 min incubation
MUSCIMOL	10^{-9}	108 ± 7.96	107 ± 4.3
	10^{-7}	110 ± 2.22	73.2 ± 3.27**
	10^{-5}	116 ± 1.11*	79 ± 1.71*
BICUCULLINE	10^{-9}	117 ± 1.1**	111 ± 1.3**
	10^{-7}	131 ± 2.73**	124.7 ± 3.0**
	10^{-5}	106 ± 5.6*	87 ± 4.17*
BACLOFEN	10^{-9}	113 ± 4.21*	110.6 ± 1.41*
	10^{-7}	83.4 ± 4.4**	79.1 ± 0.99**
	10^{-5}	93.5 ± 2.6	94.1 ± 4.52

* $p < 0.05$, ** $p < 0.02$ compared with basal release.

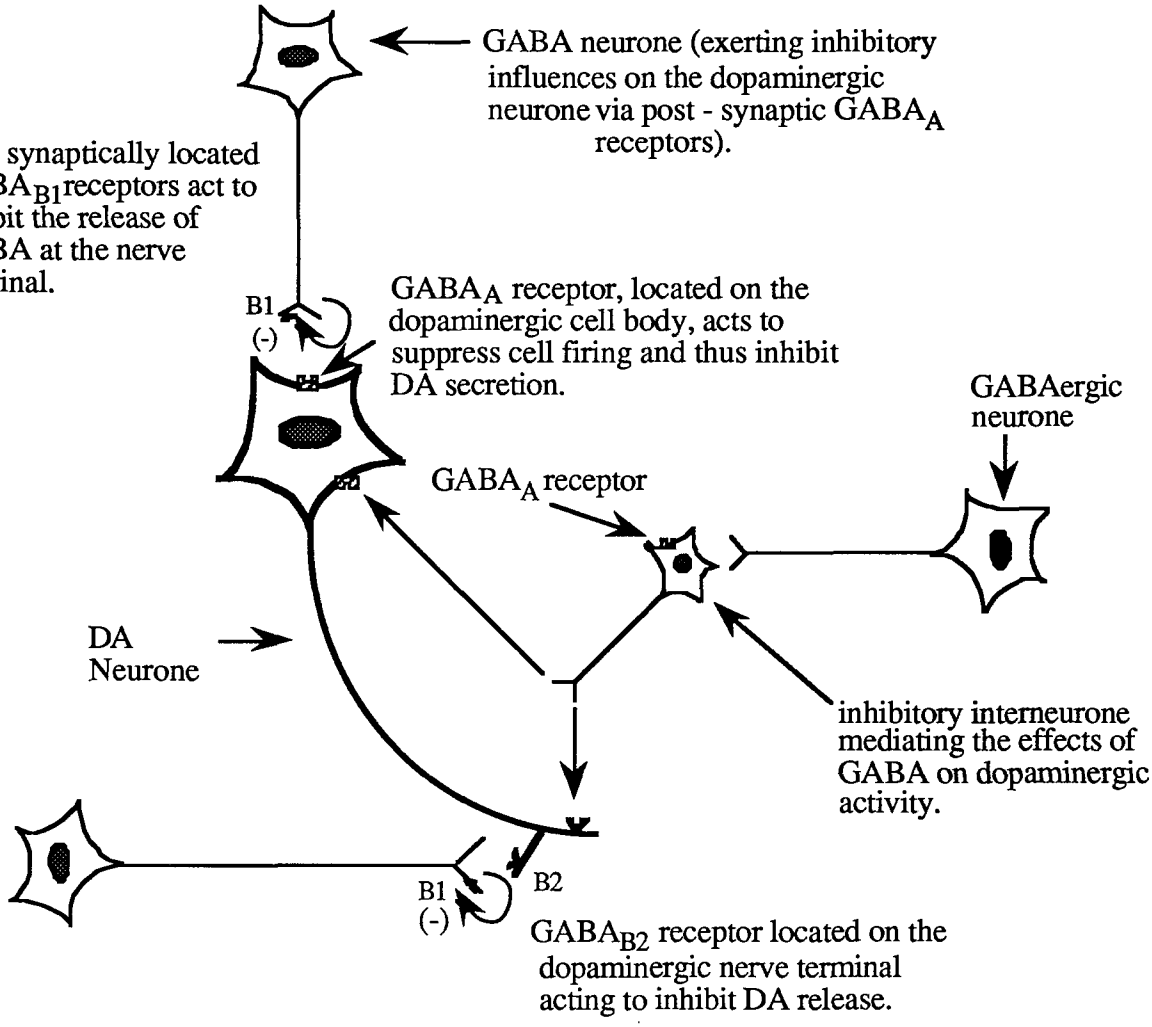
3.6.8. Discussion.

The literature provides support for an intimate relationship between the dopaminergic and GABA-ergic systems within the hypothalamus. Like DA, GABA is found in relatively high concentrations throughout the hypothalamus and especially in the preoptic, anterior and dorsomedial nuclei. Due to the fact that as many as 80% of all the neurones within the hypothalamus possess $GABA_A$ receptors it is likely that at least one population of DA containing cells within the hypothalamus are GABA - responsive.

In this series of experiments the release of endogenous DA was significantly modulated by agents which influence GABA-ergic transmission. The results suggest that there may be a complex mixture of $GABA_A$ and $GABA_B$ receptors located both on the cell bodies and terminals of the dopaminergic and GABA-ergic cells within our culture preparation (Fig. 3.41). Bicuculline elicited a dose - dependent increase in

DA output suggesting that the GABA-ergic neurones intrinsic to the cultures exert an inhibitory tone on DA secretion, via a $GABA_A$ receptor mediated action.

Fig. 3.41. Schematic representation of the putative locations of $GABA_A$ and $GABA_B$ receptors on the dopaminergic neurones within the hypothalamic culture model.



This was confirmed by the action of muscimol which effectively attenuated DA secretion at all concentrations tested. The $GABA_B$ agonist baclofen also suppressed DA release, and was in fact, considerably more effective than muscimol, virtually abolishing DA release at the highest concentration. The $GABA_B$ antagonist phaclofen however, failed to enhance output at any concentrations tested from 10^{-8} to 10^{-5} (data not shown). This may imply that $GABA_B$ receptors are not involved in the

inhibitory tone exerted by endogenous GABA on dopaminergic activity. However, in view of the potent effects of baclofen, plus our knowledge that significant amounts of GABA are released from these cultures over the incubation time studied (Gillies et al 1992), it is more likely that the $GABA_B$ type receptors influencing DA secretion from these perinatal neurones are not effectively blocked by phaclofen.

$GABA_A$ receptors are classically located post-synaptically, and cause the inhibition of neurotransmitter release via an action at chloride channels (Bormann 1988, Fig. 3.41). We therefore propose that endogenously produced GABA continually activates $GABA_A$ receptors on the dopaminergic cell body and in this way suppresses cell firing and DA secretion. The actions of $GABA_B$ receptor agonists/antagonists are however, more likely to be pre-synaptic, thus DA release is inhibited via a decrease in Ca^{2+} conductance and an increase in K^+ conductance (Bormann 1988, Bowery, 1989) at the dopaminergic nerve terminal (Fig. 3.41).

While the GABA receptor agonists/antagonists demonstrated classical inhibitory/ disinhibitory effects on DA secretion, the uptake inhibitor SKF 89976 A modulated DA activity in a biphasic manner. Significant stimulation of DA release was elicited by low concentrations of the inhibitor while higher concentrations reversed this effect and caused a marked decrease in DA output. We propose that this pattern of modulation of DA is due to the dual action of the uptake inhibitor at the GABA-ergic nerve terminals. It has been reported that a number of neurotransmitter uptake inhibitors, whose mechanism of action involves a disruption of the movement of the transporter protein, display a secondary action by preventing neurotransmitter release (Westernick et al 1985). Furthermore, a component of GABA release is considered to be via a carrier - mediated and Ca^{2+} - independent mechanism (Arias & Tapia 1986). Thus, it is feasible that the action of SKF 89976 A may disrupt both the release and uptake of GABA at the nerve terminal by nature of its interaction with the transporter protein. At lower concentrations SKF 89976 A may thus act predominantly to prevent GABA release acting presynaptically via $GABA_B$ receptors located on GABA-ergic nerve terminals, resulting in a decreased concentration of GABA and a subsequent rise in DA release due to disinhibition. This effect is then reversed by the higher concentrations of SKF 89976 A where the inhibition of uptake becomes the predominant property of the drug and thereby elevates transynaptic GABA levels and elicits classical inhibitory actions on DA output.

Alternatively, SKF 89976 A may have a local action viz. at the nerve terminal and thus it is possible that the minor increase in synaptic levels of GABA caused by low concentrations of the inhibitor may only be sufficient to act pre-synaptically, causing

activation of $GABA_B$ receptors located on their own nerve terminals (autoreceptors Anderson & Mitchell 1986). This results in a decrease of GABA release at this site with disinhibition of the dopaminergic neurones as argued above. However, as the concentration of SKF 89976 A is increased, the levels of GABA within the synaptic cleft are significantly elevated and act post - synaptically on $GABA_A$ receptors thus attenuating DA output. In addition, the GABA which has failed to be re - uptaken may also diffuse to $GABA_B$ receptors located on dopaminergic nerve terminals and may also exert an inhibitory influence on DA release at this site (Fig. 3.41).

When this series of experiments was repeated to investigate the effects of the agonists/antagonists on the release of [^{3}H]DA, we were made aware of the importance of the time - course over which certain GABA - ergic agents had their effect. Thus, an inhibitory action of muscimol on [^{3}H]DA release was not observed during a 7 min incubation period but this does not necessarily imply that the agonist was not having an effect. It is possible that over this time period both endogenous and [^{3}H] DA are inhibited by muscimol. However, if the $GABA_A$ receptor agonist acts preferentially on **endogenous DA** the inhibition of its output would result in an increase in the intraneuronal concentration of the amine which could subsequently cause the displacement of **[^{3}H]DA** from the nerve terminals. Thus at lower concentrations the action of muscimol on [^{3}H]DA may be "cancelled out" by the action of endogenous DA causing its displacement, while at the highest concentration (10^{-5}M) the increase in endogenous DA concentrations could be sufficient to cause an apparent rise in [^{3}H]DA output. When the cultures were subjected to a further 20 min exposure an inhibition of [^{3}H]DA release was noted, suggesting that the agonist was now acting equally on the stores of both endogenous and [^{3}H] DA. It was however, less potent on [^{3}H]DA release than on endogenous DA release which suggests that the store into which the [^{3}H]DA is sequestered is not as susceptible to an inhibitory influence as endogenous DA stores.

The action of bicuculline on [^{3}H]DA release was however evident within the first 7 min incubation period but the response was markedly different to that shown by endogenous DA. First, the antagonist was more potent on [^{3}H] than endogenously synthesised DA with concentrations of 10^{-9}M and 10^{-7}M significantly enhancing [^{3}H]DA output (these concentrations were ineffective in stimulating endogenous DA release). This adds further evidence to our proposal that [^{3}H]DA and possibly endogenous re-captured DA, is located in different intracellular pools to those for endogenously, newly synthesised amine and that [^{3}H]DA may be more readily mobilized for release. Second, while the release of endogenous DA continued to increase with higher concentrations of bicuculline, [^{3}H]DA secretion was

significantly attenuated by the highest concentration of the antagonist employed. As bicuculline is a potent stimulator of endogenous DA release we propose that at the higher concentrations the degree of receptor blockade and therefore disinhibition is sufficient to cause the release of newly synthesised DA, in preference to the [^{3}H]DA held in "older" stores (Cerrito, 1980; Tauc 1982). Thus, while the total amount of DA output may have increased with high concentrations of bicuculline (as was seen in experiment 3.6.2), the amount of [^{3}H]DA released (and measured) experienced a decline.

While baclofen was very potent on endogenous DA release and reduced output to virtually zero, the pattern of its effect on [^{3}H]DA release was significantly different and more complex. The apparent and significant stimulation of [^{3}H]DA release at 10^{-9}M baclofen may again be due to the action of the agonist on the endogenously produced transmitter, causing an increase in endogenous DA stores and the displacement of [^{3}H]DA from its storage sites. The inhibition of [^{3}H]DA seen with higher concentrations of baclofen may be due to direct actions of the agonist acting postsynaptically on dopaminergic nerve terminals.

The differences in the profiles of actions of these various GABA - ergic agents are likely to be reflective of the differences in behaviour of [^{3}H] and endogenous DA over short periods of incubation. It appears that [^{3}H]DA is in a more readily releasable pool than endogenous DA and that the endogenously produced amine is more sensitive to inhibition than [^{3}H]DA. Differences in response could also depend on whether inhibitory influences operate through post - synaptic (perikarya/dendritic) or pre-synaptic (axonal/nerve terminals) receptors. Ideally these experiments should be repeated for 7 and 20 min incubations for endogenous DA, but they do however, serve to caution against interpreting results from pharmacological studies involving [^{3}H]DA release alone. The complexity of the interactions between GABA - ergic and dopaminergic systems appear to demand that any investigation into their activity is as close to the "normal" condition as possible, monitoring changes in the behaviour of the naturally occuring neurotransmitter. Furthermore, the time frame in which these drugs exert their pharmacological action appears to be crucial and is complicated by either short (7 min for [^{3}H]DA), or long (60 min for endogenous DA) incubation periods. The discrepancies highlighted in this series of studies mark the major differences in these two approaches for studying dopaminergic activity and merit further investigation.

ICC studies reveal that neurones exhibiting immunoreactivity for GAD and TH lie in close proximity within the region of the zona incerta (Oertel et al 1982).

Furthermore, electron microscopical studies used in conjunction with ICC, provide ultrastructural evidence that GAD - immunoreactive neurones form synaptic contacts with TH - immunoreactive neurones in the region of the Arc and axoaxonic and dendritic connections between the two neuronal systems are indicated in the Arc, periventricular zone and also at the level of the ME. Moreover, co-localization of the two neurotransmitters within the Arc has been reported, from which both the tuberoinfundibular dopaminergic and GABA-ergic systems are considered to emerge (Everitt et al 1984).

Functional evidence for an interaction between hypothalamic dopamine and GABA - ergic tracts stems from their involvement in the regulation of neuropeptide release. For example, GABA, DA and GnRH, nerve terminals are reported to lie in close proximity both in the preoptic area and within the Arc/ME complex, as revealed by immunocytochemical studies (Van den Pol, 1986; Sakaeu et al 1988). In addition, a technique combining anterograde tracing coupled with acute neurodegeneration of DA neurones with the neurotoxin 6-OHDA, revealed that DA containing cells emerging from the area of the zona incerta impinge upon both GnRH and GABA immunoreative neurones. It is widely believed that all three neurochemicals interact at the level of the hypothalamus to control the episodic release of LH and also the preovulatory LH surge (MacKenzie et al 1988). Interestingly, it appears that DA and GABA display opposing effects on GnRH release depending on the neuronal population under investigation i.e. which GABA/dopamine - ergic system is involved, and the area of the hypothalamus at which they are acting. Demling et al (1985) reported that the release of GnRH at the level of the MPOA is inhibited by GABA whereas by contrast, DA stimulates GnRH release at this site (MacKenzie et al 1988). Moreover, DA has an inhibitory influence on GnRH at the level of the Arc/ME complex (McCann et al 1986; Jarry et al 1986), while GABA has a paradoxical effect on GnRH neurones at this site, stimulating its secretion. Direct evidence may also be found in the literature for a modulatory action of GABA on dopaminergic activity. Thus, i.c.v. injection of muscimol reduces DA turnover in the anterior mediobasal hypothalamus (Fuchs et al 1984) and also the release of [^{3}H]DA from synaptosomal preparations of median eminence tissue (Anderson & Mitchell 1986). Moreover, a tonic inhibition of dopaminergic transmission by GABA via $GABA_A$ receptor activation has been reported for other brain regions including the striatum (Kalivas et al 1990).

To add further complexity to the proposed interactions between DA and GABA, it is likely that they may both serve as mediators for steroid feedback processes occuring at the level of the hypothalamus. In certain regions of the CNS it is reported that TH -

immunoreactive neurones do not possess oestrogen receptors. For example, in the preoptic area of the hypothalamus, the dopaminergic neurones which were once assumed to be the direct target of steroid mediation of neuroendocrine responses, appear not to contain E_2 receptors (Herbison et al 1992). However, in the same region there are GABA - ergic neurones which do possess oestrogen receptors which lie in close proximity to these THIP, E_2 receptor - negative cells. It is therefore possible that the hormonal influences exerted by gonadal steroids on certain sub - populations of dopaminergic neurones within the hypothalamus are not direct, but rather are mediated via steroid responsive intermediates such as the intrahypothalamic GABA - ergic systems.

Thus, although there appear to be some fundamental differences in the behaviour of [^{3}H] and endogenous DA in response to drugs affecting GABA-ergic transmission, there is still a body of evidence emerging from this series of experiments to support an interaction between dopamine and GABA containing systems intrinsic to this hypothalamic cell culture model. The close anatomical and functional association of the dopaminergic and GABA-ergic neurones in the hypothalamus may be the basis by which these two neurotransmitters act in conjunction to regulate certain parameters of neuroendocrine function.

3.7. Other Modulatory Factors.

We have previously discussed the role played by DA in the regulation of both PRL and GnRH secretion (Section 1.8). We therefore determined to investigate whether either of these factors exerted a feedback influence on hypothalamic dopaminergic neurones.

3.7.1. Prolactin.

We investigated the effect of prolactin on the release of endogenous DA from the hypothalamic cultures, at a range of concentrations from 10^{-11} to 10^{-7}M but the release of endogenous DA from the cultures was not significantly affected. The experiment was repeated at a later time-point in culture (DIV 15) where prolactin also failed to modulate release.

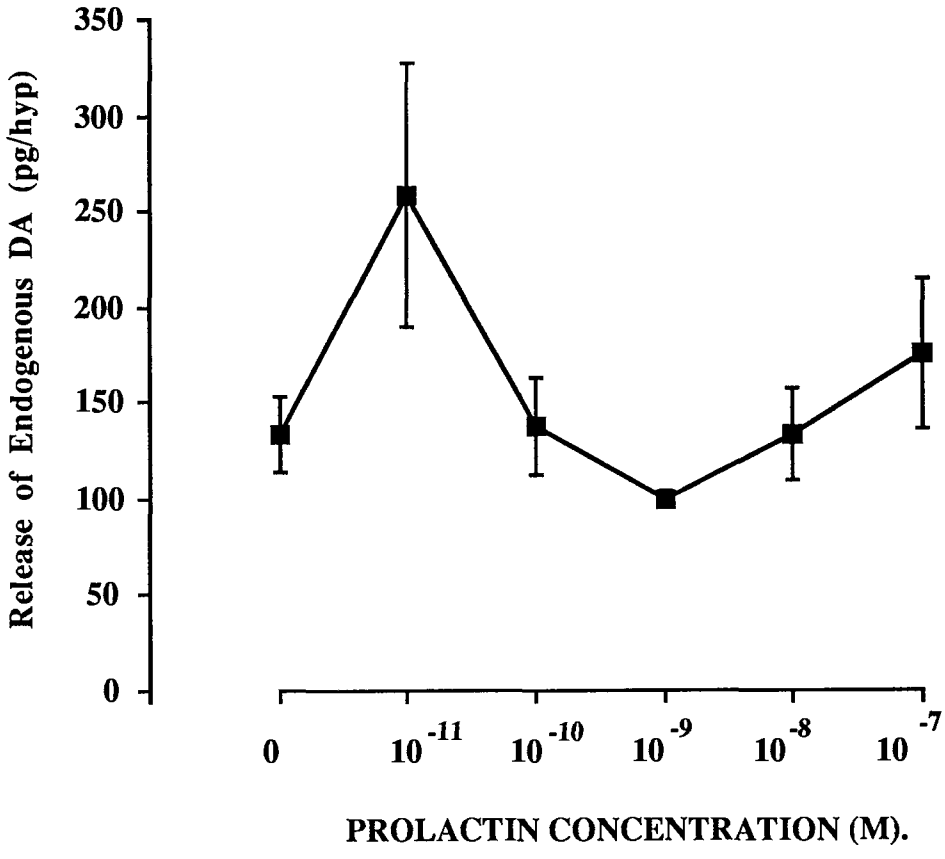


Fig. 3.42. Lack of modulation of the basal release of endogenous DA from rat foetal hypothalamic cells in culture, by exogenous prolactin. Cells were incubated in sEBSS containing a range of concentrations of prolactin from 10^{-11} to 10^{-7}M for 1 hour durations (DIV 7). Results represent the mean ± s.e.m. n=4.

3.7.2. Gonadotrophin - Releasing Hormone (GnRH).

We also investigated the action of GnRH at a range of concentrations from 10^{-10} to 10^{-6}M on the release of endogenous DA from the hypothalamic cells in culture. On DIV11, GnRH at low concentrations (10^{-10} to 10^{-8}M) significantly elevated endogenous DA release while the higher concentrations were without effect. However, on DIV16, release was unaffected by the lower concentrations of the peptide i.e. 10^{-10} and 10^{-9}M, while, in this instance higher concentrations of the peptide from 10^{-8} to 10^{-6}M, were stimulatory on DA output which was optimal at a GnRH concentration of 10^{-7}M. Thus, with increasing culture age there was a decrease in the sensitivity of the DA neurones to modulation by this neurohormone.

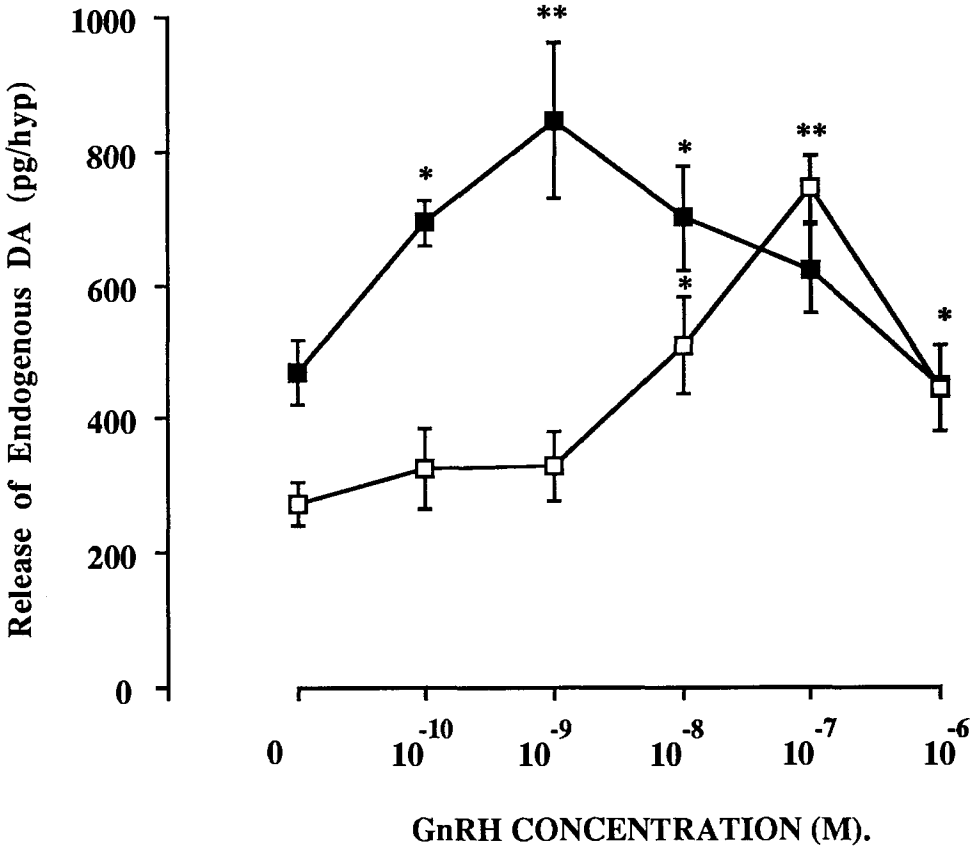


Fig. 3.43. Modulation of the basal release of endogenous DA from foetal rat hypothalamic cells in culture, by exogenous GnRH. Cells were incubated in sEBSS containing a range of concentrations of the neurohormone from 10^{-10} to 10^{-6}M for 1 hour durations on DIV 11 (—■—) and DIV 16 (—□—). Results represent the mean ± s.e.m. n=4. * $p < 0.05$, ** $p.< 0.025$.

3.7.3. Discussion.

Prolactin binding sites are present at the level of the ME (Barton et al 1989) and many reports suggest that PRL exerts a negative feedback on its own release, via an action on the synthesis and secretory mechanisms for DA (Gudelesky et al 1979; Nicholson et al 1980; Demarest & Moore 1980; Arita et al 1989). Thus hypoprolactinaemia, induced by chronic DA administration, decreases DA synthesis and turnover in TIDA neurones while the regulation of the expression and activation of TH is enhanced by hyperprolactinaemia, produced by pituitary implants under the kidney capsule. In our investigations however, we were unable to demonstrate any significant effect of PRL on the release of endogenous DA. Recently, it has been proposed that an uncharacterized cytotropic factor, extracted from the pituitary gland, enhances the expression of TH in cultured brain cells (Porter et al 1990). This implies that PRL as a pituitary factor, is not solely responsible for regulating DA synthesis/release at hypothalamic level and that results from studies where whole pituitary gland/pituitary gland extracts have been employed to activate DA synthesis may be misleading. It is possible therefore that our negative results are due to the fact that PRL is not the major factor which regulates dopaminergic function.

It is also important to consider the time course of our studies. There is a degree of controversy concerning the speed of onset of the action of PRL on TIDA activity. The release of [^{3}H]DA from medial basal hypothalamic fragments is reported to be augmented by acute exposure to PRL (Foreman & Porter 1981). However, when DA turnover is used as the index for TIDA activity, there is a delay in the onset of action of PRL of between 10 and 26 hours (Gudelesky et al 1979). The latency in the effect of the neurohormone is considered to be reflective of the alterations in dopaminergic activity after the induction of transcriptional/translational processes, as a similar time-course has been proposed for the induction of TH expression. This may account for the lack of responsiveness of DA release in our experimental model and longer incubation periods in the presence of PRL may result in the emergence of a modulatory influence on the dopaminergic activity.

The action of PRL in the regulation of DA release may also be age - dependent. Chronic hyperprolactinaemia induced by haloperidol (a DA antagonist) does not alter the expression or amount of TH in the median eminence of young intact female rats, although there is a marked increase in TH mass in mature, ovariectomized animals (Rossi 1992). As the tissue in our experimental model is derived from a foetal source, it is feasible to suggest that at this stage of development the TIDA neurones are not equipped to respond to elevated levels of PRL.

The effect of PRL on dopaminergic activity is also considered to be tract specific as little evidence exists in favour of a modulatory influence on the behaviour of the ICHDA tracts. PRL has no effect on TH expression in the A13 group of cells where there is a predominance of DA perikarya from which the rostral and caudal portions of the ICHDA tract originate. To date, regulation of this system is poorly understood, but it is generally accepted that any effect of PRL is negligible compared with its marked action on the TIDA pathway. However, the ICHDA pathway constitutes the major dopaminergic tract in the hypothalamus, and hence in our culture model, and it is therefore possible that the lack of responsiveness of these neurones may conceal any modulatory influence being exerted by PRL on TIDA release.

An obligatory role for PRL in regulating DA neurone function is exemplified in the Ames dwarf mouse which has a marked GH and PRL deficiency and significant impairment of TIDA development. It is generally accepted that the deficiency in PRL is responsible for the poor development of the TIDA pathway as replacement of the hormone restores, in part, the levels of DA synthesis. Furthermore, in female Ames dwarf mice, the number of THIP cell bodies within the Arc are significantly lower than in any other mouse strains, with fewer THIP nerve terminals innervating the external layer of the ME (Morgan & Besch 1990). Yet if PRL is administered to such animals there is a marked increase in the number of THIP neurones to a level comparable with that observed in phenotypically normal animals. It thus appears that it is not an absence of TIDA neurones, but a dormancy of the neurones in the absence of PRL, and that the hormone activates DA synthesis in otherwise inactive cells. Continued PRL treatment of our cultures might therefore, activate DA neurone development further. Alternatively, factors which substitute for this role of PRL may either be produced by the cultures themselves or may be present within the defined medium.

Although we were unable to demonstrate an effect of PRL on DA release in our hypothalamic culture model, the overall consensus found in the literature suggests that PRL is responsible for maintaining the functional integrity of TIDA neurones via an action on synthetic/secretory apparatus. Slight modifications to the experimental design i.e. isolation of the TIDA tract and increasing the period of incubation, may reveal a modulatory effect of the hormone and experiments incorporating these changes are planned for future studies.

In contrast to PRL, GnRH was effective in biphasically modulating the release of endogenous DA. On DIV 11, GnRH concentrations of less than 10^{-9}M significantly enhanced the release of DA, while higher concentrations reversed the stimulatory

effect. However, by DIV 16, the responsiveness of the dopaminergic neurones to GnRH was markedly altered. Thus, concentrations of GnRH, which previously had been stimulatory on DA release now failed to increase dopaminergic activity and 100 - 1000 fold higher concentrations of the hormone were required to elicit a response on DA secretion. These results suggest that while GnRH acts as a potent secretagogue for DA at early stages of *in vitro* development, the effect becomes less pronounced as the neurones matured.

There is both anatomical and functional evidence to suggest an association between dopaminergic and GnRH - containing systems within the hypothalamus and the role played by DA in regulating GnRH secretion is well established. The results of this investigation however, suggest a regulatory effect of GnRH on dopaminergic activity resulting, presumably, in the modulation of its own release. Other workers have proposed a direct effect of this neurohormone on dopaminergic activity. Thus, in synaptosomal preparations of the corpus striatum, the biosynthesis of [^{3}H]DA from radiolabelled precursors is significantly attenuated by GnRH at a concentration of 5 x 10^{-6}M (Wang et al 1982). Furthermore, in the teleost retina, DA containing interplexiform cells receive input from GnRH - containing, so called, centrifugal fibres, which are reported to stimulate DA release (Umino & Dowling 1991) and changes in the acquisition of conditioned avoidance responses (CARS) in adult male rats is reduced by injection of GnRH into the hippocampus and nucleus accumbens via an inhibitory action on DA release in this brain region (Mora et al 1991). Although an autoregulatory negative feedback mechanism for GnRH at hypothalamic level has been suggested (Paolo et al 1987; Valencca et al 1987), to our knowledge this is the first direct evidence for a modulatory action of the neurohormone on the activity of intrahypothalamic dopaminergic systems *in vitro*.

It is difficult to determine whether the effect of GnRH is direct, or is exerted via other neurotransmitter/neuropeptide systems. For example, endogenous opioidpeptides are known to influence the release of GnRH and morphine inhibits LH secretion and suppresses the proestrous LH surge and ovulation. Moreover, ß - endorphin and long-acting enkephalin analogues attenuate both the amplitude and frequency of LH release via an action which is reversed by nalaxone. The influence of the opioid peptides is considered to be mediated via an enhancement on DA biosynthesis and release and it is thus feasible to suggest that these peptides may be involved in the action of GnRH on DA secretion observed in this study. Furthermore, the close association of GnRH - containing neurones to GABA-ergic neurones and the functional relationship existing between these two neurochemicals, suggests that

GABA could also be involved in any potential feedback mechanism for the regulation of GnRH release.

The change in the sensitivity of the dopaminergic neurones to GnRH with increasing age of culture may be reflective of its involvement in the organizational processes which occur prior to and immediately after birth. Hypothalamic GnRH expression occurs early in foetal life and the neurohormone demonstrates cytotrophic actions well in advance of the commencement of LH biosynthesis (Aubert et al 1985). Thus, it is likely that GnRH acts primarily as a trophic factor during early stages of development. Moreover, it is possible that one or several of the functional changes occurring in the perinatal CNS is mediated via an action on central catecholaminergic systems and thus the shift in responsiveness of DA release to GnRH may represent a physiological alteration in the behaviour of dopaminergic neurones at a pre-programmed point in foetal/postnatal development. A shift in DA responsiveness was also observed for E_2 on DIV 7 and DIV 28 (Figs. 3.30 and 3.31) and it is thus possible that these observations may reflect critical changes in intrahypothalamic dopaminergic cell function in response to alterations in the steroid and neuropeptide environment which could critically influence organisational events in the hypothalamus.

3.8. Summary

Immunocytochemical Studies.

1. On the basis of TH ICC, perinatal hypothalamic dopaminergic neurones are relatively immature at the time of initiating the cultures but continue on a pre-programmed pattern of morphological development over a period of 2 weeks *in vitro* in a manner similar to that observed *in vivo*.

2. We hypothesise that the observed morphological differences may represent differences in the original anatomical location of these dopaminergic neuronal populations and consequently represent functionally distinct pathways.

3. THIP neurones with bipolar morphology may represent dopaminergic neurones of the TIDA system which are involved in the regulation of PRL and GnRH secretion.

4. Multipolar THIP neurones may derive from the rostral component of the ICHDA tract *in vivo* which may also be involved in the regulation of certain aspects of neuroendocrine function.

5. The functional development of the diencephalic dopaminergic systems appears to be retarded in relation to the development of those of the mesencephalon.

6. On the basis of GABA ICC, perinatal GABA-ergic neurones very rapidly (< 3 days) attain a mature morphology in culture.

Studies with [^{3}H] Neurotransmitters.

1. Investigations on the uptake and release of [^{3}H]DA provide functional evidence in support of the conclusions made in 1-5 above.

2. Investigations with [^{3}H]GABA provide functional evidence in support of conclusion 6 above and, when compared with DA studies, suggest that hypothalamic dopaminergic and GABA-ergic systems exhibit pre-programmed, differential developmental patterns *in vitro*.

3. Studies with [^{3}H] and "cold" DA suggest that a D_2-like receptor mediated mechanism may influence DA uptake and hence synaptic concentrations of DA.

4. On the basis of the above findings we propose that hypothalamic neurones *in vitro* exhibit developmental patterns which are specific for individual neurotransmitter systems and distinct from developmental patterns of similar neurotransmitter systems located in other brain regions. Such distinct developmental patterns are therefore, unlikely to reflect, solely an artefact of the culture conditions, but may represent important developmental processes occuring in brain-specific regions in discrete neuronal populations and at different developmental stages, many of which may be critical to the adequate functioning of the neuroendocrine system in adult life.

Studies with Endogenous DA.

Similarities in the responsiveness of endogenous and [^{3}H]DA to non-specific and neurochemically evoked release do exist but not in all situations. These results emphasize the complex balance between release, uptake, synthesis and storage and cautions against conclusions made from studies of [^{3}H]DA release alone.

Studies with Gonadal Steroids.

Investigations on the uptake and release of [^{3}H]GABA and DA and the release of endogenous DA suggest that gonadal steroids may specifically and differentially influence the maturation and/or function, during the perinatal period, of discrete populations of hypothalamic neurones involved in the control of endocrine function.

DA-GABA Interactions.

1. Endogenous GABA appears to exert potent inhibitory influences on DA release via type A and B receptors.

2. Differences in [^{3}H] and endogenous DA release in response to pharmacological manipulation of GABA-ergic transmission suggest that DA may be located in more than one intracellular pool.

3. Endogenous DA appears to be more sensitive to inhibitory GABA-ergic influences than recently accumulated [^{3}H]DA.

4. [^{3}H]DA appears to be stored in a pool which is released more readily when inhibitory GABA-ergic influences are lifted.

5. These results support the importance of DA-GABA interactions in the regulation of neuroendocrine function and demonstrate that they are active in the perinatal period.

Prolactin-DA Interactions.

Prolactin appears to have no influence on hypothalamic dopaminergic neurones during the perinatal period.

GnRH-DA Interactions.

1. These studies provide evidence for a novel, potent stimulatory effect of GnRH on hypothalamic dopaminergic systems during the perinatal period which declines as the dopaminergic neurones undergo *in vitro* development.

2. This phenomenon could reflect either a developmental stimulus by GnRH to the maturation of a population(s) of dopaminergic neurones which will ultimately be involved in the control of the reproductive system or, an early short-loop feedback system (positive on dopaminergic neurones exerting a negative influence of GnRH secreting cells) which is highly active in the immature animal in order to repress reproductive function.

CHAPTER FOUR: GENERAL DISCUSSION AND FUTURE DIRECTIONS.

The major purpose of this project was to investigate the development of the various dopaminergic neuronal populations of the hypothalamus, employing a primary cell culture model and monitoring changes in both the morphology and function of this neurotransmitter system. To a great extent, these aims have been realized and, in addition, the results have raised a number of further questions which we would hope to address in future studies.

It is widely accepted that the development of individual neurones and indeed of whole neuronal populations *in vivo,* are dependent on cell -to- cell associations, with various soluble trophic, inhibitory and migratory factors determining the exact phenotype that each cell adopts. Thus, the very identity of a neurone is believed to be exacted by its environment. Even with the use of defined media and increasingly more complex systems for supporting neuronal growth, cells maintained in such culture are unlikely to be exposed to the same developmental signals and inputs that they experience in the 3-dimensional, *in situ* situation. In the absence of extrahypothalamic regulatory factors it might therefore be expected that neurones would have very limited capacity to survive and develop in culture. However, we have demonstrated that the dopaminergic neurones within our culture model pursue distinct processes of both morphological and functional development, the patterns of which closely resemble that reported for *in vivo* development. We propose, therefore, that a degree of programming of neuronal hypothalamic cells occurs during foetal development, at least prior to E18 in the rat strain employed, and that this pre - programming resulted in the well defined pattern of morphogenesis demonstrated by THIP neurones (Section 3.1.1.3) and also the obvious pattern of functional development as indicated by the biochemical studies (Section 3.2). Furthermore, the *in vitro* behaviour of dopaminergic neurones derived from animals at different ages relative to conception, emphasised important changes in neuronal activity occurring over the perinatal period. As argued in 3.4.6, it is likely that at term certain processes of neuronal development are suppressed and this inhibition is re-exerted within the culture system. In contrast, postnatally such restraining influences are lifted, permitting the completion of neuronal development and allowing the neurones to perform at their optimal level. If our hypothesis is correct it suggests that even the different patterns of neuronal development may to some extent be reproduced in culture.

This is further evidenced by the GABA-ergic system studies. While the dopaminergic neurones within the culture model displayed a degree of morphological and functional immaturity at early time points in cultivation, from as early as DIV 3 intrahypothalamic GABA-ergic neurones were both morphologically developed and

functionally competent. This was especially apparent if one compares the values obtained for K_m in our uptake studies, where those for GABA were of the same order of magnitude as those found in adult brain, but those for DA suggested immaturity of the DA system. It is thus interesting to speculate that much of the developmental process for GABA-ergic systems occurs prior to birth and that under culture conditions they rapidy re-attain their advanced state of development viz. rapid regrowth of neurites and the expression of transporter proteins. In contrast, the dopaminergic neurones are reported to be only partially developed at birth (Olson & Seiger 1973), and this neuronal retardation (in comparison to the GABA - ergic systems) is carried over into the *in vitro* situation. Thus, although we are aware of the limitations of the tissue culture approach, it appears that, at least for intrahypothalamic dopaminergic and GABA-ergic systems, many of the characteristics of *in vivo* neuronal development are sustained and that culture models are likely to be more reflective of the *in vivo* situation than was once thought.

It would be interesting to cultivate foetal hypothalamic tissue at earlier gestational periods (viz. prior to E18), in an attempt to pin-point the time at which this pre-programming occurs. Repetition of the biochemical studies may reveal that the rate and degree of development of intrahypothalamic cultures is impaired the earlier the tissue is taken, indicating that the development of these neuronal populations is highly dependent on their exposure to certain signals which may only occur at a distinct time - point in the ontogeny of the hypothalamus. Parallel investigations involving the mesencephalic dopaminergic cell system would indicate whether the phenomenon was region specific or general to all central dopaminergic pathways. Following this, an attempt to isolate the factors involved in regulating the development of these dopaminergic systems would be beneficial. We believe that the identification of the substances which inhibit/facilitate the growth and survival of dopaminergic neurones, would be more readily achieved within a culture model system and will greatly improve our understanding of the development of the brain, the factors controlling its function and also the aetiology of age-related CNS and neuroendocrine abnormalities. Although far off, such an approach has potential for the development of new types of therapy for the treatment of endocrine, neurological and neurodegenerative diseases in a manner similar to that of the recently identified glial cell line - derived neurotrophic factor which has already been targeted for possible use in the treatment of Parkinsons' Disease (Lin et al 1993).

Further investigation into the ontogeny of the uptake and release mechanisms in play in the foetal hypothalamus is also important in order to shed light on the transition of neurotransmitter systems from their inactive to functional states. As a pronounced

change in K_m values for both the dopaminergic and GABA-ergic systems was demonstrable in this study, it is likely that physiological and therefore relevant changes in intrahypothalamic transmitter function occur around the perinatal period. The use of molecular biological techniques may allow further investigation into the ontogeny of the expression of the dopamine transporter on nerve terminals. Furthermore, a similar approach may be used to study the proposed D_2-like autoreceptor which may play a more important role in regulating dopaminergic activity in the foetal than in the adult CNS. The apparently transient nature of the Na^+ dependency of GABA uptake and also the loss of effectiveness of SKF 89976 A with increasing age may also be further explored employing molecular biological techniques to identify structural changes in the GABA transporter protein. As GABA is the major inhibitory neurotransmitter in the CNS and the major route for the termination of its transmission is via re-uptake, any perinatal changes in the nature of the uptake protein may have important implications in the development of the CNS.

One of the major limitations in this experimental model is the fact that we are dealing with a heterogeneous population of dopaminergic neurones which display very different profiles in terms of their functional capabilities and their responsiveness to peripheral steroids, neurohormones, drugs etc. In general, the activity of ICHDA systems is more readily modulated by pharmacological manipulation of neurotransmitter systems whereas, in the adult the TIDA neuronal system is more responsive to gonadal steroids and hormones such as prolactin (Lookingland & Moore 1984a, 1984b, 1985). Furthermore, adult TIDA neurones are highly resistant to the actions of 6 - OHDA, the neurotoxic effect of which is dependent on it gaining access to the nerve terminals via the uptake mechanism. By contrast, the ICHDA tract, like other dopaminergic neuronal systems, is susceptible to 6 - OHDA and it has been suggested that the differences in effectiveness of this toxic agent on these two hypothalamic tracts is reflective of the absence of a high affinity uptake mechanism on TIDA nerve terminals (Demarest & Moore 1979) although a possible difference in the ligand specificity of the TIDA transporter molecule ought also to be considered. Such contrariety makes absolute interpretation of our results difficult as we are unable to discriminate between actions exerted on TIDA neurones from those being exerted on ICHDA populations. Neither are we able to definitively identify those actions which are influencing the activity of both systems and to what extent each pathway contributes to the observed effect. In future studies, we would hope to refine the culture model used by further dissection of the hypothalamic tissue to isolate the arcuate nucleus - median eminence (Arc - ME) complex from the remainder of the structure. Thus, by culturing each portion separately we will be able to monitor the activity of the TIDA and ICHDA neuronal systems more effectively. It must be

remembered however, that some THDA neurones are known to innervate the median eminence and other, as yet uncharacterized, dopaminergic populations are present within the hypothalamus and thus, obtaining a culture model containing a totally homogeneous population of DA - containing cells would be difficult to achieve. Therefore, we would hope in the future to adopt an additional culture system in order to further investigate some of the findings reported here. A culture model has been described whereby slices of hypothalamic tissue derived from newborn animals may be maintained for up to 6 weeks (Bertini et al 1993). In such a system the structural integrity of the hypothalamic tissue is retained including the various intrahypothalamic neuronal systems and their functional connections with each other. Thus, by taking slices from the appropriate region of the hypothalamus, this model allows the investigation of discrete neuronal populations both in terms of their topography (ICC studies revealing their site of origin and termination) and also their relationship to other neuropeptide and neurotransmitter systems (release studies in the presence of different physiological factors and pharmacological agents).

The modulatory influence of E_2 and Prog on dopaminergic function allows us to speculate on the role played by the peripheral sex steroids in the developing CNS. The specific actions of E_2 on the functioning of both the perinatal and adult HP-axis have already been discussed, but it is believed that such hormones also have a non-specific influence on neuronal development. E_2 appears to promote neurite growth in mesencephalic THIP neurones *in vitro* and has also been shown to increase neuronal survival in a number of brain regions including the hypothalamus (Reisert et al 1987). *In vivo* such actions may play a critical role in establishing the anatomical and functional sexual dimorphism demonstrated in several brain regions. Thus, androgens, after their conversion to oestrogens by aromatases, are believed to regulate synaptogenesis and the phenotypic nature of cells which form synaptic connections. This is exemplified in the mPOA where neonatal castration of the male animal leads to a permanent alteration in the morphology and presumably the neuronal circuitry because gender - specific patterns of LH secretion become disrupted (Arnold & Gorski 1984). It is however, yet to be elucidated exactly how the structural changes elicit the functional changes exhibited in the adult. In future studies it would be valuable to investigate the effect of neonatal androgen exposure on the development/ differentiation and activity of the intrahypothalamic dopaminergic neurones. Morphometric studies employing ICC coupled to image analysis would allow determination of the effects of E_2/Prog and aromatizable androgens on THIP neuronal growth and survival *in vitro* and any changes in neuropeptide output which coincided with changes in dopaminergic activity could simultaneously be investigated. Again, the use of slice cultures may prove useful in such a study as it would be possible to

relate steroid induced changes in the neuroendocrine system with alterations in intrahypothalamic neurotransmitter activity. Such changes are widely believed to have implications in the activity of the HP-axis both at puberty and in the adult. In addition, the individual action of E_2 and Prog on either ICHDA or TIDA neurones could be studied more discretely, as could the synergism existing between the two hormones. Such a model would also allow us to investigate gender related differences in the development of the dopaminergic and GABA-ergic populations by using hypothalamic tissue from both sexes in isolation. As sexual dimorphism has been reported for the distribution of dopaminergic neurones in the hypothalamus (Simerly & Swanson 1987) and the activity of TIDA neurones in the adult male is lower than in the female, such differences may well be responsible for the gender - specific differences in the release of neuropeptides such as prolactin and LH in the adult intact animal.

The hypothalamus, like all other regions of the CNS, exhibits a considerable degree of diversity in its morphological, biochemical and physiological properties and this diversity literally demands a cellular approach, such as tissue culture technology, for the investigation and correlation of these properties. We have demonstrated that the morphological and functional development of intrahypothalamic dopaminergic and GABA-ergic systems, in addition to their anatomical and functional interactions and their responsiveness to neuropeptides and gonadal steroids, may be readily studied using a dissociated cell culture model. We propose that such an approach to the investigation of the development of the hypothalamus will continue to enhance our understanding of the neuroendocrine system and the myriad of factors involved in the regulation of its ontogeny and function.

APPENDIX I

Addresses of Suppliers.

i. Consumbles and small equipment:

SUPPLIER	PRODUCT
Amersham Life Science Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA	[^{3}H]gamma aminobutyric acid
AMS Biotechnology Unit 6, Tannery Yard, Whiney Street, Burford, OX8 3DN	tyrosine hydroxylase antibody
Anderman and Co Ltd 145 London Road, Kings-Upon-Thames, Surrey, KT2 6NH	Sterile Pipette Tips
Arterial Medical Services 313 Chase Road, Southgate, London, N14 6JH	1ml syringes
BDH/MERCK Ltd Freshwater Road, Dagenham, Essex	acetonitrile 7ml Bijou bottles citric acid calcium chloride diethylether D-Glucose EDTA Hamilton syringes (HPLC Type) hydrochloric acid di-sodium hydrogen phosphate potassium chloride magnesium sulphate paraformaldehyde perchloric acid picric acid methanol "Ralmount" (mountant) sodium chloride sodium dihydrogen orthophosphate sodium hydrogen carbonate sodium hydroxide

Bibby Sterilin Tilling Drive, Stone, Staffs ST15 OSA	Sterile 10ml pipettes
Boeringher Manheim U.K. Bell Lane East Sussex, BN7 1LG	dispase (Grade I)
Chance Propper Spon Lane, Smethwich, Warley	glass coverslips (15mm) microscope slides
Charing Cross Hospital (Pharmacy) Fulham Palace Road Hammersmith W6 8RF	sterile water sterile saline aprotinin
Citifluor Ltd Connaught Building (Room 303) Northampton Square, London, EC1V 0HB	Citifluor (mountant)
Dako Ltd 16 Manor Court, Hughenden Avenue, Highwycombe, Bucks, HP13 5RE	normal goat serum
DuPont Ltd Wedgewood Way, Stevenage, Hertfordshire, SG1 4QN	[^{3}H]dihydroxyphenylamine hydrochloride ([^{3}H]DA)
Gelman Sciences 10 Hanover Road, Brackmills, Northampton, NN4 OE2	Acrodisc 0.2μ filters
Gibco Life Technologies 3 Fountain Drive Inchinan Business Park, Inchinan, PA4 9RF	Dulbecco's Modified Eagles Medium foetal calf serum fungizone Ham's F-12 Nutrient Mixture horse serum Nunc 4x4 multiwell plates (15mm) Nunc 6 well culture plates (35mm)
Hoechst UK Ltd Pharmaceutical division, Hoechst House Salisbury Road, Hounslow, Middx, TW4 6JH	benztropine nomifensine

A.R. Horwell Ltd 73 Haygrove Road, West Hampstead, NW6 2BP	Sterilin petridishes
Hughes and Hughes Ltd Elms Industrial Estate, Church Road, Harold Wood, Romford Essex	scintillation vial inserts and caps
ICN Biomedicals Pereguine Business Park, High Wycombe, Bucks, HP13 7DL	HEPES
Lab Impex Research 111-113 Waldegrave Road, Teddington, Middlesex TW11 8LL	Speed-Cat Cartridges
Labsystems Unit 5, The Ringway Centre, Edison Road, Basingstoke	Finpipettes
Lancaster Synthetic Ltd Eastgate, White Lund, Morcambe, Lancs, LA3 3OH	sodium-n-octylsulphate
National Institue of Diabetes and Digestive and Kidney Diesease. Suite 505 210 West Fayette Street, Baltimore, Maryland, 21201-3472	prolactin
Nova Pharmaceutical Corporation 6200 Freeport Centre Baltimore, Maryland, 21224-2788	SKF-89976A (GABA uptake inhibitor)
Nuclear Enterprise Sight Hill, Edinburgh, Scotland, EH11 4BH	scintillation cocktail
Phillip Harris Scientific 618 Western Avenue, Park Royal, London W3 OTE	1ml disposable syringes

Penninsula Laboratories Ltd Box 62, 17K Westdale Industrial Estate, Jackson Street, St Helens, Merseyside, WA9 3AJ	gonadotrophin hormone-releasing hormone
Sigma Chemical Company Fancy Road, Poole, Dorset BHM 7NH	aminooxyacetic acid ascorbic acid bacitracin baclofen hydrochloride bicuculline bovine serum albumin DNA-ase dopamine diaminobenzedine (free base) 3, 4,- dihydroxybenzylamine 3, 4, -dihydroxyphenylacetic acid epinephrine Hanks balanced salt solution insulin muscimol pargyline poly-L-lysine trizma base trizma hydrochloride tri-iodthyronine
Stratech Scientific 61-63 Dudley Street, Luton, Bedfordshire	Texas Red conjugated goat anti-mouse IgG Fluoroscein conjugated goat anti mouse IgG Fluoroscein conjugated goat anti rabbit IgG
UniPath Ltd Wade Road, Basingstoke, Hants, RG 0PW	Phosphate buffered saline (tablets)
Vector Laboratoies 16 Wulfric Square, Bretton, Peterborough, PE3 8RF	Unconjugated horse anti-mouse IgG peroxidase anti peroxidase conjugated antibody
Walter Starstedt 68 Boston Road, Leicester, LE4 1AW	5ml tubes 3.5ml tubes

ii. Large Equipment.

SUPPLIER	PRODUCT
Biotech Instrument Ltd 183 Camford Way, Luton, Beds, LU3 3AN	HPLC-EC Equipment
Decon Ultrasonics Ltd Conway Street, Hove, Sussex	Water sonicator for cleaning glassware
Envair York Avenue Haskingden, Rosendale, Lanc, BB4 4HX	Class II biological safety cabinet
Fison's Instruments Sussex Manor Park, Gatwick Road, Crawley, Sussex	MSE "coolair" benchtop centrifuge MSE "soniprep" 150 sonicator
Heraeus Equipment Ltd 9 Wales Way, Brentwood, Essex, CM15 9TB	CO_2 incubator
Lab Impex Research 111-113 Waldegrave Road, Teddington, Middlesex TW11 8LL	"Speed-mate" vacuum pump

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