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CHICK BRAIN CHOLINERGIC RECEPTORS STUDIED BY ANTAGONIST LABELLING ~~:~~ DISTRIBUTION, ONTOGENY & FUNCTION.

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The Open University

Presented to the Open University in June 1988, in part fulfil ment of the requirements for the Degree of Doctor of Philosophy.

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Abbreviations and alphabetical index of structures.

ABBREVIATIONS

Acetylcholine	ACh
Acetylcholinesterase	AChE
Choline acetyltransferase	CAT
Cyclic adenosine monophosphate	cAMP
Cyclic guanosine monophosphate	cGMP
Dopamine	DOPA
Gamma amino butyric acid	GABA
Muscarinic receptor field	MRF
Muscarinic cholinergic receptor (as labelled by ^{3}[H] antagonists)	MAChR

Nicotinic receptor field	NRF
Nicotinic cholinergic receptor (as labelled by ^{3}[H] antagonists)	NAChR
N-Methyl scopolamine	NMS
Tritiated α Bungarotoxin	^{3}[H] α BTX
Tritiated *l* Quinuclidinyl benzilate	^{3}[H] *l* QNB
Tritiated Propylbenzilylcholine mustard	^{3}[H] PrBCM

STRUCTURE	ABBREVIATION
Ansa lenticularis	AL
Archistriatum	A
Archistriatum, pars dorsalis	Ad
Archistraitum, pars ventralis	Av
Area entorhinalis	AE
Area corticoidea dorsolateralis	CDL
Area hypothalami posterioris	AHP
Area parahippocampalis	APH
Area parahippocampalis pars linearis	PHL
Area pretectalis	AP
Area temporo-parieto-occipitalis (Edinger, Wallenberg & Holmes)	TPO
Area ventralis (Tsai)	AVT
Brachium colliculi superioris	BCS
Brachium conjunctivum	BC
Brachium conjunctivum descendens et tractus tectospinalis (tractus tectospinalis (TS) Papez)	BCTS
Bulbous olfactorius	BO

STRUCTURE	ABBR.
Capsula interna occipitalis	CIO
Cerebellum	Cb
Chiasma opticum	CO
Commissura pallii	CPa
Commissura anterior	CA
Commissura posterior	CP
Commissura tectalis	CT
Commussura cerebellaris ventralis	CCV
Corpus pineale	P
Cortex	C
Decussatio brachiorum conjunctivorum	DBC
Decussatio supraoptica	DS
Ectostriatum	E
Fasciculus diagnalis Brocae	FDB
Fasciculus longitudinalis medialis	FLM
Fasciculus prosencephali lateralis	FPL

STRUCTURE	ABBR.
Fasciculus prosencephali medialis	FPM
Formatio reticularis medialis mesencephali	FRM
Hippocampus	Hp
Hyperstriatum accessorium	HA
Hyperstriatum dorsale	HD
Hyperstriatum intercalatus superior	HIS
Hyperstriatum ventrale	HV
Hyperstriatum ventrale dorso-ventrale	HVdv
Hyperstriatum ventrale ventro-ventrale	HVvv
Infundibulum	Inf
Lamina frontalis superior	LFS
Lamina frontalis suprema	LFM
Lamina hyperstriatica	LH
Lamina medullaris dorsalis	LMD
Lemniscus lateralis	LL
Lemniscus spinalis	LS
Lobus parolfactorius	LPO
Locus ceruleus	LoC
Neostriatum	N
Neostriatum caudale	NC
Neostriatum frontale	NF
Neostriatum intermedium	NI
Nervi glossopharyngeus et vagus	NIX.X
Nervus hypoglossus	NXII
Nervus oculomotorius	NIII
Nervus trigeminus	NV
Nervus trochlearis	NIV
Nucleus accumbens	Ac
Nucleus ansea lenticularis	nAL
Nucleus anterior medialis hypothalami	AM
Nucleus archistrialis anterior	AA
Nucleus basalis	Bas
Nucleus cerebellaris internus	Cbl
Nucleus cerebellaris intermedius	CbM
Nucleus cerebellaris lateralis	CbL
Nucleus commissuralis septi	CoS
Nucleus geniculatus lateralis (pars dorsalis principalis)	GLdp
Nucleus geniculatus lateralis pars centralis	GLv
Nucleus dorsointermedius posterior thalami	DIP
Nucleus dorsolateralis anterior thalami	DLA
Nucleus dorsolateralis anterior thalami pars lateralis	DLL
Nucleus dorsolateralis anterior thalami pars medialis	DLM
Nucleus dorsolateralis posterior thalami	DLP
Nucleus dorsomedialis anterior thalami	DMA
Nucleus dorsomedialis posterior thalami	DMP
Nucleus reticularis pontis oralis	RPO
Nucleus reticularis superior pars dorsalis	RSd
Nucleus reticularis superior pars ventralis	Rsv
Nucleus rotundus	Rt
Nucleus ruber	Ru
Nucleus semilunaris	SLu
Nucleus intercollicularis	ICo
Nucleus intercalatus	IC
Nucleus intercalatus thalami	ICT
Nucleus interpeduncularis	IP
Nucleus interstitio-pretecto-subpretectalis	IPS
Nucleus intrapeduncularis	INP
Nucleus isthmi, pars magnocellularis	Imc

STRUCTURE	ABBR.
Nucleus isthmi, pars parvocellularis	Ipc
Nucleus isthmo-opticus	IO
Nucleus lateralis anterior thalami	La
Nucleus lateralis hypothalami	LHy
Nucleus lemnisci lateralis, pars dorsalis (Groebbels)	LLd
Nucleus lemnisci lateralis, pars ventralis (Groebbels)	LLv
Nucleus lentiformis mesencephali	LM
Nucleus lentiformis mesencephali, pars magnocellularis	LMmc
Nucleus lentiformis mesencephali, pars parvocellularis	LMpc
Nucleus mamillaris lateralis	ML
Nucleus ectomamillaris	EM
Nucleus et tractus descendens nervi trigemini	TTD
Nucleus habenularis	Hb
Nucleus habenularis lateralis	HL
Nucleus habenularis medialis	HM
Nucleus nervi oculomotorii, pars dorsalis	OMd
Nucleus nervi oculomotorii, pars ventralis	OMv
Nucleus nervi oculomotorii, pars dorsolateralis	ODL
Nucleus nervi trochlearis	nIV
Nucleus ovoidalis	Ov
Nucleus papillioformis	Pap
Nucleus paragiantocellularis lateralis	PGL
Nucleus parahippocampalis pars linearis	PHL
Nucleus pontis lateralis	PL
Nucleus pontis medialis	PM
Nucleus posteroventralis thalami, (Kuhlenbeck)	PV
Nucleus propticus anterior	POA
Nucleus propticus medialis (Van Tienhoven)	POM
Nucleus pretectalis	PT
Nucleus pretectalis diffusus	PD
Nucleus pretectalis medialis	PTM
Nucleus principalis precommissuralis	PPC
Nucleus raphes	R
Nucleus reticularis lateralis	RL
Nucleus reticularis pontis caudalis	RP
Nucleus mesencephalicus lateralis, pars ventralis	MLv
Nucleus motorius dorsalis nervi vagi	nX
Nucleus motorius nervi trigemini	MV
Nucleus nervi abducentis	nVI
Nucleus nervi facialis	nVII
Nucleus nervi glossopharyngei et nucleus motorius dorsalis nervi vagi	nIX.X
Nucleus nervi hypoglossi	nXII
Nucleus sensorius principalis nervi trigemini	PrV
Nucleus septalis lateralis pars dorsalis	SLd
Nucleus septalis lateralis pars ventralis	SLv
Nucleus septalis medialis	SM
Nucleus spiriformis lateralis	SpL
Nucleus subpretectalis	SP
Nucleus subrotundus	SRt
Nucleus superficialis parvocellularis (Nucleus tractus septomesencephalic)	SPC
Nucleus supraopticus (Ralph)	SO
	Tn

STRUCTURE	ABBR.
Nucleus taeniae	Tn
Nucleus tegmenti (Gudden)	Te
Nucleus tegmenti pedunculopontinus, pars compacta	TPc
Nucleus triangularis	T
Nucleus ventrolateralis thalami	VLT
Nucleus vestibularis medialis	VeM
Nucleus vestibularis superior	VS
Paleostriatum augmentatum	PA
Paleostriatum primitivum	PP
Periectostriatum	Ep
Polus caudalis telencephali	PCT
Radix mesencephalicus nervi trigemini	RxVM
Stratum album centrale	SAC
Stratum cellulare externum	SCE
Stratum cellulare internum	SCI
Stratum griseum centrale	SGC
Stratum griseum et fibrosum superficiale	SGF
Stratum opticum	SOp
Stria medullaris	SMe
Substantia gelatinosa Rolandi (trigemini)	SG
Substantia grisea centralis	GCt
Substantia grisea et fibrosa periventricularis	SGP
Tectum opticum	TeO
Torus semicircularis	ToS
Tractus cortico-habenularis et cortico-septalis	CHCS
Tractus archistriatalis dorsalis	DA
Tractus cortico-habenularis	CH
Tractus fronto-archistriatalis	FA

STRUCTURE	ABBR.
Tractus fronto-thalamicus et tratus thalamo-frontalis	FT
Tractus habenculo-interpeduncularis	HIP
Tractus infundibularis	IN
Tractus isthmo-opticus	TIO
Tractus nuclei ectomamillaris (basal optic root)	TrEM
Tractus nuclei ovoidalis	TOv
Tractus occipitomesencephalicus	OM
Tractus opticus	TrO
Tractus pretectosubpretectalis	PST
Tractus quintofrontalis	QF
Tractus septomesencephalicus	TSM
Tractus spinocerebellaris dorsalis	Cbd
Tractus spinocerebellaris ventralis	Cbv
Tractus tectothalamicus	TT
Tractus thalamostriaticus	TTS
Tuberculum olfactorium	TO
Ventriculus	V
Ventriculus olfactorius	VO

Abstract.

By means of an *in vitro* labelling technique and methods of light microscope autoradiography the distribution of muscarinic cholinergic receptor, labelled by the tritiated antagonists *l*-Quinuclidinyl benzilate or Propylbenzilylcholine mustard, and the distribution of the nicotinic cholinergic receptor, labelled by α-Bungarotoxin, was shown in thin tissue sections of the young post hatch chick brain. The distribution of muscarinic cholinergic receptor in the chick brain was found to be widespread. The highest density concentrations of muscarinic receptors, for example in the paleostriatum augmentatum and hyperstriatum ventrale of the forebrain, thalamic and mesencephalic relay nuclei, optic tectum and brain stem nuclei, were found to be highly regionally localised. In contrast, nicotinic receptors were found to be concentrated to mesencephalic and diencephalic regions of the brain, in particular to colliculi and the principal optic relay nuclei of the diencephalon. The density of nicotinic receptor in the forebrain, apart from the olfactory lobe, was found to be very low.

The concentration of muscarinic receptor throughout the brain was found to be at least one order of magnitude greater than the concentration of nicotinic cholinergic receptor. Without exception, all regions populated by substantial concentrations of nicotinic receptor were also populated by substantial concentrations of muscarinic receptor. On the other hand, a number of brain regions populated by high densities of muscarinic receptor were found to be almost devoid of specific α-bungarotoxin labelled nicotinic cholinergic receptor, eg. the hyperstriatum ventrale.

^{3}[H] antagonist labelled muscarinic and nicotinic cholinergic receptors in chick brain tissue slices were shown to be similar to values given by other reports for alternative tissue preparations. The great majority of ^{3}[H] muscarinic antagonist binding sites were shown to be specific, ie. atropine 'sensitive' or displaced. Evidence has been given which suggests that muscarinic antagonists are labelling a heterogeneous population of receptor. Questions concerning the identity of muscarinic antagonist labelled receptor are discussed.

The distribution of antagonist labelled muscarinic receptor in the chick brain was compared with *in vitro* labelled autoradiographed brain sections of the 50 day post natal rat brain, with the objective of discovering whether homologous neurons of the rat and chick brain showed similar patterns of distribution and concentrations of muscarinic receptor. For the great majority of established and assumed homologous neuronal populations between these species of vertebrate brain, a correspondence of distribution and density of receptor was shown. Where differences were apparent, eg. cell layers of the olfactory bulb, these differences have been suggested to reflect the increase, or alternatively decrease, of particular cholinoceptive cell types, possibly micro circuit interneurons, subserving the greater or lesser emphasis of particular sensory modalities between these species of vertebrate.

The distribution of antagonist labelled muscarinic receptor, again using *in vitro* labelling autoradiographic localisation proceedures, was measured in the in ovo and early post hatch chick brain. High densities of muscarinic receptor were shown in those regions of 10 days in ovo brains which, post hatch, are populated by (regionally comparative) high densities of receptor. In addition almost all regions of the post hatch chick brain, shown to be populated by low densities of muscarinic receptor, were shown during the latter stages of in ovo development to be populated by transient moderate to high densities of muscarinic receptor, eg. ectostriatum, hyperstriatum and intercalatus superior. Between 12 and 19 days in ovo, all regions of the developing chick brain, but in particular regions of the mid- and forebrain, were characterised by patterns of muscarinic receptor distribution which were found to occur only during in ovo brain development. Antagonist labelled muscarinic binding sites, apparently localised to parallel arrays of cells, disappeared around 19 days in ovo for reasons which may reflect a change in muscarinic ligand binding properties or a change in access of the radiolabelled ligand to receptor binding sites, restricted around this age by oligodendrogliogenesis and subsequent neuronal process myelination.

1. Introduction

1.1. A perspective.

The brain is essentialy composed of two cell types,neurons and glia. Neurons,discrete functional units,transmit their information at specialised points of contact,the synapse,by means of chemical or electrical signals.The synapse is probably the main site of decision and modulation behind the transmission of signal information in the brain (Sherrington 1906).

The number of different chemical substances released by neuronal and non neuronal cells which have been shown to excite,inhibit or modulate the electrical conductance or resistance properties of brain cell membranes is growing rapidly (see Hotzfeld et al., 1980). Many of the more recently discovered neurologically active endogenous molecules have been grouped under a general heading of neuromodulators.The distinction between neuromodulators (eg. amino acid and peptide hormones) and neurotransmitters (eg. acetylcholine,adrenaline etc.) is becoming harder to define (see Dismukes,1979).

Neurotransmitters produce a physiological response in brain cells through changes brought about by binding to receptor It is thought that for each neurotransmitter there is a unique receptor recognition site and that in the binding of transmitter some second portion of the receptor complex 'translates' transmitter recognition into changes in cell ion permeability (Robinson et al., 1971; Greengard, 1978).

The pharmacological identity of receptors, for example cholinergic as opposed to adrenergic, or muscarinic as opposed to nicotinic, is based upon ligand recognition specificity (although the physiological response of cells to the action of each neurotransmitter is also distinct in character (see Krnjevic, 1974)). The assumption is that there exists a definable relationship between the structure of the ligand and the site to which it binds, such that, by a correlation of the changes in biological activity with molecular changes in the ligand, some structural definition of the binding site on the macromolecular surface may be achieved. With almost no exception, it has been found necessary to propose more than one recognition site for each endogenous neurotransmitter. This study in reporting the distribution of cholinergic receptor (both muscarinic and nicotinic) in the early post hatch and in ovo chick brain employs receptor labels whose selectivity is based upon pharmacological criteria of receptor identity.

It is a commonly held view that receptors and neurotransmitters are 'genetically prespecified'. For example, the early appearance of cholinergic molecules during vertebrate muscle and brain development (apparently largely neuronal interaction independent) is suggested by Fambrough and Rash (1971), amongst others, to be a 'gene expression'. The commitment of cells to differentiate activates the 'set of genes' encoding all special protein characteristics of the differentiated state. During the course of this report, two analytical approaches concerning cholinergic receptor distribution in the brain contribute to the above view and to the ongoing nature-nurture debate, namely a comparison of receptor distribution between species of vertebrate brain and the regional and whole brain development of tritiated antagonist labelled muscarinic receptors during chick brain ontogenesis. Certain schools of thought have based their experimental approaches on the belief that the presence of a given receptor presupposes that the adequate transmitter (hormone) is present as well (Snyder and Matthysse, 1975, Goldstein, 1976). However, in all probability the hormone-transmitter and receptor have not arisen simultaneously during the course of evolution (see Sakharov, 1974; Csaba, 1980) and that those influences serving to specify the character of particular receptors during evolution may also be observed during development.

An opinion with which many are in accord is that the schematic opposition between 'experience' or 'gene' prescription of form, does not hold up under close examination of the biological processes occuring during neurogenesis (Changeux and Danchin, 1976). For example, a neural organisation of performance at birth, prior to 'actual' experience with the outside world, is often referred to as genetically determined (see above).

. Moreover, the cerebral cortex of the mammal is electrically active days or even weeks before birth and often before anatomically differentiated synapses can be recognised (Corner and Kwee, 1976; Adrian, 1976)

. Accordingly, Changeux and Danchin (1976) suggest that the acivity evoked by the interaction of the newborn/newly hatched with its environment may be viewed more as a modulation of this spontaneous activity than as an entirely de novo process. Receptors are ideal points of experimental focus with regard to the nature of influences upon brain development and functional capabilities. For example, it has been proposed that receptors may be 'specified' in ovo/utero by factors related to the 'activity' of the developing nervous system (see Csaba, 1980), how these 'influences' are possibly related to the 'selective stabilisation' hypothesis of Changeux and Danchin (1976) will be returned to during the course of this report.

What remains of this introduction will be directed to discussing the nature and identity of ligand labelled receptors, the brain distribution and functional relationship of other cholinergic molecules, their time of appearance and rate of increase during the course of brain development, and the anatomical and functional organisation of the avian brain, with particular consideration given to the equivalence of chick forebrain neuronal populations with those of the forebrain of other vertebrate species.

1.2 Cholinergic receptor identity.

The identity of receptors remains a problem of semantics because the term 'receptor' ('receptive substance') was introduced by Langley (1907) to account for a phenomenon rather than to name a specific physiological entity.

The muscarinic-nicotinic distinction for cholinergic receptors was classically demonstrated by Dale (1914) who in examining a series of choline derivatives noted that two types of activity could be observed - a muscarinic action which tended to be slow in onset and prolonged, essentially mimicking the effects of parasympathetic stimulation and the alkaloid muscarine, and a nicotinic action possessed by nicotine and many quaternary ammonium ions which tended to be fast in onset and short lasting and exerted at skeletal muscle and autonomic ganglia. Examination of the activities of a series of choline derivatives revealed them to be a spectrum of these two actions with the extremes representing purely muscarinic or nicotinic action. Furthermore, atropine could abolish the muscarinic actions of a given derivative leaving the nicotinic action largely unaffected.

Dale declined to explain these differences in terms of two different receptors for acetylcholine (ACh), but his study does indicate the criteria most commonly employed in receptor classification, namely the selective action of agonists and in particular antagonists. Agonists can induce maximal response, ie. they possess a high "efficacy" which is unrelated to affinity (Stephenson, 1956). Antagonists prevent the stimulatory action of agonists through occupancy of the receptor, with minimal efficacy.

The primary division of cholinergic receptors into muscarinic and nicotinic can be broken down further, since the latter category consists of receptors sensitive to the antagonist d-tubocurarine as in skeletal muscle and receptors quite insensitive to d-tubocurarine, but sensitive to hexamethonium as in the autonomic ganglia (see Triggle and Triggle, 1976; and ref. therein). Furthermore, Kehoe (1972) has shown that the visceral ganglion cell of Aplysia has three distinct cholinergic receptors on a single cell. These receptors mediate a fast depolarisation and a fast and slow hyperpolarisation. The two fast responses appear to be mediated through nicotinic receptor, since they are stimulated by nicotine and nicotine-like agents, blocked by d-tubocurarine and unaffected by muscarine. The receptor mediating the excitatory depolarising response is blocked by hexamethonium, Only this receptor resembles that of autonomic ganglia, whereas the receptor mediating the fast inhibitory response more closely resembles that of skeletal muscle. The third cholinergic receptor mediating the slow hyperpolarisation response (in CNS, in particular cortical cells, typically muscarinic) is apparently quite insensitive to a variety of classic muscarinic and nicotinic agents, tentatively identified by Kehoe (1972) as neither typically muscarinic nor nicotinic.

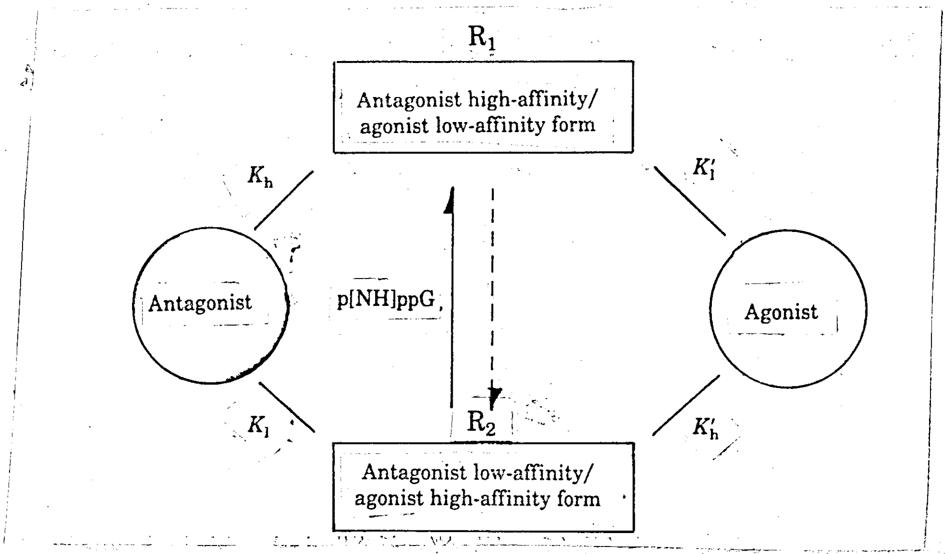
In the last decade evidence has highlighted different muscarinic receptor binding characteristics of agonists compared to antagonists (see Hulme et al., 1978). The binding of transmitter or analogues to receptors is usually taken to be described by the Langmuir absorption isotherm, formally equivalent to the Michaelis-Menten equation (eg. see Karlin, 1974). The binding of agonist to CNS has been suggested to best fit a model of two major agonist affinity sites, termed 'high' and 'low', with a third minor 'super high' affinity site (Birdsall and Hulme, 1976). Antagonists, on the other hand, have been suggested to bind to all three agonist sites with one affinity

(Birdsall et al., 1978). Other reports, however, suggest that antagonist binding is not to a single class of muscarinic receptor in the periphery of CNS (Paton and Rang, 1965; Szerb, 1977; Gupta et al., 1976; Hammer et al., 1981). The different agonist affinity forms of muscarinic receptor have been suggested to reflect induced changes in the coupling of receptor with a guanine nucleotide regulatory protein (see Rodbell, 1980; Hulme et al., 1981) accompanying changes in receptor 'efficacy'. Hanley and Iversen (1978) suggest that c GMP response accompanied by an increase in c AMP in rat brain tissue slices, appears to be linearly related to muscarinic receptor occupancy. Curiously, muscarinic antagonists were shown by Hanley and Iversen to be one order of magnitude more potent in blocking c GMP response than in displacing the potent tritiated muscarinic antagonist $[^3H]$ Quinuclidinyl benzilate ($[^3H]$ QNB). The importance of agonist affinity heterogeneity and of c GMP change in determining overall physiological response of tissue to muscarinic agonist remains unclear. However, the apparent strong generic similarity between heart muscarinic receptor (where antagonist binding is potentiated by guanine nucleotides), opiate and α adrenergic receptor in the brain, in that all inhibit the activity of adenylate cyclase and that all three receptor systems are inhibited by guanine nucleotides with similar nucleotide specificities (see Hulme et al., 1981; and ref. therein), supports the suggestion of Greengard (1978) that c GMP and c AMP are common membrane metabolic 'effectors' for a wide range of neurotransmitter types.

The differences between heterogenous binding sites for agonists and homogenous sites for antagonists (see above) have been suggested to be compatible with the existence of 'spare receptor' (Birdsall and Hulme, 1976) invoked to explain the discrepancy between drug potencies in binding assays and biological test systems (see Stephenson, 1956; Kebabian et. al,1975; but in addition Albuquerque

Figure 1 Schematic diagram of a reciprocal binding model showing two distinct forms of R_1 and R_2 of the receptor distinguished by antagonists and agonists. The form R_1 has a higher affinity (Kh) for antagonists than does the form R_2; reciprocally, R_2 has a higher affinity (K_h') for agonists than does R_1. The two forms are interconvertable in the presence of a guanine nucleotide (eg. p(NH) ppG. K_l, low affinity (From Burgiser et al., 1982)

et al., 1973; Snyder, 1975; Hanley and Iversen, 1978). For example, it has been calculated that when present in concentrations at which they produce half maximal ileal muscle contrations, strong agonists occupy only between 0.5 and 5.3% the number of $[^{3}H]$ QNB binding sites. (Aronstam et al., 1979). Snyder (1975) has suggested that the above phenomenon and the observations of agonist-antagonist binding heterogeneity are compatible with a model whereby antagonist have high and low affinities respectively for the antagonist and agonist form of receptor with the reverse true for agonist (see figure 1).


R_1
Antagonist high-affinity/ agonist low-affinity form
K_h
K_l'
Antagonist
p[NH]ppG
Agonist
K_l
R_2
K_h'
Antagonist low-affinity/ agonist high-affinity form

The importance of a lack of correspondence between the efficacy of potent agonists and the percentage of receptor sites labelled by antagonists and also the possibility of antagonist binding heterogeneity cannot be underestimated with respect to the observations of this study in reporting the distribution and development of cholinergic receptor in the chick brain. For example, the antimuscarinic ligands phencyclidine (PCP) and phencyclidine methiodide (PCP-MeI) exhibit moderate antagonism of ACh induced contractions of smooth muscle (Maayani et al., 1974) and inhibit binding of the potent muscarinic antagonnist $[^{3}H]$ N-methyl-4-piperidyl benzilate (Kloog et al., 1977) and $[^{3}H]$ DL QNB to muscarinic receptor in mammalian brain (Vincent et al., 1978; Jim et al., 1979). However, according to evidence given by Aronstam et al. (1981), PCP and PCP-MeI exhibit "properties associated with muscarinic antagonists but not agonists", or in other words 'muscarinic receptors' are not the only sites of interaction of these ligands (see Albuquerque et al., 1980).

1.3 Cholinergic molecules - functional relationships and distribution.

In spite of the wealth of published evidence pertaining to the distribution of various cholinergic functional molecules and markers in the vertebrate and in particular mammalian brain (see Butcher, 1977; Lewis and Shute, 1978; Kuhar and Atweh, 1978; McGeer and McGeer, 1979), our understanding of the organisation of cholinergic systems in the brain remains unclear. According to some (see McGeer et al., 1974; Rossier, 1975) the reason is that mapping of cholinergic structures has been handicapped by the lack of specific and routinely workable histochemical methods by which to localise functional cholinergic molecules. Histochemical methods, in preserving the intercellular geometry of the brain in relation to the neurochemicals under study are a necessary methodological constraint, if we are to understand the 'chemical coding' of brain neuronal circuits.

The uncertainty of brain cholinergic 'pathways' stems from repeated discrepancies in distribution and regional concentration between different cholinergic markers and molecules. These discrepancies do not correspond with established criteria for proof of chemical neurotransmission, namely: 1) the presence of a presynaptic biosynthetic pathway for the transmitter, eg. *acetylcholine* (ACh) is formed by *choline acetyltransferase* (CAT) catalyzing the alcolysis of acetyl CoA; 2) the presence of presynaptic transmitter, eg.

ACh; 3) the presence of postsynaptic receptor, eg. muscarinic or nicotinic or both and 4) the presence of a specific mechanism functioning to terminate transmitter action, eg. ACh is hydrolysed by *acetylcholinesterase* (AChE). An underlying assumption is that evidence for the regional preponderence of cholinergic-cholinoceptive cells requires that the ' functionally operative' concentrations of all cholinergic molecules should show a close correlation.

Choline uptake, CAT and AChE activity for the whole brain apparently show a good correlation (see Hebb and Silver, 1956; McGeer and McGeer, 1979). However, studies employing microdissection biochemical assay techniques or alternatively histochemical methods of localisation consistently report a lack of correlation between concentration of cholinergic molecules (markers or ligands). Curiously, these discrepancies are frequently reported for the same regions of the brain, eg. cerebellar and cerebral cortex, globus pallidus and hippocampus. For example, data shown by table I,recalculated from Yamamura et al. (1974) and taken from Lewis and Shute (1978), show that the number of antagonist labelled muscarinic cholinergic receptors are considerably in excess of choline uptake CAT and AChE activity in the frontal and pyriform cortex, hippocampus and cervical chord, as a percentage of mean values for the caudate putamen.

Many cells in the cortex are responsive to the iontophoretic application of ACh (Legge et al., 1966) and the response is typically muscarinic (Krnjevic and Phillis, 1963). Cortical cell layers I, II, III and V have been shown to be populated by high densities of antagonist labelled muscarinic receptor (Rotter et al., 1979; Walmsley et al., 1980). Despite the observations of Krnjevic (1965) and Krnjevic and Silver (1965) for a good correlation between AChE staining cells and ACh responsive cells, many other reports have shown that AChE stain in cortical layers is very light (see figure 2, taken from Lewis and Shute, 1978; also Parent and Oliver, 1969; Cotman and Nadler, 1978; Paxinos et al., 1980; Johnston et al., 1981; Arimatsu et al., 1981). The density of antagonist labelled muscarinic receptor in the cortex is identical to that observed in the basal ganglia (Yamamura et al., 1974; Hiley and Burgen, 1974; Rotter et al., 1979a).

The only cortical layer to stain moderately for AChE is layer IV the only cortical layer populated by low densities of antagonist labelled muscarinic receptor (Walmsley et al., 1980) and populated by a substantially higher *percentage* of high muscarinic agonist affinity binding sites compared to other cortical layers. Kloog and Sokolovsky (1979) have shown that cortical muscarinic receptors have an affinity for antagonists which is twice that of the medulla pons and cerebellum with the reverse true for agonists. Dawson and Jarrot (1980) reported that the I_{50} of acetylcholine is $^{1}/_{60}$th of that of the hippocampus and cortex at approximately the same concentration of ^{3}[H] QNB. Walmsley et al. (1980) have suggested that the different ratios of high and low agonist affinity sites for muscarinic receptors may be interpreted as indicative of subclasses of muscarinic receptor distributed to different regions of the brain. This would not present a problem to the present study, if antagonists labelled all agonist forms of receptor with one affinity (see Birdsall et al., 1976; and section 1.2). However, Szerb (1977) has suggested that while the affinity of pre and post synaptic muscarinic receptor for agonists is identical, the affinity of presynaptic muscarinic receptor for antagonists is 10-20 times less than for postsynaptic receptor, and he further suggests that presynaptic muscarinic receptor in the forebrain, in particular in the absence of nicotinic receptor, have a physiological role in the autoregulation of ACh release, ie. autoreceptors

Figure 2. *Frontal section of the rat forebrain (adult) stained for acetylcholinesterase (after the method of Shute and Lewis ,]963). The caudate putamen (C and Pu), globus pallidus (GP), and anterior thalamic nucleus (AT) and olfactory nucleus (ON) are shown to stain intensely for acetylcholinesterase . The cortex (C) and hippocampas (HC) are shown to be almost completely devoid of acetylcholinesterase activity. (Taken from Lewis et.al.,]967).*

Figure 3, *(A to C.) A parasagittal (A) and frontal (B) section through the brain of Uroloncha domestica, stained for acetylcholinesterase after the method of Koelle ( ]954) and taken from Kusunoki , ( ]97]); C is a frontal section through the forebrain of Colubia stained for acetylcholinesterase. (Taken from Karten and Dubbeldam,]973).*

ha = hyperstriatum; hd = hyperstriatum dorsale; hv = hyperstriatum ventrale; es = ectostriatum; psa = paleostriatum augmentatum; psp = paleostriatum primitivum; rt = nucleus rotundus; dg = diagonal band; in = interpeduncular nucleus; and nX = nervi vagi .

Figure 2.

Acetylcholinesterase in the rat forebrain

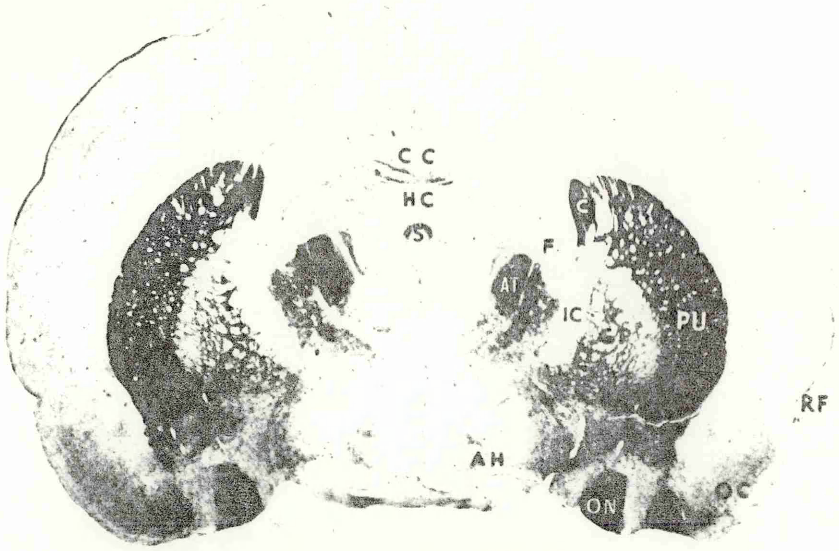

CC
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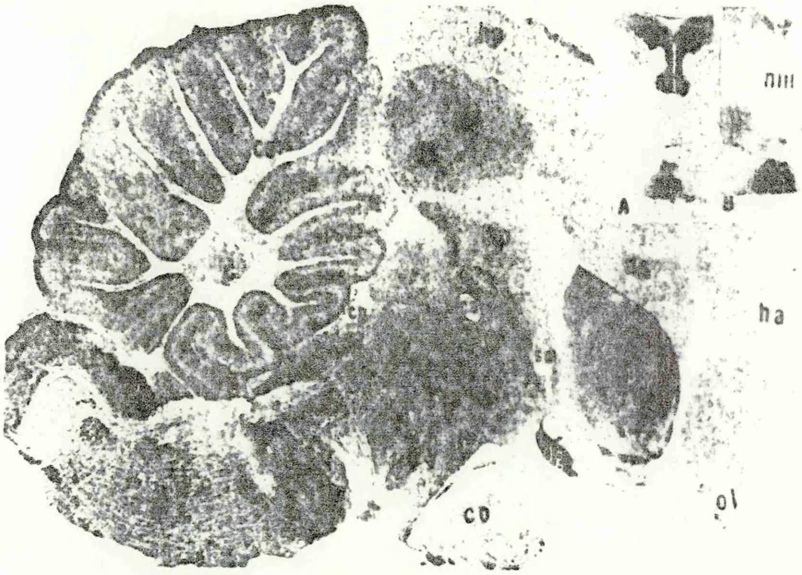
Figure 3

Acetylcholinesterase in the duck brain

A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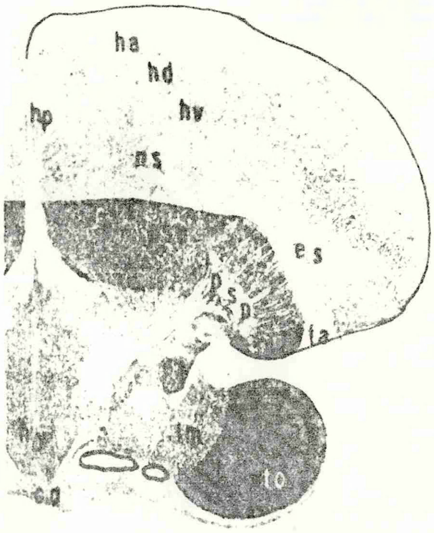
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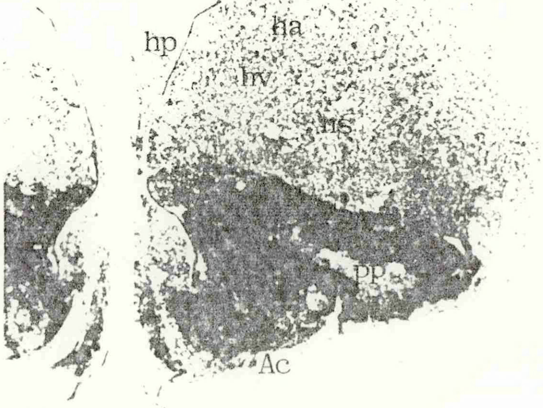
C Acetylcholinesterase in the pigeon forebrain.


hp
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Ac

(see also Langer, 1978). Presynaptic muscarinic receptors have been reported on cholinergic terminals in the rat hippocampus, striatum, and cortex (Giorguiff et al., 1977, Hadhazy and Szerb, 1977). Hiley and Burgen (1974) reported substantial concentrations of muscarinic receptor to callosal fibres of mammals and suggested that muscarinic receptors are distributed along the fibres and are not necessarily confined to cell soma,dendritic regions or synaptic terminals.

A lack of correlation between AChE distribution and antagonist labelled receptor is more curious for the observation that in muscle the number of catalytic AChE centres per endplate is almost 1:1 with the number of α bungarotoxin labelled nicotinic receptor (Barnard et al., 1971). And although the α BTX labelled cholinergic receptor and AChE molecules have been shown to have a different amino acid profile (Eldefrawi and Eldefrawi, 1971) and to have been extracted separately (Miledi, 1971), these two molecules are very closely associated at the synaptic membrane (Changeux et.al., 1976).

The number of muscarinic receptors in the rat brain has been shown be at least an order of magnitude greater than the number of nicotinic antagonist labelled receptors (Poltz-Tejera et al., 1975, Francis et al., 1981). Nicotinic receptors have been shown to be principally localised to midbrain colliculi of mammals (Poltz-Tejera et al., 1975; Arimatsu et al., 1981). While pharmacologists continue to reinforce the distinction between muscarinic and nicotinic receptors,physiologists are less than convinced. For example, the characteristic nicotinic response, a fast excitatory depolarisation, has as a rule not been easily reproduced in the central nervous system (see Krnjevic, 1974). Bird and Aghajanian (1975) have shown that ACh excited pyramidal cells of the mammalian hippocampus are completely and apparently specifically inhibited to ACh excitation by either muscarinic (eg. QNB) or

Table 1. *Regional concentration of ^{3}[H] Ouinuclidinyl benzilate labelled muscarinic binding sites* ^{a}*, high-affinity choline uptake and choline acetyltransferase and acetylcholinesterase activity in the monkey brain. (Taken from Lewis and Shute,]978; a, data recalculated from Yamamura et.al.,( ]974) and expressed as percentages of the mean values for the caudate-putamen.*

Table 2. *Presumed (and suggested) cholinergic pathways in the vertebrate, but in particular, mammalian brain. (Taken from McGeer and McGeer,]979).*

Table 3. Withdrawn.

Concentration of cholinergic markers in the monkey brain.

Region	Muscarinic sites	Choline uptake	ChAT	AChE
Frontal cortex	42	4	5	6
Pyriform cortex	45	15	20	11
Caudate-putamen	100	100	100	100
Globus pallidus	16	6	10	27
Hippocampus	48	16	14	17
Superior colliculus	36	26	37	45
Inferior colliculus	26	12	11	17
Cerebellar cortex	12	4	1	20
Inferior olive	45	10	3	25
Medulla oblongata	11	11	11	14
Cervical cord	45	8	9	8

Table 2. Presumed (and suggested) cholinergic pathways

Anterior horn cells to all voluntary muscles and to Renshaw cells
Lateral horn cells to all autonomic ganglia
Nuclei of cranial nerves III–VII, IX–XII
Postganglionic parasympathetic fibers
Occasional postganglionic sympathetic fibers
Septo-hippocampal tract
Septo-cingulate cortex
Habenulo-interpeduncular tract
(Diagonal band of Broca to interpeduncular nucleus)
Striatal interneurons
Interneurons in nucleus accumbens
(Interneurons in olfactory tubercle)
Some mossy fibers of the cerebellum
(Some retinal amacrine cells)
(Tuberoinfundibular fibers)
(Pallido-cortical fibers)
(Thalamo–head of caudate)
(Cortical interneurons)

Table 3

nicotinic (eg. dihydro-β-erithroidine) antagonists, and they suggest that, unlike autonomic ganglia and Renshaw cells, pyramidal cells do not possess two separate and independent cholinergic receptor (see Kehoe, 1972; section 1.2).

In reporting the distribution of nicotinic cholinergic receptor in the chick brain, this study has used as a tritiated label the snake venom polypeptide α bungarotoxin (α BTX) which, though structurally completely unrelated to the transmitter acetylcholine (Changeux et al., 1970), has been reported to bind specifically and essentially irreversibly to peripheral nicotinic cholinergic receptor (Chang and Lee, 1963; Changeux et al., 1970; Miledi et al., 1971; Fambrough and Hartzell, 1972). Table 2 taken form McGeer and McGeer (1979) summarises the established , presumed and suggested cholinergic pathways in the vertebrate, but in particular, mammalian brain. Of particular interest is the number of cholinergic systems which are thought to be regionally intrinsic.

1.4 Cholinergic systems in the avian brain.

Aprison et al. (1964) and Aprison and Takahashi (1965) have shown that the concentrations of ACh,CAT and AChE in the pigeon brain are considerably higher than those of serotonin, dopamine and noradrenalin , and they suggest after Whittaker (1953) that cholinergic systems in the avian brain are predominant and of singular importance. The concentrations of ACh,CAT and AChE are high in the pigeon diencephalon, mesencephalon and medulla pons in comparison to concentrations in the telencephalon (Aprison et al., 1964). This observation was confirmed by Wächtler (1979) for the pigeon brain. Wächtler

(1979) in comparing AChE activity across a range of vertebrate species from amphibians to mammals noted that cholinergic activity in pallial regions of the forebrain was low in all species studied.

Perhaps as a consequence of the early finding of high cholinergic activity in the mesencephalon, the majority of studies investigating cholinergic pathways in birds have concentrated on visual systems. The optic tectum of birds, for example, has been shown to be rich in all cholinergic molecules, including antagonist labelled muscarinic and nicotinic cholinergic receptor (Poltz-Tejera et al., 1975; Henke and Fonnum, 1976; Woolston et al., 1980). Monocular deprivation and/or retinal afferent ablation studies have proved inconclusive in determining whether retinal ganglion cell input is cholinergic or not (see Brecha et al, 1979; Oswald and Freeman, 1980). It would seem unlikely, since the concentration of CAT in the optic nerve is very low (Hebb, 1963). The distribution of α toxin labelled nicotinic cholinergic receptor in the chick tectum (Poltz-Tejera et al., 1975) does not correspond with regional distribution of AChE or CAT in the pigeon tectum (Palkovits and Jakobowitz 1974; Henke and Fonnum, 1976). According to Poltz-Tejera et al. (1975), the highest densities of α BTX nicotinic receptor are localised to layer 7 of the tectum which apparently contains no neurons, but radial dendrites, suggested by Hunt and Kunzle (1979) to be gabaergic. Tectal circuitry may exhibit certain similarities with that of the mammalian hippocampus, where hippocampal input is muscarinic on pyramidal cells, and nicotinic on golgi (gabaergic) inhibitory interneurons, afferent to pyramidal cells (Kuhar and Yamamura, 1976; Lewis and Shute, 1978).

A recent study by Ryan and Arnold (1981) suggests that the efferent projections of most vocal controle areas of the zebra finch brain may be cholinergic.

That study reported high densities of muscarinic receptor and AChE stain to the lobus parolfactorius magnocellular nucleus of the anterior neostriatum, caudal nucleus of the hyperstriatum ventrale (HVc), intercollicular nucleus, archistriatum and hypoglossal nerve nucleus (XII) (see section 1.5). It should be stressed, however, that Ryan and Arnold (1981) presented evidence for muscarinic receptor distribution and AChE stain to selected (auditory) regions of the brain, and the presence of cholinergic molecules in itself is insufficient evidence to suggest, for example, that auditory systems per se, are cholinergic-cholinoceptive.

By far and away the most detailed and comprehensive study of cholinergic distribution in the avian brain is that by Kusunoki (1969) in reporting, histochemically, the distribution of AChE in the brains of *Uroloncha domestica* and *Anas platyryncha v domestica*. Kusunoki (1970) showed, as do other reports (eg. Karten and Dubbeldam, 1973), that the basal ganglia of birds stain intensely for AChE, in addition to many midbrain relay nuclei, the optic tectum and cranial nerve nuclei of the hindbrain (see figure 3). Apart from the recent study of Rainbow et al. (1982), reporting autoradiographically the distribution of [^{3}H] QNB labelled muscarinic receptor across one level of the zebra finch brain, the only directly comparable studies to the present report for muscarinic receptor distribution in the chick are those reporting, histochemically, the distribution of acetylcholinesterase.

1.5 Ontogeny of brain cholinergic systems.

A major problem in neurobiology is the way in which genetic and environmental factors interact to form the intricate pattern of neurons that comprise the mature nervous system. Young (1957) proposed that the mature synapse represents the endproduct of a continuous process of growth and degeneration of the terminal part of nerve fibres and further suggested that the "modification of such daily growth by functional activity would provide the basis of plasticity of the nervous system".

A frequent comment accompanying observations on development of cholinergic systems is that cholinergic molecules may serve a function distinct from their role in chemical neurotransmission. This 'extra' to transmission function is rarely speculated on (but see Changeux and Danchin, 1976; Freeman, 1977; Giacobini, 1979). For example, evidence showing temporal, spatial or rate changes for cholinergic molecules out of 'synchronisation' with the onset of functional chemical transmission,or morphological evidence for the appearance of anatomical synapses has on occasions been somewhat dismissively labelled as 'extrasynaptic', 'non functional' or yet another expression of histogenetic memory (eg. see Silver,1974;Rotter et al.,1979). And yet it is quite probable that the information held by these apparent ambiguities in cholinergic molecule development and brain distribution may provide vital clues as to the maturational processes which underly the correct ordering and patterning of neuronal systems.

As with other aspects of this study, evidence for the ontogeny of cholinergic systems in the brain is taken from reports for a number of different vertebrate species and in particular mammals. With regard to brain development and early experience in particular, one fact cannot be overemphasised. Development of behaviour in the chick, ie. movement, is precocial. Spontaneous motility has been reported in all vertebrate embryos, but for the chick, beginning from day 17 in ovo (Hamburger, 1968), complex coordinated movements are rehearsed in preparation for hatching, the process of breaking out of the shell requiring coordination of all parts of the body. According to Hamburger (1968) these coordinated patterns of activity are fundamentally different from the random movements seen up to day 16 and 17 in ovo.

In the developing nervous system of the chick the greatest weight of evidence is for a close correlation between the increase in concentration/activity of cholinergic molecules and the onset of neural function (Nachmansohn, 1939; Rogers et al., 1960; Birdick and Strittmatter, 1965; Marchand et al., 1977; Enna et al., 1976; Leah et al., 1980). However, a number of reports contradict such a correlation. For instance, Burt (1968) reported that maximal AChE activity in the spinal chord preceded the commencement of synaptogenesis and in addition preceded maximum CAT activity in ovo by 11 days; a similar observation was made by Turbow and Burthalter (1968).

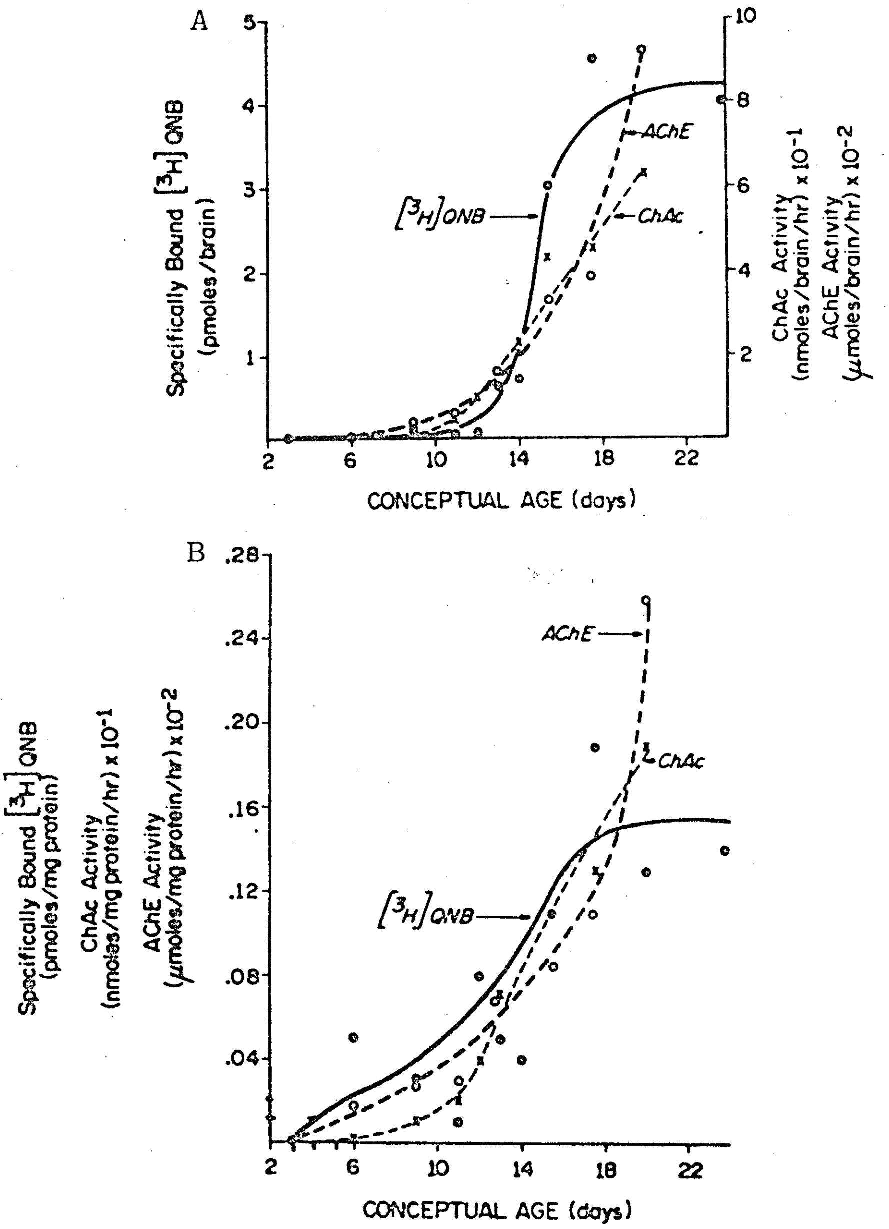
In the chick ciliary ganglion it has been shown that the highest rate of appearance of α BTX labelled nicotinic receptor occurs later than the onset of ganglion transmission, but simultaneously with the rise in both AChE and CAT activity in cell bodies (see figure 4c taken from Giacobini, 1979). However, in this regional example the picture is a little more complex. When values for α BTX labelled receptor, AChE and CAT are expressed relative to those recorded at 7 days post hatch (see figure 4 e, taken from Chiappinelli and Giacobini, 1978), AChE and α BTX binding sites reach approximately 100% of 7 day post hatch values at 14 days in ovo, while CAT activity is much lower and increases slowly over this period. In the chick iris, between 7 days in ovo and 7 days post hatch, α BTX binding increases 70 fold, AChE activity 60 fold and CAT activity 825 fold (Giacobini, 1979).

In contrast to the above observations, Enna et al. (1976) report a very close

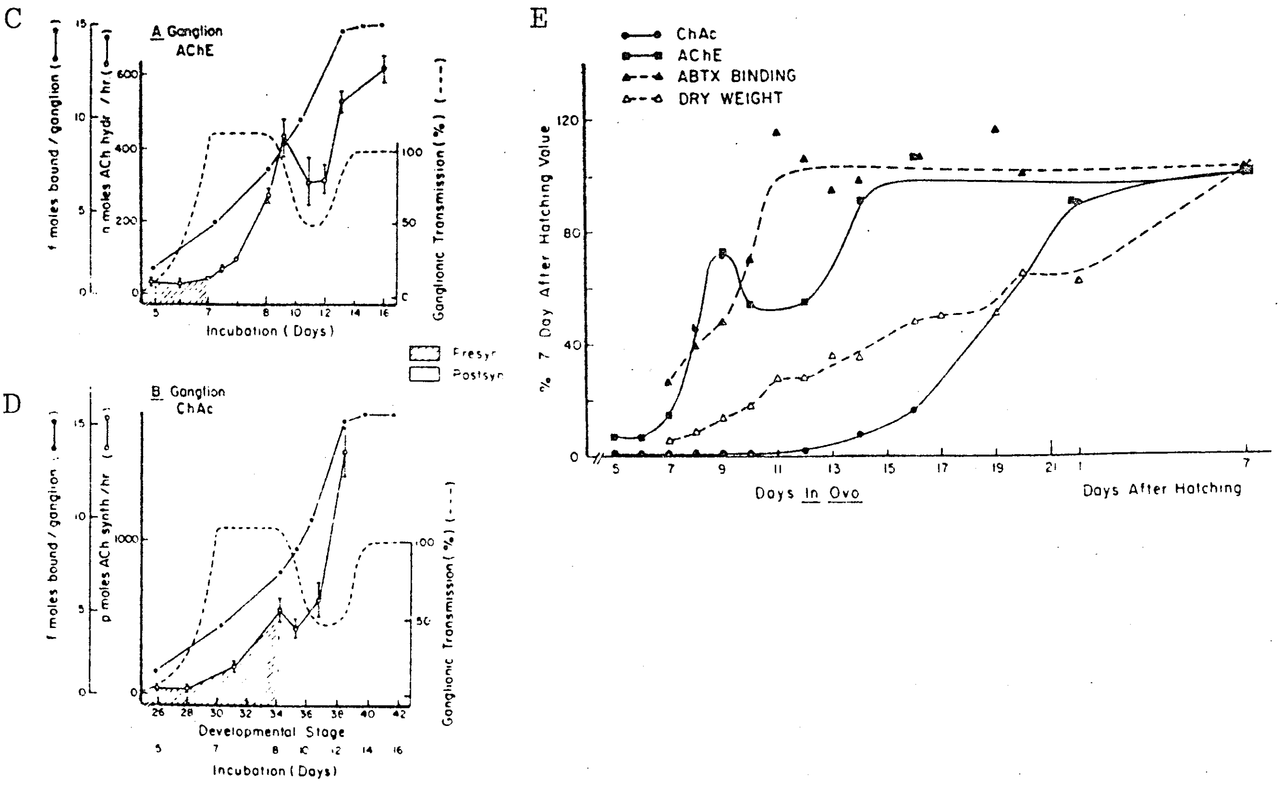
Figure 4. a and b. *Development of acetylcholinesterase (AChE) cholineacetyltransferase (ChAc), and ^{3}[H] Quinnuclidinyl benzilate (^{3}[H] QNB) labelled muscarinic receptor in the chick brain, expressed per whole brain (A) and as a function of protein (B). (Taken from Enna et. al., 1976). Per whole brain, muscarinic receptor are not detected until 10 days in ovo, but substantial concentrations are evident at 6 days in ovo when the same data is recalculated as a function of protein (b).*

c, d and e. Developmental relationship between choline acetyltransferase (ChAc), acetylcholinesterase (AChE) activity, α Bungarotoxin labelled nicotinic receptor and percentage transmission in ciliary ganglion of the chick (C and D). (Taken from Chiapinelli et. al., 1978), and biochemical development of ciliary ganglion (E) where values for α toxin labelled receptor , AChE and ChAc activity are expressed relative to values recorded at 7 days post hatch (Taken from Giacobini , 1979).

Figure 4.



Cholinergic molecules: development in the CNS of birds.



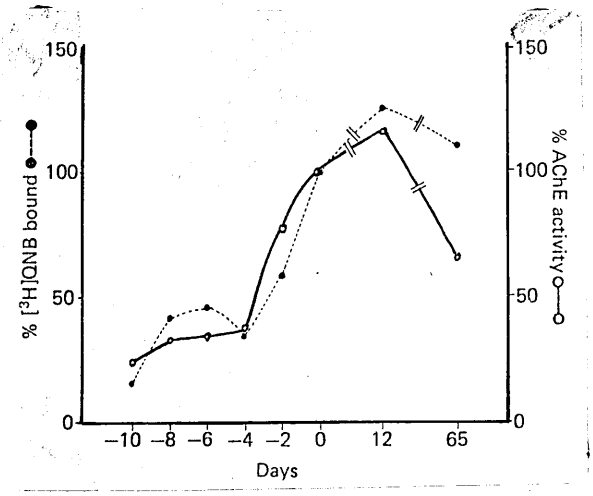
correlation between the appearance and rate increase of CAT, AChE and ^{3}[H] QNB labelled muscarinic receptor for the whole chick brain (see figure 4*A* and *B*). The observations of Chiappinelli and Giacobini (1978) and Enna et al. (1976) highlight a number of analytic and methodological considerations in reporting transmitter-enzyme-receptor development in the nervous system. In the first place, figure 4 *A* and *B*, taken from Enna et al. (1976) reveal considerable differences in the time of appearance and rate of development of cholinergic molecules between values expressed per mg brain protein or for the whole brain. This is not surprising because the neonatal/in ovo brain has a considerably higher water content than the adult brain. (Vernadakis and Woodbury, 1965). In addition it is clear that at a regional level in the central nervous system, the ontogeny of cholinergic functional molecules can differ substantially from that shown for the whole brain. This is further demonstrated by the observations of Kouvelas and Greene (1976) for α toxin labelled nicotinic receptor and by Jerusalinsky et al. (1981) for ^{3}[H] QNB labelled muscarinic receptor and AChE concentrations during chick brain ontogenesis (see figure 5 A to **C**).

Jerusalinsky et al. (1981) report that the rate of increase in muscarinic receptor and AChE activity show, in general, a good correlation, apart from the cerebellum. It is also interesting to note that Jerusalinsky et al. report equivalent concentrations of AChE activity between the paleostriatum augmentatum and hyperstriatum of the chick brain, an observation which contradicts reports for the histochemical localisation of AChE in adult forebrain of birds (see section 1.4).

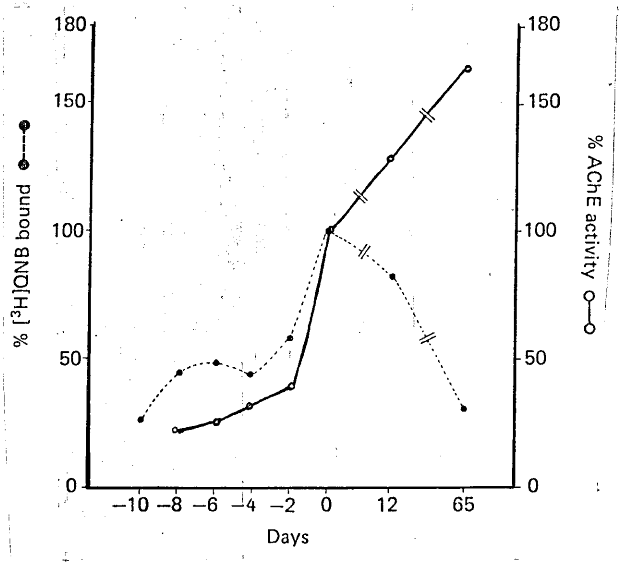
Comparatively recent evidence has demonstrated AChE molecular forms, specifically associated with cholinergic postsynaptic membranes (Courand et al., 1979;

Figure 5. *Development of acetylcholinesterase (AChE) and ^{3}[H] Quinuclidinyl benzilate (^{3}[H] QNB) labelled muscarinic receptor in the chick hyperstriatum (A), cerebellum (B), and paleostriatum (C) in the in ovo (-10 days in ovo to11 days post fertilisation and 0 = 21 days post fertilisation, the day of hatch) (Taken from Jerusalinsky et. al., 1981).*

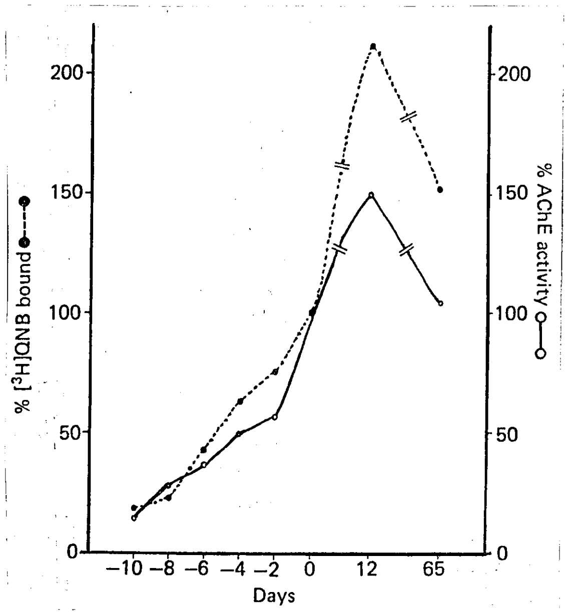
Figure 5. A AChE development in the chick hyperstriatum



B AChE development in the chick cerebellum



C AChE developement in the chick paleostriatum



Rotundo and Fambrough, 1979; Scarsella et al., 1978). During development, for example in the chick optic tectum, the combined specific activities of two slow sedimentation forms of AChE (6 and 4 S) reach maximum activity prior to 8 days in ovo and thereafter remain constant, while there is a sharp increase in the 11 S AChE molecular species, indentified as mainly axonal, from day 16 in ovo onwards (Courand et al., 1979; Villafruela et al., 1981). In addition different regions of the brain show different rate increases in these AChE sedimentation forms during chick brain ontogenesis (Marchand et al., 1977; Villafruela et al., 1981).

Curiously similar to the number and temporal pattern of developmental forms of AChE, recent evidence for the ontogeny of muscarinic receptor in the rat brain has revealed several different agonist affinity binding forms of muscarinic receptor which differ substantially in order of appearance during development (Aronstam et al., 1979b; Walmsley et al, 1981). Walmsley et al. (1981) report a six to seven day lag in the appearance of high affinity sites following the appearance of low affinity muscarinic receptor sites in the neonate rat. The affinity of muscarinic receptor for antagonists on the other hand does not change during brain ontogenesis (Aronstam et al., 1979b). The functional significance of different rates of appearance of muscarinic receptor agonist affinity forms is not known, but it is curious that it is the appearance of high affinity agonist receptor which corresponds more closely with the major period of synaptogenesis (Walmsley et al., 1981). And yet it is the low affinity agonist form of muscarinic receptor which is thought to be important for neurotransmission (Birdsall et al., 1978). Walmsley et al. (1981) have suggested that the more rapid maturation of muscarinic receptor densities in caudal regions of the rat brain compared to cortical regions is compatible with the observation that cell division ceases and synaptogenesis begins in the hindbrain in advance of cortical areas (Altman, 1969).

It has been known for some time that experimental conditions which 'cause' mature muscle cells to develop extrajunctional receptor also makes them receptive for further innervation. Incidentally, junctional and extrajunctional receptor apparently exhibit slight but reproducible differences in ligand recognition properties (see Triggle and Triggle, 1976; and ref. therein). Freeman and Lutin (1975) and Freeman (1977) have proposed that receptor formation in the brain, like peripheral muscle (Sytkowski et al., 1973), not only preceeds synaptogenesis, but may play a critical role in guiding incoming fibres and in the maintenance of neuronal contacts. Such a role for receptor is particularly attractive in view of the observation that most neuronal cell death occurs around the time when most brain cells are establishing their synaptic connections. Cell death is apparently related in some critical way to the magnitude of the available innervation field from which it has been postulated that outgrowing cells compete with each other for a limited number of synaptic (or receptor) sites or a diffusable "trophic factor" essential for their maintenance (see Cowan, 1978).

1.6 Avian brain, anatomical and functional organisation.

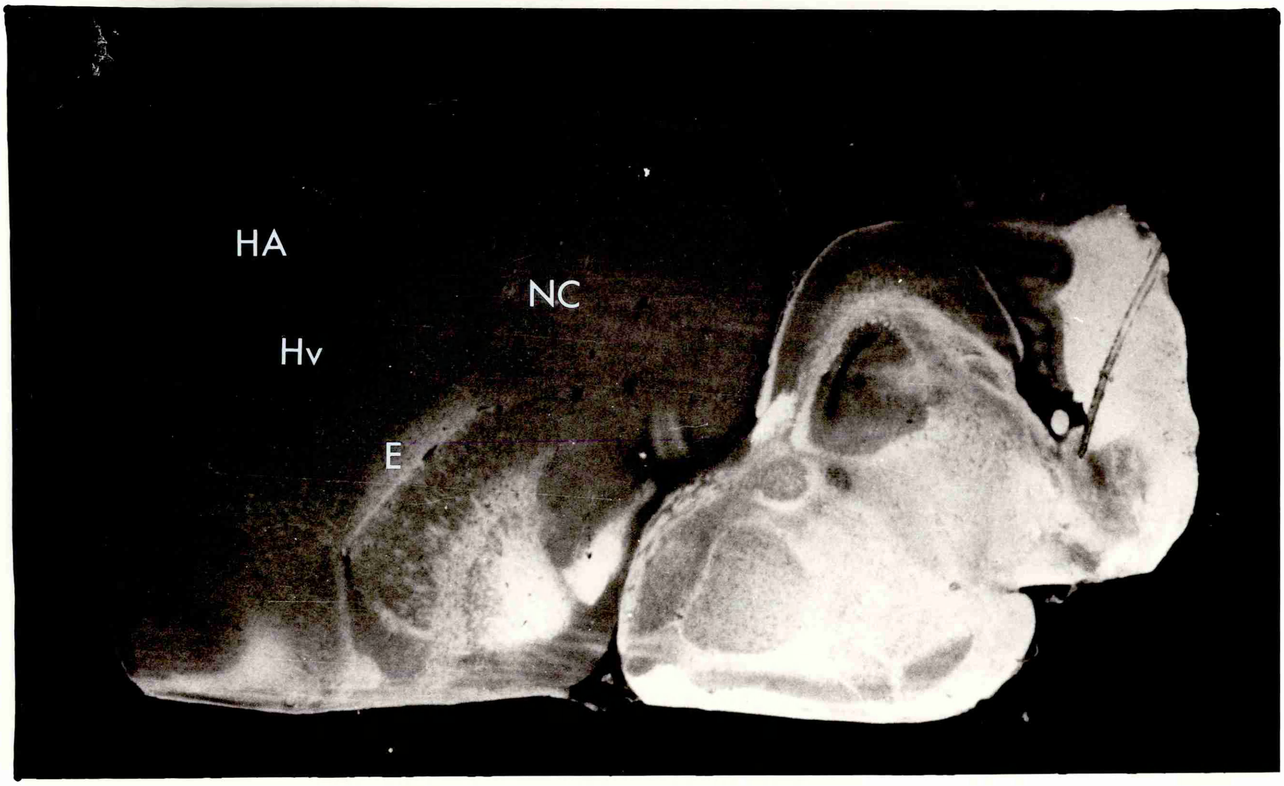
According to Herrick (1948) the cortical evolution of birds occupies

> "an anomalous position. They are much more highly differentiated than reptiles, (...), but with no mammalian affinities. In most of them the olfactory system is greatly enlarged. There is extensive local differentiation of the thalamic nuclei, but not in the mammalian pattern. The system of ascending thalamic projection fibres is larger than in reptiles, and most of these fibres end in the enormously enlarged and complicated corpus striatum. Correlated with the latter point is the fact that, despite the great increase in thalamic projection fibres, the cortex of many birds is scarcely more extensive than in reptiles and in some species is less well differentiated. Birds are more highly specialised in both structure and behaviour than are the lower mammals, and yet their cerebral cortex is rudimentary in comparison with even the most primitive mammals. The explanation for this is that the bird's most diversified behaviour is largely stereotyped in instinctive patterns adequately served by subcortical apparatus, while the patterns of mammalian behaviour, even in the lowest members of the class, are in larger measure individually learned. And enhancement of learning ability goes hand in hand with cortical differentiation."

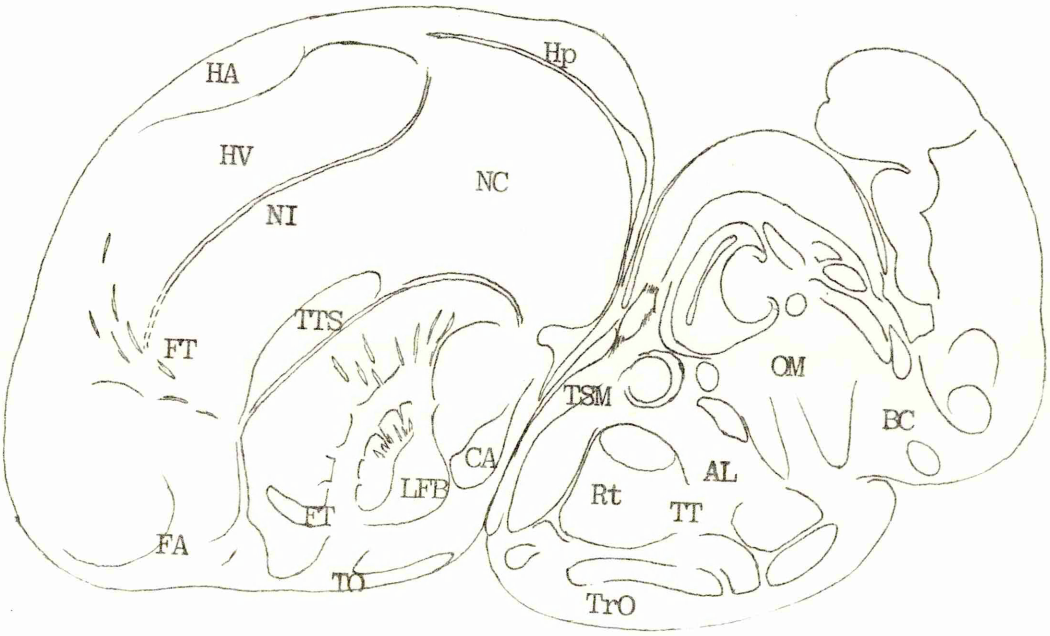
This view of avian behaviours and forebrain capabilities has changed dramatically in the last twenty years. The evolutionary principle of corticalisation of function, unless a vaguely teleological definition of 'function' is accepted, is certainly incorrect (Jensen, 1976). The behavioural capabilities of the brain are distributive, emerging from complex interactions of the universal metazoan information processing unit, the neuron and more

Figure 6.

A Fibrous regions of the chick brain



B


Hp
HA
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LFB
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Rt
FT
TT
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TO
TrO

particularly local populations of neuronal circuits capable of simple learning (eg. habituation) (see Bullock, 1967), upon which more complex and elaborate neural systems are constructed. Any misconceptions in Herrick's synopsis (see above) of the functional capabilities of the avian forebrain probably stem from the dogma that the telencephalon of birds is an enormously enlarged mammalian basal ganglion with a thin overlying cortex.

In (apparent) contrast to the laminar organisation of the mammalian cortical mantle, the avian telencephalon is characterised by broad fields of cells, some nuclear clusters and a thin overlying laminated zone. Yet, despite the morphological dissimilarities, comparative embryological, anatomical and histochemical studies all suggest that most of the bird's telencephalon is comparable with elements in the mammalian cortex (see Cohen and Karten, 1974; Benowitz, 1980). Cross sections through the telencephalon of the developing chick (see figure 7 , redrawn form Kuhlenbeck (1938) and Kallen (1962)) show essentially two zones, dorsal and ventral to the sulcus (s), giving rise to pallial (D) and basal (B) telencephalic structures. In mammals all tissue arising from the zone dorsal to this sulcus becomes part of the cortical mantle (Kallen, 1951). The avian structures which have a comparable embryological origin include not only the dorsalmost portions of the telencephalon which are laminar in appearance (ie. the corticoid, parahippocampal and wulst regions), but also the cell masses of the hyperstriatum ventrale (HV), neostriatum (N), ectostriatum (E) and portions of the archistriatum (β) which suggests that they may have a common phyletic origin and therefore may be homologous with portions of the mammalian cortex. (see Karten and Dubbeldam, 1973; Northcutt, 1978). The observations of the present study for cholinergic receptor distribution in the post hatch and developing chick brain contribute to the above view.

Based primarily on morphological considerations, Kappers et al. (1936) regarded non laminar regions of the telencephalon to be elaborations of the basal ganglia and thus their use of the suffix "striatum". Although this nomenclature is still used, it should be emphasised that it is only the paleostriatal complex that is probably comparable with the mammalian striatum (Karten, 1969; Karten & Dubbelbam, 1973; Brauth et al., 1978).

Figure 6A & B (page 26) show some of the anatomical features of the chick brain in parasagittal section.The first point to notice is the continuous mass of gray,undifferentiated by fibre tracts,dorsal to the striatal regions. The dorsal pallium originating from D2 zone shown in figure 7 includes the hyperstriatum dorsale (HD), hyperstriatum intercalatus (HIS) and hyperstriatum accessorium (HA), collectively known as the wulst. A major portion of the wulst is a visual projection area,comparable with the visual cortex of mammals (Cuenod, 1974; Karten et al., 1973; Pettigrew and Konishi, 1976). Ascending projections to this area arise from a nuclear complex of the anterior dorsolateral thalamus, the principal optic nucleus (Karten & Nauta, 1968; Hunt & Webster, 1972; Miceli et al., 1979; see figure 9).

Projections from the visual wulst descend down the medial hemispheric. wall as a component of the tractus septomesencephalicus (TSM) projecting to several thalamic nuclei in receipt of retinal and optic tectum afferents as well asto the brain stem and cervical spinal cord. Similarities between these efferents of the HA and visual & somatosensory cortices of

Figure 7 a to e. *Development of regions of the Avian forebrain. Cross sections through th telecephalon of the developing chick (a-c), showing the origin of the pallial (D) and basal (B) telencephalic structures seperated by the sulcus (S). The right hand side of (a) shows the same regions drawn according to the system of Kallen (1962). Cross sections through the adult pigeon brain (d and e) indicating some major structures and their embryological origin (in parentheses). (Taken from Benowitz , 1980).*

Figure 8. *Diagramatic representation of origins of somatomotor efferents in birds and mammals (a and b). In birds the hyperstriatum ventrale (HV) and neostriatum (N) project on two efferent zones: the paleostriatum augmentatum (PA) and the archistriatum (anterior division, Aa shown in figure). Pa projects on the paleostriatum primitivum (PP) and intrapeduncular nucleus (INP), a projection equivalent to the projection in mammals (b) from the caudate putamen (Pu) to the globus pallidus (GP). Birds PP-INP and mammals GP give rise to the descending "extrapyramidal" pathway, the ansa lenticularis. Pyramidal tract efferents in mammals (b) arise from layer V cells in the neocortex;the comparable efferent pathway in birds arises from two sources: the hyperstriatum accesorium (HA), which projects out by way of the tractus septomesencephalicus (TSM),and the archistriatum which sends its efferents out by way of the occipitomesencephalic tract (OM)(from Karten and Duddeldam, 1973).*

Subdivisions of the archistriatum (c), mediale (Am), posterior (Ap) and nucleus taeniae (Tn) project to the hypothalamus by way of the tr. occipitomesencephalicus (pars hypothalami (HOM); these regions appear to be equivalent to the mammalian amygdala. The division intermedium *(Aid) and anterior (Aa) are the somatosensory portions of the archistriatum. Their input comes from the contralateral archistriatum via the the anterior commissure (CA), from the medial hemispheric wall, and from the overlying N and HV via the tractus* dorsoarchistrialis *(DA). OM, the lateral portion of the birds pyramidal tract, originates only in somatomotor portions of the archistriatum (Zeir and Karten, 1971). (Figure and legend taken from Benowitz , 1980).*

Figure 7. Development of regions of the avian forebrain

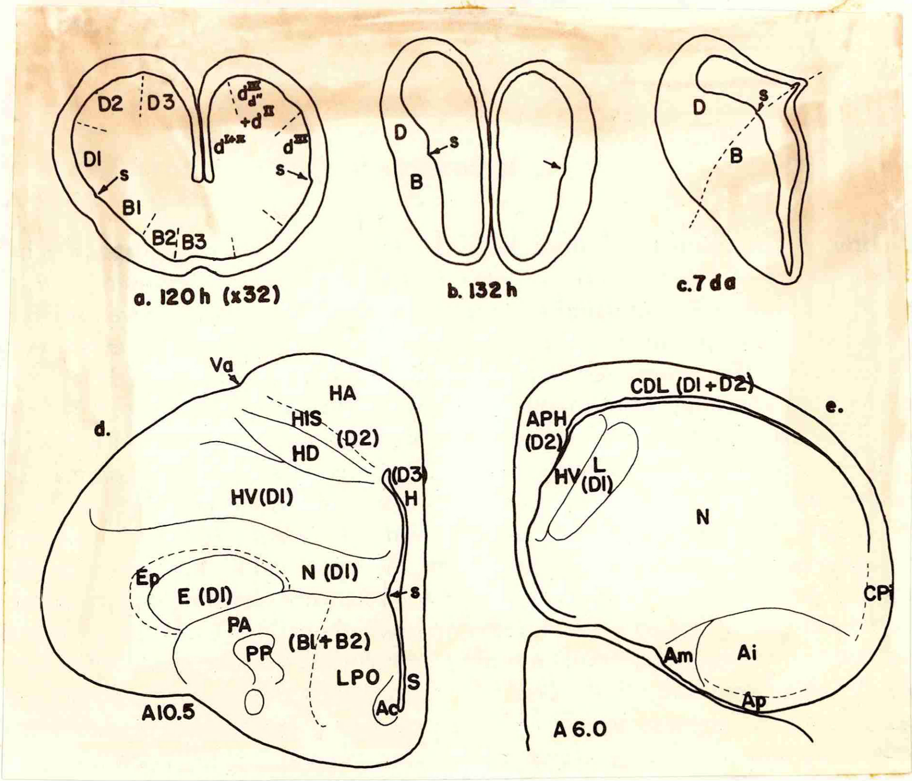
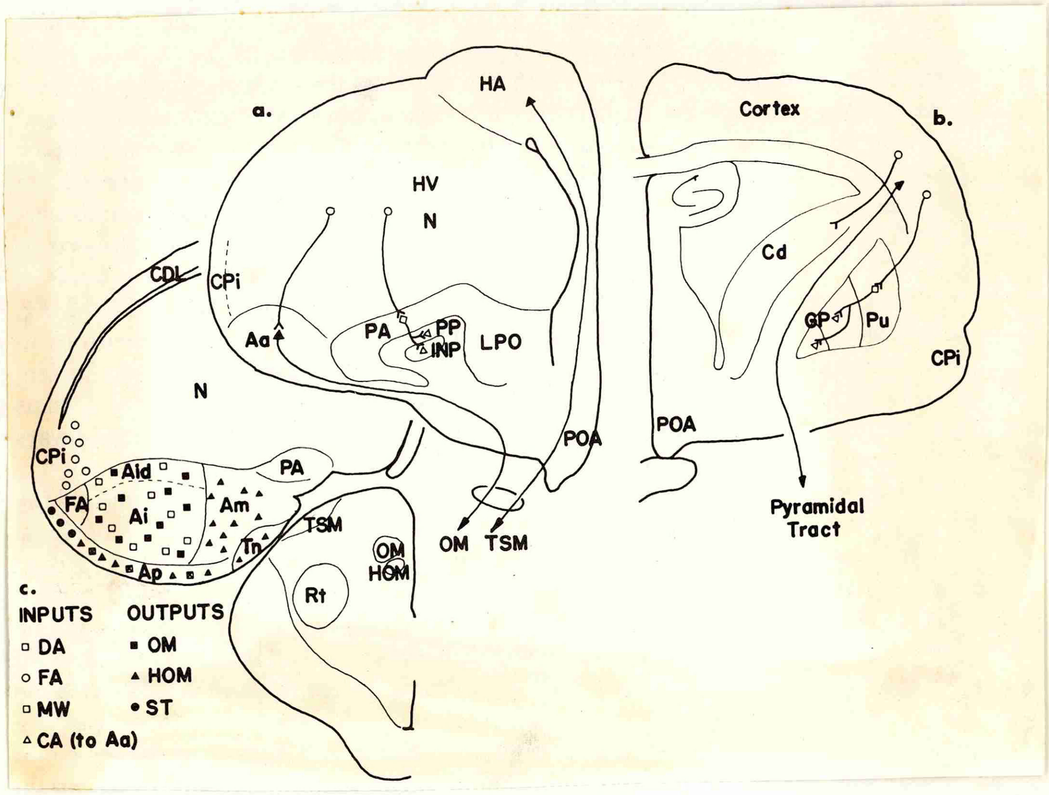

D2
D3
D1
s
B1
B2
B3
$d^{III}_{d''}$
$+d^{II}$
d^{I+II}
d^{III}
s
a. 120 h (x32)
D
s
B
b. 132 h
D
s
B
c. 7 da
Va
HA
HIS
(D2)
HD
d.
(D3
H
HV(D1)
Ep
N (D1)
E (D1)
s
PA
PP
(B1+B2)
LPO
S
Ac
A10.5
CDL (D1+D2)
e.
APH
(D2)
HV
L
(D1)
N
CPi
Am
Ai
Ap
A 6.0

Figure 8. Pathways of the avian forebrain


a.
HA
HV
N
CDL
CPi
Aa
PA
PP
INP
LPO
N
POA
CPi
Aid
PA
FA
Ai
Am
Tn
TSM
Ap
OM
HOM
Rt
OM
TSM
Cortex
b.
Cd
GP
Pu
CPi
POA
Pyramidal
Tract
c.
INPUTS
□ DA
○ FA
□ MW
△ CA (to Aa)
OUTPUTS
■ OM
▲ HOM
● ST

mammals have been noted (Zeier and Karten, 1971). The accessorium possibly receives 'limbic' and somato-motor inputs arising from caudal nuclei of the dorsal thalamus (Hunt and Webster, 1972). A descending projection from the nonvisual wulst down the medial hemisphere wall in the brain stem a medial division of the bird's "pyramidal tract" (Karten, 1973; see figure 8).

Pallial structures below the wulst,derived from Dl zone of figure 7 , recieve sensory specific projections from the thalamus, ie. ectostriatum (E) and field 'L' (Rose), while the archistriatum (A) projects out of the telencephalon. Interposed between the sensory and motor areas of the forebrain is the hyperstriatum ventrale and neostriatum whose connections are all intrinsic to the telencephalon (Benowitz, 1980, but see below). The ectostriatum (E), the core of which is composed of heavily myelinated fibres(see figure 6), is the telencephalic projection zone of an elaborate visual pathway arising from the tectum (Karten and Hodos, 1970) (see figure 9). Visual information from the deep tectal neurons project onto the nucleus rotundus thalami (Rt) (see figure 9) which in turn projects ipsilaterally to the palaeostriatum and ectostriatum (Karten and Revzin, 1966; Karten and Hodos, 1970). The E, unlike the visual wulst, does not possess long efferents leaving the telencephalon,but projects to the periectostriatal belt which in turn projects to parts of the neostriatum, hyperstriatum ventrale and archistriatum (Ritchie and Cohen, 1977). Efferents from the N and HV go to two regions, the palaeostriatum and archistriatum, which give rise to two major extratelencephalic pathways. The palaeostriatal complex of birds is organised much like the mammalian basal ganglia (see figure 6 and 8). Efferents from this region form the "extrapyramidal" pathway projecting to a variety of thalamic and subthalamic nuclei (Karten and Dubbeldam, 1973;

Brauth and Kitt, 1978). The pathway arising from the archistriatum, the occipitomesencephalic tract (OM), projects to a variety of di- and mesencephalic centres, motor nuclei and the medial reticular formation down to spinal levels. Based on these projections, Zeier and Karten (1971) have suggested that the caudal and medial archistriatum is homologous to the mammalian amygdala, the anterior and intermediate archistriatum are homologous to parts of the mammalian cortex, and the main portion of the OM homologous to the mammalian pyramidal system (see figure 8).

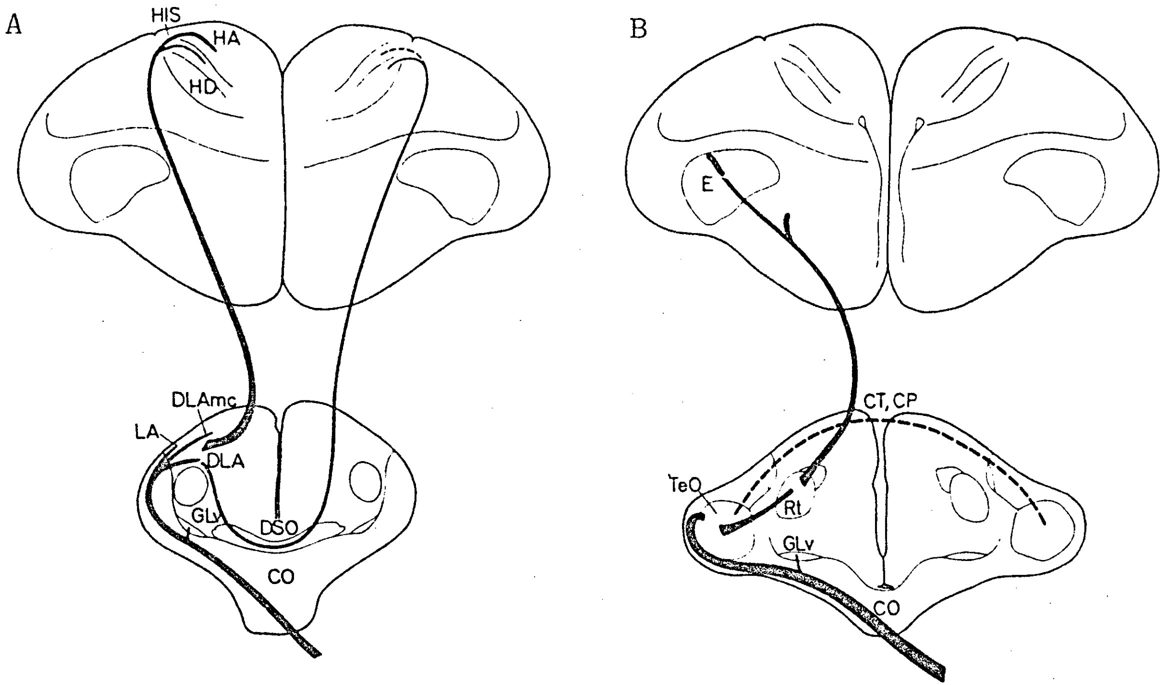
An additional subpallial telencephalic nucleus (nucleus basalis) occurs rostral to the paleostriatum in birds (see figure 6). This nucleus recieves a direct bilateral input from the principal sensory trigeminal nucleus (PrV) via a quintofrontal tract (QF) (Wallenberg, 1903, Zeigler et al., 1969; Dubbeldam et al., 1981). It is not clear whether the nucleus basalis (BAS) should be considered a thalamic, telencephalic or even a pallial structure. Electrophysiological analysis of the BAS reveals the existence of a distinct somatotopic representation of areas of the bill and tongue (Berkhoudt et al., 1981).

Avian auditory pathways are organised in a similar manner to the visual ecto-striatal system. The pars dorsalis of the lateral mesencephalic nucleus (MLD), (see figure 6), situated dorsal and central in the optic lobe, recieves auditory input from a number of medullary auditory nuclei (Boord, 1968) and projects bilaterally to the thalamic nucleus ovoidalis (Karten, 1967) which in turn projects ipsilaterally to the paleostriatum and a caudal portion of the neostriatum termed field L (Karten, 1967; Nottebohm et al., 1976; Bonke et al., 1979) situated immediately beneath the medial hyperstriatum ventrale (see figure 10). The avian field L exhibits tonotopic organi-

Figure 9 a and b. *Schematic summary of two major recognised ascending visual projection pathways in birds, the thalamofugal pathway (retino-thalamo-hyperstriatal system in figure A) and tectofugal (B). These terms refer to pathways ariseing from regions in direct receipt of retinal efferent connections. The tectofugal pathway projects upon the nucleus rotundus (Rt) thalami which in turn projects upon the core regions of the ectostriatum (E). The thalamofugal pathway projects upon the core regions of the ectostriatum E). The thalamofugal pathway arises from the principle optic nucleus of the thalamus, which includes the nucleus dorsolateralis anterior thalami (DLAmC)and nucleus lateralis anterior thalami (LA), which projects upon the visual Wulst, ie. the hyperstriatum intercalatus superior (HIS) and the hyperstriatum accessorium (HA). (Taken from Cuenod , 1974).*
HD: hyperstriatum dorsale; GLv: nucleus geniculatis lateralis pars ventralis; DSO: decussatio supra optica; CO: optic chiasma and tract; TeO : optic tectum.

Figure 10. *Schematic summary of the ascending auditory system* → *and descending pathways and regions concerned with vocalisation in birds* ⇢. *The principle ascen-pathway arises from the nucleus magnocellularis lateralis pars dorsalis (ML) which projects via the nucleus ovoidalis (Ov) upon "L" an auditory area of the neostriatum. The hyperstriatum ventrale p. caudale (HVc), the 'highest structure whose destruction in the canary results in a loss of song, projects on two other telencephalic centres: nuleus robustus archistrialis (RA) and area X in the lobus parolfactorious (LPO). RA projects to the nucleus intercol-icularis (ICo), a region implicated in vocalisation, and to the motor nucleus innervateing the syrinx , n Xll ts. (modified from Boord , 1969; Cohen and Karten, 1974; and Benowitz , 1980).*

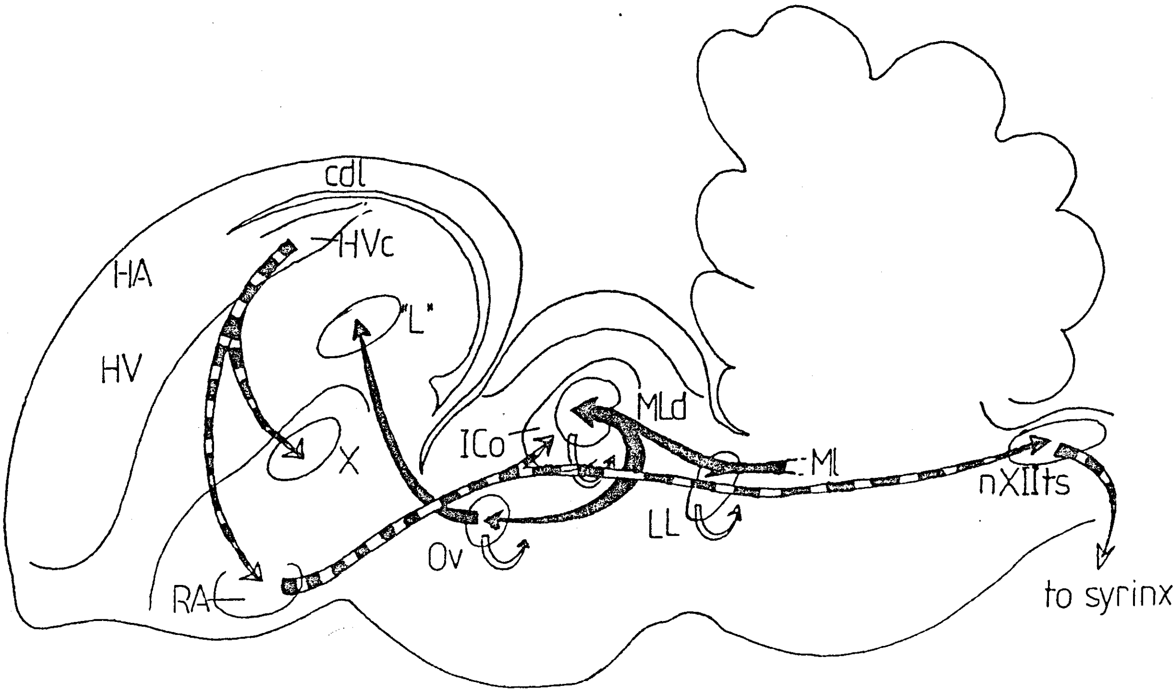
Figure 9. Visual pathways in birds


A
HIS
HA
HD
DLAmc
LA
DLA
GLv
DSO
CO
B
E
CT, CP
TeO
Rt
GLv
CO

RETINO - THALAMO -
HYPERSTRIATAL SYSTEM

RETINO - TECTO - ROTUNDO -
ECTOSTRIATAL SYSTEM

Figure 10. Auditory and song pathways in birds


cdl
HA
HVc
"L"
HV
X
ICo
MLd
Ml
nXIIts
RA
Ov
LL
to syrinx

sation (Zaretsky and Konishi, 1976; Scheich et al., 1979) with close similarities to higher order auditory cortices in mammals (Karten, 1969; Scheich et al., 1979). Field L in turn projects to the hyperstriatum ventrale and part of the archistriatum (Nottebohm et al., 1976; Bonke et al, 1979). Field L and the hyperstriatum both project to the paleostriatum and magnocellular nucleus of the neostriatum (see figure 10 , and section 1.4).

The hippocampal and parahippocampal cortices recieve afferents from the hypothalamus, the diagonal band, and septal nuclei (Benowitz and Karten, 1976; Kraniak and Siegel, 1978 a) and project back to septal nuclei and the nucleus of the diagonal band via a precommissural fornix system (Kraniak and Siegel, 1978 b). The septal nuclei are characterised by extensive descending projections to the hypothalamus, ventrolateral and dorsomedial thalamic nuclei, lateral habenular nucleus and midbrain tegmentum (Kraniak and Siegel, 1978 a).

Much of avian behaviour is visually dominated, and the central pathways associated with visual function are elaborate. The retina projects to many portions of the di- and mesencephalon, including the nucleus geniculatis lateralis ventralis pars ventralis (GLv), nucleus lentiformis mesencephali, the pretectal region, nucleus ectomammilaris, hypothalamus, principle optic complex of the anterior dorsolateral thalamus and the optic tectum.

From the preceeding description it is clear that the avian telencephalon recieves sensorial representation essential for associative processes. Interposed between sensory and motor regions of the forebrain is the complex, nonlemniscal network of the neostriatum and hyperstriatum ventrale. The neural organisation of these regions suggests that they may be sites of polysensory integration and associative plasticity (Benowitz, 1980). Lession of the hyperstriatum ventrale severely disrupts learned associations (Zeigler, 1967; Benowitz, 1972;)Greif, 1976). This region apparently recieves neither direct ascending sensory information from the brain stem nor projects out of the telencephalon (Zeier and Karten, 1971; Karten, 1969). However, recent studies suggest that medial regions of the hyperstiatum ventrale are in receipt of afferents from the optic tectum as well as from the visual wulst, neostriatum, hippocampus, septal nuclei, paleostriatum augmentatum and intermediodorsal part of the archistriatum (Bradley and Horn, 1978; Davies and Bradley, 1981; Bradley et al., 1982).

Studies involving decerebration in birds indicate that, while many behavioural capacities are organised at subtelencephalic levels, coordination and elicitation by appropriate environmental stimuli, and perhaps the ability to form associations,requires the cerebral hemispheres. Curiously, in'neonatal'chicks decortication does not have so nearly a devastating effect on behaviour as in adults; more spontaneous activity is seen and reactivity levels are less impaired(Martin and Rich, 1918), classical conditioning still takes place, a capacity lost following comparable surgery in the adult (Tuge and Yueh, 1962). The parallel between the above observations and those, for example, of Goldman-Rakic (1982) in showing that deficit learning in the rhesus monkey depends on subcortical functions (ie. basal ganglia) in the infant, which become 'more essentially' cortical in the adult, should not be underestimated with respect to how we view the functional capabilities and organisation of the chick brain.

1.7 Aims and objectives of this thesis.

The principal objective of this thesis is to report as accurately as possible the distribution of tritiated antagonist labelled cholinergic receptor in the young post hatch and in ovo chick brain. As with any experimental study, the observations, inferences and conclusions rest upon the validity of approach and technique by which the objective is achieved. With regard to the observations and conclusions of this report the balance between validity and inference is a particularly delicate one.

In selecting to study the whole brain, correspondences of functional molecular distribution with principles of brain organisation must be more concerned with general features of brain morphology and topography than with detailed regional examples of cell circuitry. Nevertheless, an objective of this study is to search for the links between the distribution of a particular functional molecule and the morphological , anatomical and physiological properties of the brain. And,by comparing between brains of different vertebrate species and by studying the development of that particular functional molecule, it is hoped that something can be discerned of the influences serving to specify brain organisation and function.

2. Methods; Light microscope autoradiographic proceedure for the localisation of tritiated antagonist-labelled chick brain cholinergic receptor.

2.1 Preparation & sectioning of the chick brain.

At the same time each day, chicks (type Ross Chunky) of various ages were taken from a communal incubator or pen and with the minimum of handling decapitated. The brain was immediately dissected free hand from the skull and placed ventral surface down on dry ice. A small concave glass bowl was placed over the exposed dorsal surface of the brain to prevent dessication and to accelerate the time taken for the brain to freeze.

Once frozen, individual brains were mounted on cryostat sectioning chucks (see figure 11) using Tissue-Tek II O.C.T. (Miles Laboratories) mounting medium, in quantities sufficient to adhere the brain firmly to the cryostat chuck, but not to enclose that part of the brain to be sectioned. O.C.T. mountant has a different viscosity to brain tissue and, if sectioned with the brain results in tissue folding and tearing. In order to prevent dessicatiom, the chuck mounted brain was wrapped in parafilm and immediately placed into the body of the cryostat in order to equilibrate the temperature of the brain to that of the sectioning temperature.

Consecutive parasagittal or frontal plane sections of the brain (8-20 microns thick) were cut at varios knife/brain temperatures (-15°C to -30°C) using a Slee (South London Electrical & Engineering Co.) cryostat operated manually. The reason for this variation in sectioning temperature is that the brains of younger chicks have a higher fluid-to-tissue content, and to prevent the tissue slice from fracturing, the brain section must be cut at a warmer temperature than that of older brains. The brain sections were immediately freeze-thaw mounted (see Rogers, 1967 and figure 11) on cool, clean microscope slides which had been precoated with a solution containing 5% gelatin and 0.5% chrome alum hardener. The slide mounted sections were placed in a slide rack in groups of 25 and stored for periods up to six hours in a liquid nitrogen freezer. The total number of sections cut, their relative sequence, the number discarded, as well as the sectioning temperature were recorded.

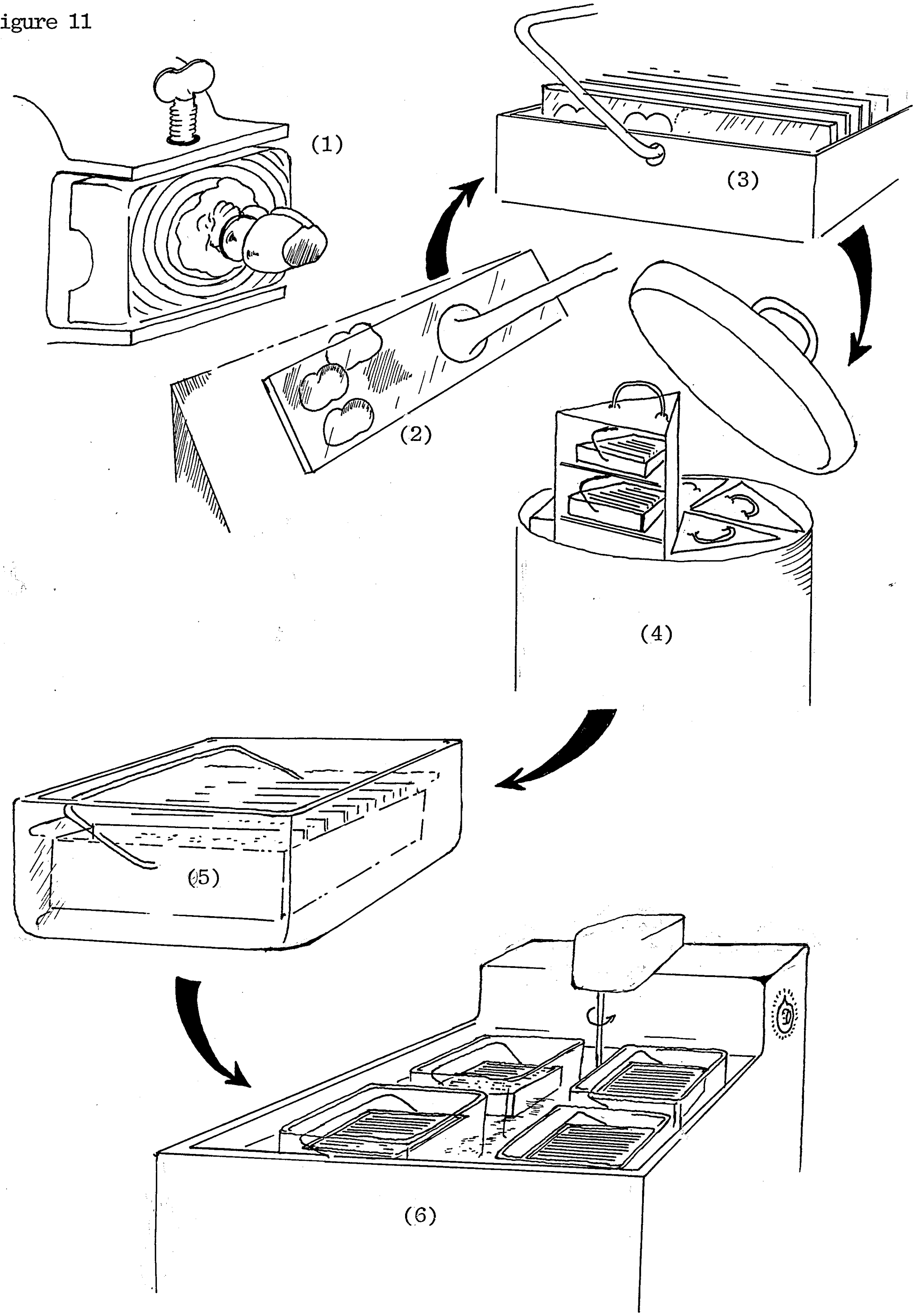
In the case of chick embryos (8 to 17 days in ovo), it was found to be necessary to section the brain without removing it from the chick head. To prevent tissue fracturing problems, cartilage and bone were cut away from the brain while the head was mounted on the cryostat chuck set in the clamps of the advance arm of the cryostat. Whole brain dissection of the younger embryos proved to be difficult without damaging brain structure.

2.2 *In vitro* labelling of muscarinic cholinergic receptor in chick brain tissue slices: receptor ligand $[^3H]$Propylbenzilycholine mustard.

The following proceedure for the *in vitro* labelling of muscarinic cholinergic receptor of brain tissue slices is a modified version of that described by A. Rotter (Ph.D Thesis 1977, National Institute of Medical Research, Mill Hill, London). The muscarinic receptor ligand N-2'- chloroethyl-N (2", 3", $[^3H]_2$)propyl-2-aminoethyl benzilate

Figure 11. *Diagramatic representation of the apparatus and proceedures of chick brain sectioning and in vitro receptor labelling of cryostat sectioned tissue slices. Whole brains,mounted on cryostat chucks (1) were sectioned and freeze thaw mounted on acid clean slides (2),then stored in slide racks (3) in a liquid nitrogen freezer (4) to await in vitro labelling by* $^{3}[H]$ *antagonists in 300 ml volumes of incubationg media , contained in slide rack dishes (5) held at a constant temperature and rate of agitation in a water bath (6).*

Figure 11



($[^3H]$ PrBCM) was synthesized by Dr. N.J.M. Birdsall (MIMR, Mill Hill, London) according to the method described by Young et al. (1972).

The pharmacologically active species of $[^3H]$ PrBCM is the aziridium ion formed in aqueous solution at a pH such that the parent compound is largely in the free base form. Prior to each MAChR $[^3H]$ PrBCM labelling experiment, $[^3H]$ PrBCM was routinely cyclized at a concentration of 10^6 M in 50ml 10mm Phosphate buffer (pH 7.4) for one hour at room temperature (22^o C) and the reaction stopped by placing on ice. For convenience in describing assay additions, the solution added, termed $[^3H]$ PrBCM, is the cyclized reaction mixture, and the concentration quoted is that of the aziridium derivative calculated on the basis of a 91% yield (Burgen et al. 1974).

Slide mounted thin sections of the chick brain, held in slide racks in groups of 25 (see figure 11), were removed from a liquid nitrogen freezer and immediately prefixed in 300 ml Krebs Henseleit solution (NaCl, 118.6mM, KCl, 4.7mM, Mg SO_4, 1.2mM, $NaHCO_3$, 25mM, $CaCl_2$, 0.9mM, KH_2PO_4, 1.2mM, pH 7.4 after equilibration with 5% CO_2 / 95% 0_2), containing 0.125% gluteraldehyde (see results) for 15 minutes at 0 to 5^oC. The slide mounted sections were then briefly rinsed in ice cold Krebs Henseleit buffer.

Following prefixing, one third ($^1/3$) of the slide mounted sections were preincubated in 300 ml Krebs Henseleit buffer containing 125nM atropine sulphate for 15 minutes at 25^oC (see results). These sections were then transferred to a further 300 ml Krebs Henseleit buffer solution containing 3.18 nM $[^3H]$ PrBCM (55 Ci/m mol) and 125 nM atropine sulphate

(an in excess concentration, binding to all available MAChR ligand binding sites) for 15 minutes at 25°C (see results). The remaining two thirds of the slide mounted sections were incubated in 300 ml Krebs Henseleit solution, containing 3.18 nM $^{3}[H]$ PrBCM alone, for 15 minutes at 25°C.

In earlier studies, binding by $^{3}[H]$ PrBCM to chick brain tissue slice MAChR was terminated by postfixing in 300 ml of Carnoy's fluid(ethanol 30%, chloroform 30%, acetic acid 10%) followed by washing in 5 changes of absolute alcohol. Postfixing was abandoned in later autoradiographic experiments, having discovered that this proceedure resulted in a substantial reduction in the number of $^{3}[H]$ PrBCM binding sites ($>$ 10%) and further that the tissue section became brittle, resulting in tissue fracturing and causing unnecessary procedural difficulties in direct count measurements (see section 3.10) .Consequently $^{3}[H]$ PrBCM binding was simply terminated by washing in 5 changes of ice cold Krebs Henseleit buffer over a period of 90 minutes. Preincubation, incubation and washes were all performed under light but constant agitation. The slide mounted $^{3}[H]$ antagonist labelled tissue sections were covered and left to dry at room temperature, in preparation for either emulsion coating or apposition autoradiography.

2.3. In vitro labelling of muscarinic cholinergic receptor in chick brain tissue slices: receptor ligand $^{3}[H]$ Quinuclidinyl benzilate.

The following proceedure is a modification of that first described by Yamamura and Snyder (1974) for labelling muscarinic cholinergic receptor of vertebrate brain homogenates using the ligand $^{3}[H]$ 3-Quinuclidinyl benzilate.

3-Quinuclidinyl benzilate or benzeneacetic acid, α -hydroxy - α phenyl 1 azabicyclo (2.2.2) oct- 3-yl ester (Chem Abstr. listing) also known as BZ (Edgewood Arsenal designation) was first synthesized by Sternbach and Kaiser (1951,1952) as an atropine analogue. *l* - Quinuclidinyl (phenyl - 4 (n) - ^{3}[H]) benzilate (^{3}[H] QNB) (30 - 60 Ci/mmol, Radiochemicals Centre, Amersham) was used here for autoradiographic studies.

Slide mounted sections of chick brain (10-20 microns thick), racked in groups of 25,were removed from a liquid nitrogen freezer and immediately prefixed in 300 ml 0.05 M Na K phosphate buffer (pH 7.4) containing 0.125% glutaraldehyde for 15 minutes at 0 to $5^{o}C$ (see results). One third (^{1}/3) of these slide mounted sections were then incubated in 300 ml Na K phosphate buffer containing 1.6 nM ^{3}[H] *l* QNB (43 Ci / mmole Radiochemical centre, Amersham) and 120 nM atropine sulphate for 30 minutes at $25^{o}C$. The remaining two third (^{2}/3) slide mounted sections were incubated in 300 ml Na K phosphate buffer (pH 7.4) containing 1.6 nM ^{3}[H] *l* QNB alone. ^{3}[H] *l* QNB binding was terminated for both atropine exposed and non exposed sections by 5 washes in ice cold Na K phosphate buffer for a period of 90 minutes. The slide mounted sections were covered and left to dry at room temperature.

2.4. In vitro labelling of putative nicotinic cholinergic receptor in chick brain tissue slices: receptor ligand ^{3}[H] α Bungarotoxin.

Cryostat cut sections of chick brain , mounted on slides in groups of 25, were removed from a liquid nitrogen freezer and immediately prefixed in 300 ml 0.05 M Na K phosphate buffer (pH 7.4) containing 0.125% glutaraldehyde and 1mg/1ml bovine serum albumin (BSA) for 15 minutes at 0 to 5°C.

One third ($^1/3$) of these slide mounted sections were then preincubated in 33 ml 0.5 M Na K phosphate buffer (pH 7.4),containing 1nM d-tubocurarine and 1mg/1ml BSA,for 15 minutes at 25°C (after the method of Greene, 1976, for $^{125}[I]$ α-bungarotoxin binding to chick brain putative nicotinic receptor). These sections were then transfered to a further volume of 300 ml buffer, containing 1mM d-tubocurarine, 1mg/1ml BSA and 8nM $^3[H]$ α bungarotoxin (58 Ci/mmol, Radiochemical centre, Amersham),for two hours at 25°C. The remaining two third ($^2/3$) sections were incubated in 300 ml Na K phosphate buffer, containing 8nM $^3[H]$ α bungarotoxin and 1mg/1ml BSA, for 2 hours at 25°C. $^3[H]$ α BTX binding was terminated in all sections by four washes in ice cold buffer over a period of 15 minutes. Preincubation, incubation and washes were all carried out under light, constant agitation. The slide mounted sections were left to dry at room temperature.

2.5 Time course and equilibrium binding studies: ligands $^3[H]$ PrBCM and $^3[H]$ *l* QNB.

Methods are the same as described in sections 2.1 to 2.3, but with the following modification: 1) To ensure equivalence of sampled brain regions, consecutively cut slide mounted tissue sections (12 microns thick) were separated and placed in slide racks in numbers equal to the treatment variables. 2) $^3[H]$ MAChR ligands were added in discrete concentration increments to one 300ml volume of Na K phosphate buffer (pH 7.4) or Krebs-Henseleit buffer (pH 7.4) to give a sampled concentration range between 10^{-8} M to 10^{-9} M.

2.6 Competitive cholinergic antagonist binding studies: ligands ^{3}[H] *l* QNB and ^{3}[H] α BTX.

Methods are the same as described in sections 2.1 - 2.4, but with the following modifications. For competitive antagonist studies of ^{3}[H] *l* QNB binding, the concentration of ^{3}[H] *l* QNB was kept constant at 1.47±3nM in one 300ml volume of Na K phosphate buffer (pH 7.4), to which were added discrete concentration increments of competing antagonist over the range 10^{-13}M to 10^{-10}M. For competitive antagonist studies of ^{3}[H] α bungarotoxin binding, the concentration of ^{3}[H] α BTX was kept constant at 8nM in one 300ml volume of Na K phosphate buffer containing 1mg/1ml BSA, to which was added discrete concentration increments of competing antagonist over the range 10^{-3}M to 10^{-10}M. Prior to incubation with ^{3}[H] α BTX and competing 'cold' antagonists, chick brain tissue sections were preincubated with exactly the same concentration of competing antagonists, but in the absence of ^{3}[H] α BTX .

2.7 Direct count quantitation of ^{3}[H] antagonist binding to chick brain tissue slice cholinergic receptor.

The concentration of specifically bound cholinergic receptor antagonist to chick brain tissue slices was determined by scintillation counting. For kinetic analysis and determination of anatomical regional receptor concentration, tissue sections were scraped off a microscope slide with a single edged razor and placed into scintillation vials. To each vial were added 1 ml of distilled water to aid solubilization and, some time later, 8ml of Cocktail T (Hopkin and Williams) liquid scintillant containing 5% Protosol (New England). Sealed vials were left to stand for 24 hours in the dark at room temperature in order to reduce chemiluminescence. The number of counts per minute (CPM) were recorded on a Phillips PW 4540 liquid scintillation counter and converted to disintegrations per minute (DPM) on an interfaced Hewlett Packard computer (9815 A). DPMs were converted to pmole concentrations of specifically bound antagonist per tissue slice or brain region.

Microdissections of selected brain regions of tissue slices were made after the ^{3}[H] antagonist labelled tissue sections had been subjected to light microscope autoradiography. The silver grain patterns of autoradiograms were used to guide brain region dissection, carried out under a binocular microscope at x 4 magnification.

2.8 Protein estimation of chick brain tissue sections.

A proportion of brain sections to be subjected to autoradiographic proceedure, following ^{3}[H] antagonist *in vitro* labelling, were put aside in order to determine protein concentration. The total area of the tissue section or of regions to be microdissected were measured, using a Video Image Analyser (Kontron, West Germany). This involved tracing the section circumferance using a magnetic pen. The data was then fed into a Videoplan computer (64K working memory) which gave the surface area of the tissue section. The brain sections were scraped from the slide with a razor blade and placed in test tubes. Each section was homogenized using a Polytron tissue grinder in a 3ml of copper tartrate and alkaline carbonate in a ratio of 1:50 (after Lowry et al. 1951). Following homogenization, the homogenized tissue sections were left for 24 hours before completing Lowry's assay for protein.

A calibration curve of tissue section v protein concentration for each age of chick was used to estimate the protein concentration of autoradiographed tissue sections.

2.9 Nuclear emulsion coating proceedures for Light Microscope Autoradiography.

Selection of autoradiographic method depends on: 1) the required degree of silver grain image resolution, 2) the time of ^{3}[H] ligand exposure to nuclear emulsion and 3) the properties of the radiolabelled receptor antagonist. With these points in mind, two methods of light microscope autoradiography were used. The first, wet emulsion coating, gives the greatest silver grain image resolution, but was found to be unsuitable for ^{3}[H] reversibly bound receptor ligands, eg. ^{3}[H] *l* QNB. The second, apposition autoradiography, gives poorer silver grain image resolution, but does not affect the distribution of specifically bound ligand during the period of emulsion exposure.

Wet emulsion coating autoradiography.

The following proceedure was carried out in a photographic dark room in complete darkness, or, on occasions, under a 40 W red safelight masked by a Kodak series II filter.

Either Ilford L4 (fine grain emulsion) or G5 (coarser grain) nuclear emulsion, stored at 5°C for periods not in excess of 3 months, was ladelled with a plastic spoon into a Coplan jar containing 35 ml distilled water. Sufficient emulsion was added to the distilled water to raise the level to a mark indicating 50 ml of emulsion/water mix (an approximate 3:1 dilution). The Coplan jar was placed into a water bath of 50°C for 30 minutes, and the emulsion was mixed thoroughly with the distilled water using a Parafilm covered glass rod. It is important that,at all times, electrical static discharge is kept to a minimum, as this may increase background exposure. Surface air bubbles were removed by dipping several test slides into the diluted warm emulsion. At the same time, the flow and thickness of the emulsion was checked.

Slide mounted $[^3H]$ antagonist labelled brain tissue sections were slowly dipped 3/4 way into the emulsion and slowly withdrawn. Fast dipping results in pressure emulsion artifacts. Each emulsion coated slide was then suspended from a dog clip attached to a line and left to dry thoroughly at room temperature.

With each series of emulsion coating, two blank slides, without tissue sections, were coated with emulsion.One was used as a control for background exposure, the other was fully exposed to white light and used as an absolute positive and to check the properties of the photographic emulsion. All emulsion coated slides, after drying, were stored in a light tight slide box with silica gel crystals and, as an addional safeguard, wrapped in several photographic plastic bags. Exposure times varied from 10 to 130 days. In most experiments the slides were stored at room temperature.

Apposition autoradiography.

This autoradiographic method was applied to all ^{3}[H] receptor antagonist labelled tissue sections, but was used in particular to show the distribution of specifically bound ^{3}[H]-*l*-QNB.

Clean slides were precoated with either Ilford L4 or G5 nuclear emulsion, as described in the previous section. ^{3}[H] antagonist labelled slide mounted tissue sections were aligned with thoroughly dried emulsion coated slides, pressed firmly together and forced into single slots of light tight slide boxes. The time of exposure ranged from 100 to 300 days.

An alternative apposition method used LKB ultra sensitive tritium film. Sheets of this film were laid, emulsion side up, in a specially constructed light tight box. ^{3}[H] antagonist labelled slide mounted sections, section face down, were laid on top of the tritium sensitive film. This proceedure requires that the section comes in contact with the film only once, and precaution should be taken not to allow the slide to move against the film at any time during exposure. The light-tight box was filled with alternatively laid emulsion sheets and slides. Exposure times were initially calculated to be $^1/6$ of that required for Ilford L4 or G5 emulsion (see above), i.e. 3 to 40 days exposure. In fact, exposure times were extended in some instances to 100 to 150 days. (^{3}[H] α Bungarotoxin labelled sections)

2.10 Development of autoradiograms.

In a dark room, emulsion coated slides were removed from their storage slide box and placed in slide racks in groups of 25. The racked slides were placed in 300 ml Kodak D19 developer for 6 minutes at 18 - 20°C.

The developed autoradiograms were then fixed in either 300 ml 24% sodium thiosulphate or 300 ml Hypam (Ilford) rapid fixer for 15 minutes. The slide autoradiograms were then washed for several hours in cold running water before exposure to white light.

Development of LKB tritium film was in Kodak D19 developer for 2 to 4 minutes at 20°C. It was found that the manufacturer's recommended developing time resulted, on occasion, in a high background exposure level. The LKB autoradiograms were fixed in Ilford Hypam fixer for 4 minutes.

2.11 Densitometric analysis of ^{3}[H] receptor antagonist labelled autoradiograms.

Manual counting of silver grains using a specially constructed grid was considered, but rejected as an inappropriate means of silver grain quantitation in this study for the following reasons. Even over short periods of nuclear emulsion exposure (20 days) to ^{3}[H] antagonist labelled autoradiograms, silver grains were frequently grouped together which made objective counting difficult if not impossible. Secondly, such a method of analysis would have involved selective sampling which, if randomised, may have missed a particularly significant population of grains and, if not, would be open to criticism on the grounds of subjective representation. Finally, the aim of this study is to show whole brain photomicrographs of ^{3}[H] antagonist labelled cholinergic receptor which necessitated extensive exposure times, known to invalidate any direct correlation between the number of silver grains and concentration of tritium sources (see Rogers 1967). Consequently, optical density traces of ^{3}[H] muscarinic antagonist labelled autoradiograms were made, using a Joyce Loebl chromoscan system

(200/201) with an optimum optical density range of 0-3D between 405-610 nanometers. The light source was modified to give a scanning light beam of 0.14 mm in diameter, permitting 9-15 discreet scans of each brain tissue section autoradiogram at different levels across the brain with a high degree of resolution. At the start and end of each trace, a small ink spot marked the trace angle and level. The trace spots and autoradiogram were photographed and the density trace overlaid on the photomicrograph in order to assign brain regions to the peaks and troughs of the density trace.

3. Results: Light microscope autoradiographic localisation of chick brain cholinergic receptor.

3.1 Experimental design and preliminary studies.

To identify neurotransmitter receptor, one must first have an analogue or label which binds specifically to that receptor. This study may have been possible some 30 years ago, if the specific muscarinic cholinergic antagonist, Quinuclidinyl benzilate (QNB) (otherwise known as BZ) had not been placed on a military classified materials list (Edgewood Arsenal). In 1974, Yamamura and Snyder published evidence reporting on the selectivity of this muscarinic ligand, and a little time later, QNB became available commercially, radiolabelled to a high specific activity (15 Ci/mmole), (The Radiochemicals Centre, Amersham).

In the design of method for this study, a question was, whether to label chick brain cholinergic receptor *in vivo* or *in vitro*, in preparation for light microscope autoradiographic localisation. Yamamura and Snyder (1974) had shown that it was possible to label rat brain MAChR *in vivo*, using $[^{3}H]$ QNB. However, it was clear from that study that these authors experienced a number of methodological problems in proving receptor ligand binding specificity. Similar difficulties were reported by Dr. A. Rotter (PhD. Thesis 1977, NIMR, Mill Hill, London) in attempting to label, *in vivo*, rat brain MAChR, using the alternative muscarinic receptor antagonist $[^{3}H]$ Propylbenzilycholine mustard. This cholinergic receptor ligand was used at a later date in the present study. In view of these past apparent difficulties, it was decided to design an *in vitro* receptor labelling protocol.

It was recognized at an early stage in this study that the greatest technical problem was to maintain brain anatomical structure and tissue integrity (Dr. M. Stewart, pers. comm., The Open University), without altering the ligand binding properties of the cholinergic receptor(s). The choice of thin whole brain tissue sections, cut from fast frozen brains using a cryostat, seemed the most valid approach to the considered aim of this study (see Introduction).

Early *in vitro* labelling methods, such as freeze thaw mounting of brain sections on Poly-L-Lysine coated *coverslips*, incubated with muscarinic antagonist(s) in micro wells of tissue culture dishes, proved to be unsuccessful. The problem was tissue damage, resulting from excessive handling and/or poor tissue slice adhesion to the coverslip. Excessive handling was reduced by freeze thaw mounting brain sections on microscope *slides* which could be stored and incubated with the receptor ligand in slide racks. The problem of poor adhesion was diminished by precoating slides with a gelatin chrome alum hardener solution. However, even with these modifications, the number of damaged sections was still found to be unacceptably high.

This problem was eventually resolved by prefixing slide mounted tissue sections with glutaraldehyde (Dr. N.J.M. Birdsall, pers. comm. after Dr A.Rotter, PhD. Thesis 1977, both of NIMR, Mill Hill, London). The quality of tissue integrity following prefixing, receptor ligand incubation and washing, was subsequently found to be excellent. However, it was not known whether glutaraldehyde prefixing affected cholinergic receptor antagonist binding and what prefixing concentration of glutaraldehyde would maintain tissue structure without altering receptor antagonist binding character.

3.2 Effects of prefixing concentrations of glutaraldehyde on muscarinic receptor $^{3}[H]$ antagonist binding.

Figure 12 shows the effect of increasing concentrations of glutaraldehyde on $^{3}[H]$ muscarinic antagonist binding to 12 micron thick chick brain tissue slices over a glutaraldehyde concentration range of 0·01% to 1·3%. Specific maximal binding by $^{3}[H]$ QNB at a free ligand concentration of 6nM and $^{3}[H]$ PrBCM at a free ligand concentration of 3nM,was observed at a glutaraldehyde concentration of between 0.05 and 0·25%. Maximal receptor binding capacity for $^{3}[H]$ QNB at 6nM free ligand was determined to be 520 p moles/g at a prefixing gluteraldehyde concentration of 0·16%. Maximal receptor capacity for $^{3}[H]$ PrBCM binding was lower, at a free ligand concentration of 3nM (505 p moles/g) and, in addition, occured at slightly lower concentrations of glutaraldehyde, eg. 0·1%.

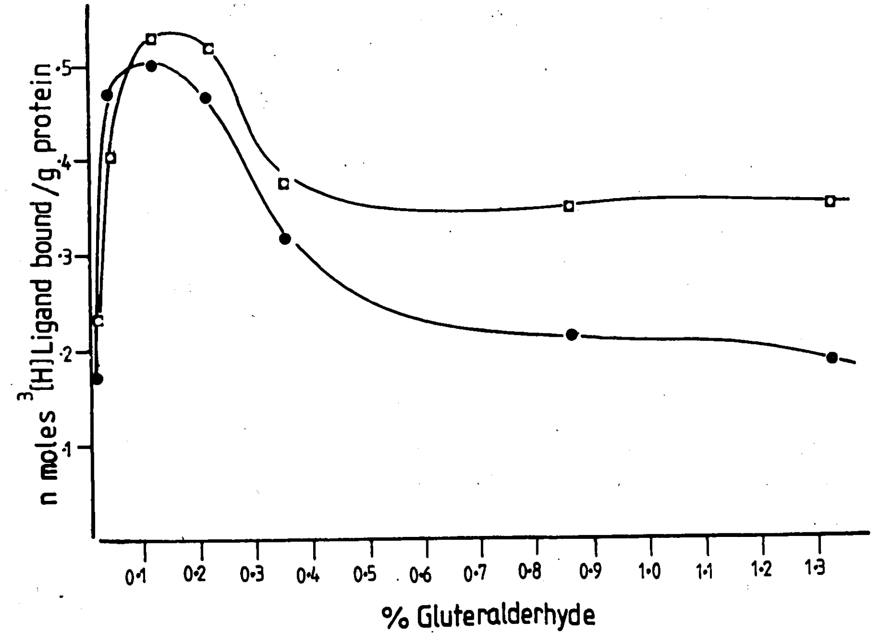
It is likely that the reduced receptor binding capacities of both muscarinic receptor antagonists between 0·0 and 0·1% glutaraldehyde prefixing concentrations reflects a loss of brain tissue and, therefore, the number of available receptor ligand binding sites during *in vitro* receptor labelling proceedure, rather than an effect of glutaraldehyde on receptor/membrane character. On the other hand tissue preservation between 0·1 and 1·3% glutaraldehyde prefixing concentrations was excellent, and therefore the observed reduced maximal binding by both antagonists over this concentration range does probably reflect some aspect of receptor/protein/membrane denaturing. It is also apparant from figure 12 that maximal binding by $^{3}[H]$ PrBCM at a free ligand concentration of 3nM is less affected by higher glutaraldehyde prefixing concentrations, than is maximal binding by $^{3}[H]$ QNB. In addition, the rate of decrease in the

Figure 12. *The concentration of specifically bound (atropine sensitive) ^{3}[H] Quinuclidinyl benzilate (^{3}[H] QNB) —□—□—□—□— and ^{3}[H] Propylbezilylcholine mustard (^{3}[H] PrBCM) —●—●—●—●— to muscarinic cholinergic receptor of chick brain tissue slices at free ligand concentrations of 1.6 ±0.2 nM and 3.0 ±0.2 nM respectively over increasing preincubating concentrations of glutaraldehyde. Chick age 5 weeks post hatch. The results are expressed as the mean of 6 determinations (ie. the concentration of bound ^{3}[H] antagonist in 6 tissue slices of 1 brain), and the standard error of the mean was found to be no greater than 4% for any data point. Binding expressed as noles ^{3}[H] ligand bound per g tissue slice protein.*

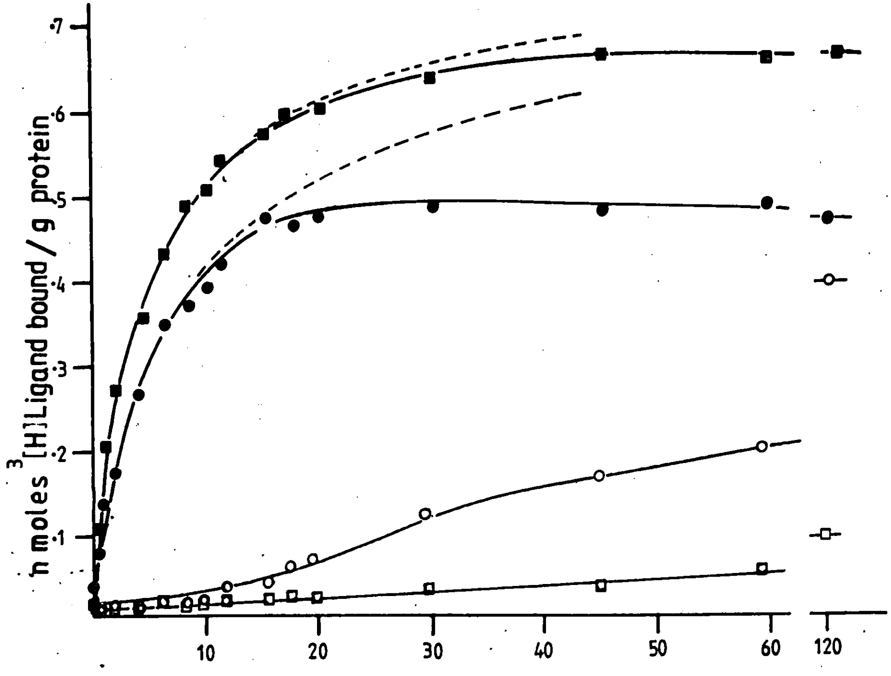
Figure 13. *The concentration of specifically bound (atropine sensitive) ^{3}[H] QNB —■—■—■— and ^{3}H PrBCM —●—●—●— , and the concentration of non specific (ie. non atropine sensitive) binding by ^{3}[H] l QNB —□—□—□— and ^{3}[H] PrBCM —○—○—○— in the presence of excess and saturating concentrations (125 nM) of atropine sulphate , to chick brain tissue slices (N= 12, ie. 12 determinants from 3 brains), at different times past the start of receotor labelling incubation. Chick age , 5 weeks post hatch. The standard error of the mean (SEM) was found to be no greater than 14% for any data point. Binding expressed as n moles ^{3}[H] ligand bound per g tissue slice protein.*

Figures 12 and 13.

12 Glutaraldehyde v $^{3}[H]$ muscarinic antagonist binding.



13 Time course of $^{3}[H]$ muscarinic antagonist binding.



number of antagonist binding sites with increasing concentrations of glutaraldehyde is less for ^{3}[H] PrBCM binding than for ^{3}[H] QNB. These slight differences of receptor binding between these two antagonists are probably related to the observation that ^{3}[H] PrBCM binds irreversibly to MAChR and most probably partitions into the receptor site membrane (N.J.M. Birdsall, pers. comm.), while ^{3}[H] *l* QNB binds reversibly to CNS MAChR. In view of the evidence shown in figure 12, a glutaraldehyde prefixing concentration of 0·125% was used for all *in vitro* labelling studies in preparation for autoradiographic receptor localisation.

3.3 Muscarinic cholinergic ^{3}[H] antagonist binding to chick brain tissue slice receptor.

There is a substantial body of evidence demonstrating that the antagonists ^{3}[H] PrBCM and ^{3}[H] *l* QNB bind specifically to muscarinic cholinergic receptor of brain homogenate and neuronal membrane enriched preparations (see Introduction). However, a number of studies have reported that these two muscarinic ligands bind in different ways to MAChR. ^{3}[H] PrBCM apparently irreversibly alkylates muscarinic receptor (Hulme et al. 1975), while ^{3}[H] *l* QNB binds reversibly (Yamamura and Snyder, 1974). Specific binding by ^{3}[H] PrBCM and ^{3}[H] *l* QNB has not been previously demonstrated for chick brain tissue slice preparations, nor has the receptor binding character of these antagonists been compared under identical conditions of brain tissue preparation, species of animal and receptor labelling proceedure. Therefore, the time course of binding, maximal receptor capacity, equilibrium dissociation constant, and competitive antagonist/agonist inhibition of muscarinic receptor ^{3}[H] ligand binding were determined prior to *in vitro* receptor labelling for autoradiographic localisation of chick brain muscarinic receptor.

3.4 Time course of muscarinic ^{3}[H] antagonist binding.

Figure 13 shows the time course of specific and non specific binding by the muscarinic antagonists ^{3}[H] PrBCM and ^{3}[H] *l* QNB to 12 micron thick sections of the adult chick brain at free ligand concentrations of $3 \cdot 18 \cdot 10^{-9}$M and $1 \cdot 6 \cdot 10^{-9}$M respectively. Specific muscarinic ^{3}[H] antagonist binding was determined by subtracting from total ^{3}[H] antagonist binding values (recorded in the absence of competing 'cold' antagonist, atropine sulphate) those ^{3}[H] antagonist binding values recorded in the presence of excess and saturating concentrations of atropine.

Specific binding by ^{3}[H] PrBCM and ^{3}[H] *l* QNB rose steeply until 20 minutes after the start of incubation, from which time the rate of increase in antagonist binding decreased to a plateau to give maximal recorded receptor bound antagonist concentrations of 630 pmoles/g protein for ^{3}[H] *l* QNB and 485 pmoles/g protein for ^{3}[H] PrBCM. In contrast to specific binding, non specific binding by ^{3}[H] *l* QNB increased slowly and proportionately with time and did not saturate up to 120 minutes after the start of incubation. Non specific binding by ^{3}[H] PrBCM increased slowly and linearly with time until 12 minutes after the start of incubation, from which time the rate of non specific binding increased substantially. From Table 4 it may be seen that the percentage of non specific to specific binding sites by ^{3}[H] PrBCM increases from 8·3% at 15 minutes to 83·3% at 120 minutes after the start of incubation. On the other hand, the ratio of non specific to specific binding by ^{3}[H] *l* QNB increases from 2·9% at 15 minutes to 14·9% at 120 minutes.

Table 4. *Concentration of specifically and non specifically bound $^{3}[H]$ PrBCM and $^{3}[H]$ 1 QNB to chick brain tissue slices at vario times post start of incubation. Results are expressed as the mean of 12 determinations. The standard error of each mean value is given.*

Table 5. *Regional concentration of $^{3}[H]$ PrBCM and $^{3}[H]$ α BTX binding site Results are expressed as the mean of 6 determinations. The standard error of each mean is given.*

TABLE 4 Time course of binding of varying concentrations of ^{3}[H] PrBCM & ^{3}H 1 QNB to chick brain tissue slices.

Time Minutes	^{3}H ligand	total binding	Non specific binding (NS)	Specific binding (S)	%NS/S
0	PrBCM	0.010±0.00	0.008±0.00	0.002±0.00	-
	QNB	0.006±0.00	0.002±0.00	0.004±0.00	-
15	PrBCM	0.480±0.06	0.037±0.00	0.443±0.07	8.3
	QNB	0.562±0.05	0.016±0.00	0.547±0.04	2.9
30	PrBCM	0.613±0.08	0.124±0.02	0.489±0.07	25.3
	QNB	0.660±0.05	0.025±0.00	0.633±0.09	3.9
60	PrBCM	0.692±0.10	0.210±0.02	0.491±0.08	42.7
	QNB	0.710±0.03	0.046±0.03	0.650±0.08	8.0
120	PrBCM	0.855±0.09	0.400±0.05	0.480±0.06	83.3
	QNB	0.764±0.08	0.098±0.00	0.654±0.08	14.9

TABLE 5 Regional concentrations of ^{3}[H] ligand labelled muscarinic and nicotinic cholinergic receptor in chick brain.

Brain Area	mAChR ^{3}[H] ligand PrBCM (Chicks 4 weeks PH)		α BTX nAChR ^{3}[H] ligand α BTX (chicks 2 weeks PH)	M/N
Whole brain	364±32 (6)		26±2 (6)	14
Forebrain	339±75 (6)		15±2 (6)	22
Optic Lobe	398±137 (6)		40±3 (6)	10
Optic tectum	859±284 (6)		92±18 (6)	9
Midbrain	310±86 (6)	Ant	160±25 (6)	2*
		post	35±15	9
Hindbrain	205±96 (6)		22±01	9
Cerebellum	139±55 (6)		19±3	7

The reason for the rate increase in non specific binding by ^{3}[H] PrBCM to chick brain tissue section, 10 minutes after the start of incubation, is not clear. It is possible that from this time point specifically bound atropine begins to dissociate from the cholinergic receptor site leaving the vacated receptor open to irreversible alkylation by ^{3}[H] PrBCM. (but see discussion). In any case, it was apparent that 75% of receptor sites bound by atropine at 10 minutes after the start of incubation had, by 120 minutes, been irreversibly alkylated by ^{3}[H] PrBCM.

In order to reduce the apparent non specific to specific ^{3}H PrBCM muscarinic receptor binding ratio, the time of incubation of chick brain tissue slices was kept to 15 minutes.

3.5 Equilibrium binding of muscarinic receptor ^{3}[H] antagonists.

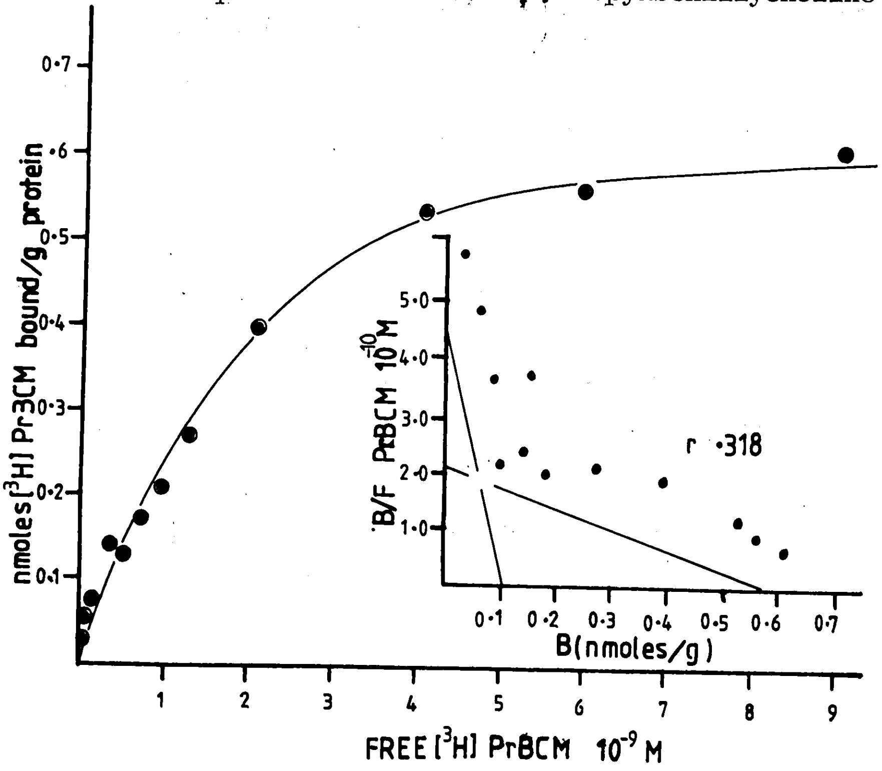
Equilibrium binding studies were carried out at 25°C. The concentration dependence for specific binding of ^{3}[H] PrBCM and ^{3}[H] *l* QNB to 12 m thick sections of the adult chick brain over a ligand concentration range of 5.5^{-10} M to 9.10^{-9} M is shown in figures 14 and 15. Specific binding by both ^{3}[H] antagonists increased rapidly until 1nM concentration of free ligand from which time the rate of binding decreased to plateau between 4.10^{-9} M and 6.10^{-9} free ^{3}[H] ligand, giving a typical saturation curve. Maximal receptor binding capacity by ^{3}[H] *l* QNB was found to be 695 pmoles/g protein and for ^{3}[H] PrBCM 585 pmoles/g protein. The reduced maximal receptor binding capacity by ^{3}[H] PrBCM compared to ^{3}[H] *l* QNB may be accounted for by reduced incubation times and higher non specific binding values. Half maximal saturation values for ^{3}[H] PrBCM and ^{3}[H] *l* QNB were determined to be $1.3.10^{-9}$M and $1.1.10^{-9}$M respectively.

Figure 14. *Specific binding of ^{3}[H] PrBCM at 25 ^{o}C to chick brain tissue slices, incubated with varying concentrations of free ^{3}[H] ligand for 15 minutes in Krebs-Henseleit Ringer (pH 7.4). Inset: a Scatchard plot of equilibrium binding data. The results are expressed as the mean of 24 determinations (ie. 6 determinants from 4 brains). The SEM for each data point was found not to exceed 5% . The lines to the data points of the Scatchard plot are fitted by eye. The regression coefficient r = 0.318. Binding is expressed as n moles ^{3}[H] PrBCM bound per g brain tissue slice protein and in the Scatchard plot as specifically bound (B) over free (F) ^{3}[H] ligand.*

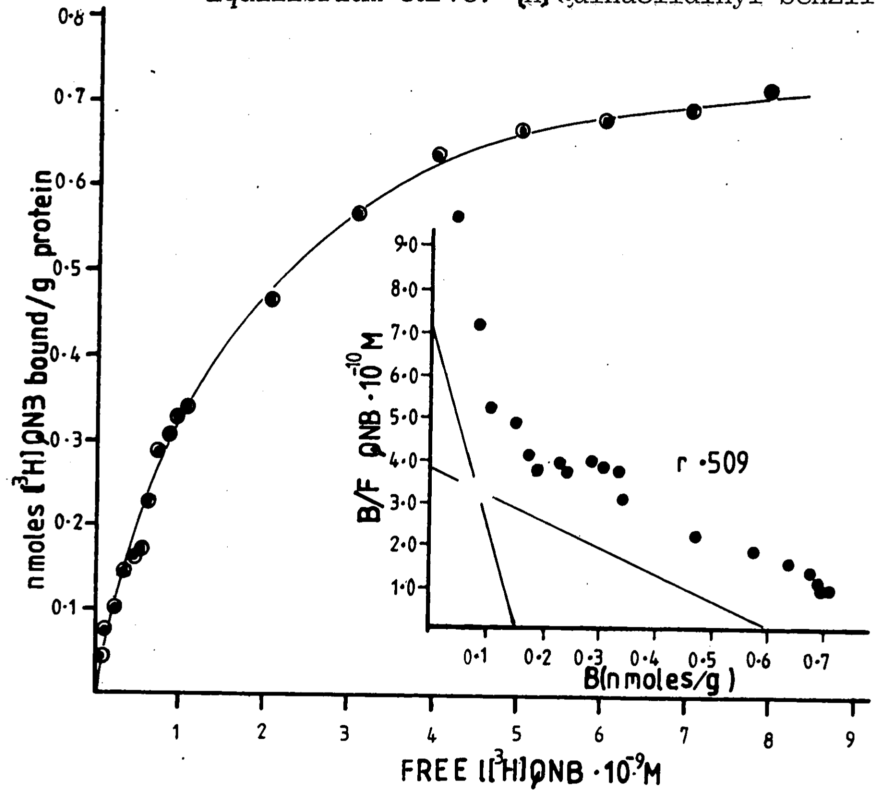
Figure 15. *Specific binding of ^{3}[H] l QNB at 25 ^{o}C to chick brain tissue slices, incubated with varying concentrations of free ^{3}[H] ligand for 30 minutes in Na-K phosphate buffer (pH 7.4). Inset: a Scatchard plot of equilibrium binding data. The results are expressed as the mean of 24 determinations, and the SEM was found not to exceed 12 % for each data point .The lines to the data points of the Scatchard plot are fitted by eye. The regression coefficient r = 0.509.*

Figures 14 and 15.

14 Equilibrium curve: $[^3H]$ Propylbenzilycholine mustard.



15 Equilibrium curve: $[^3H]$ Quinuclidinyl benzilate.



The data of figures 15, 16 , when replotted according to Scatchard (see figures 15a, 16a), can be seen to deviate clearly from linearity. Regression analysis gave a sample correlation coefficient of 0•509 for ^{3}[H] *l* QNB binding and 0•318 for ^{3}[H] PrBCM.These data therefore, suggest that both muscarinic receptor ligand antagonists are possibly not binding to a single population of equivalent non interacting binding sites, but, alternatively, muscarinic antagonist binding could be accomodated by a two-non-interacting binding site model, characterised by a high affinity site K_H (0•64±0•14)•10^{-9}M and low affinity site K_L (4•70±0•20)•10^{-9}M for ^{3}[H] *l* QNB and K_H of (0•37 ± 0•08)•10^{-9}M and K_L (4•0 ± 0•36)•10^{-9}M for ^{3}[H] PrBCM (see Discussion). The apparent affinity, determined at the point of half maximal saturation, is (1•1 ± 0•06)•10^{-9}M and (1•3 ± 0•08) •10^{-9}M for ^{3}[H] *l* QNB and ^{3}[H] PrBCM respectively. The equivalence of maximum receptor binding capacity between ^{3}[H] *l* QNB and ^{3}[H] PrBCM taken together with the equivalence of receptor affinity parameters confirms that ^{3}[H] *l* QNB and ^{3}[H] PrBCM are probably competing for the same receptor binding sites, and, considering the sensitivity of ^{3}[H] antagonist binding to atropine sulphate competition, these receptor then are probably the muscarinic acetylcholine receptor (see Discussion).

3.6 Competitive inhibition of ^{3}H *l* QNB binding to chick brain muscarinic receptor.

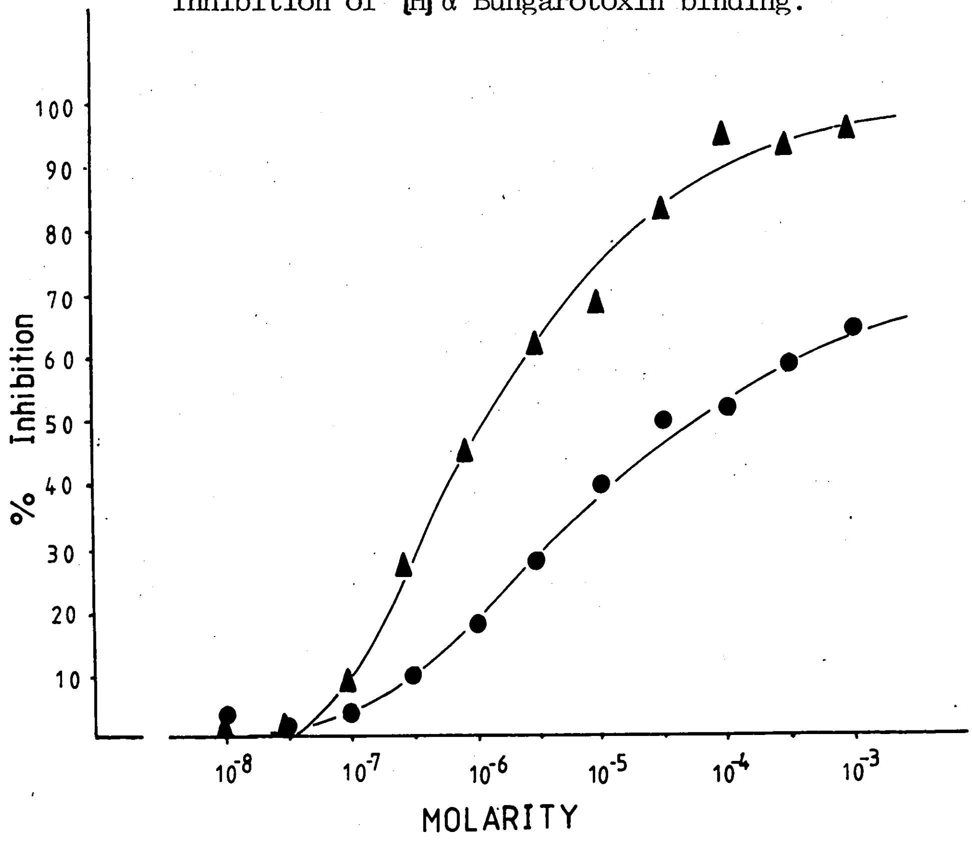
Figure 17 shows the % inhibition of ^{3}[H] *l* QNB binding at an incubating concentration of 1•6•10^{-9}M and N-methyl scopolamine (NMS) together with the nicotinic receptor antagonist D-tubocarine. The inhibition curve (or, alternatively, competition curve) for atropine is steep and is complete within 1•5 orders of magnitude, exhibiting an ID_{50} (point of 50% inhibition

Figure 16. *Competitive inhibition of ^{3}[H] α Bungarotoxin binding (8 nM free ligand concentration) at 25 °C for 2 hours in Na-K phosphate buffer (pH 7.4) containing 1 mg ml BSA, to chick brain tissue slice receptor by varying concentrations of d-tubocurarine* —▲—▲—▲—▲— *and atropine sulphate* —●—●—●— *. Each data point is the mean of 6 determinations from 2 brains. The results are expressed as a percentage of the concentration of ^{3}[H] α toxin binding in the absence of competing antagonist. The SEM was found to be no greater than 12% for each data point.*

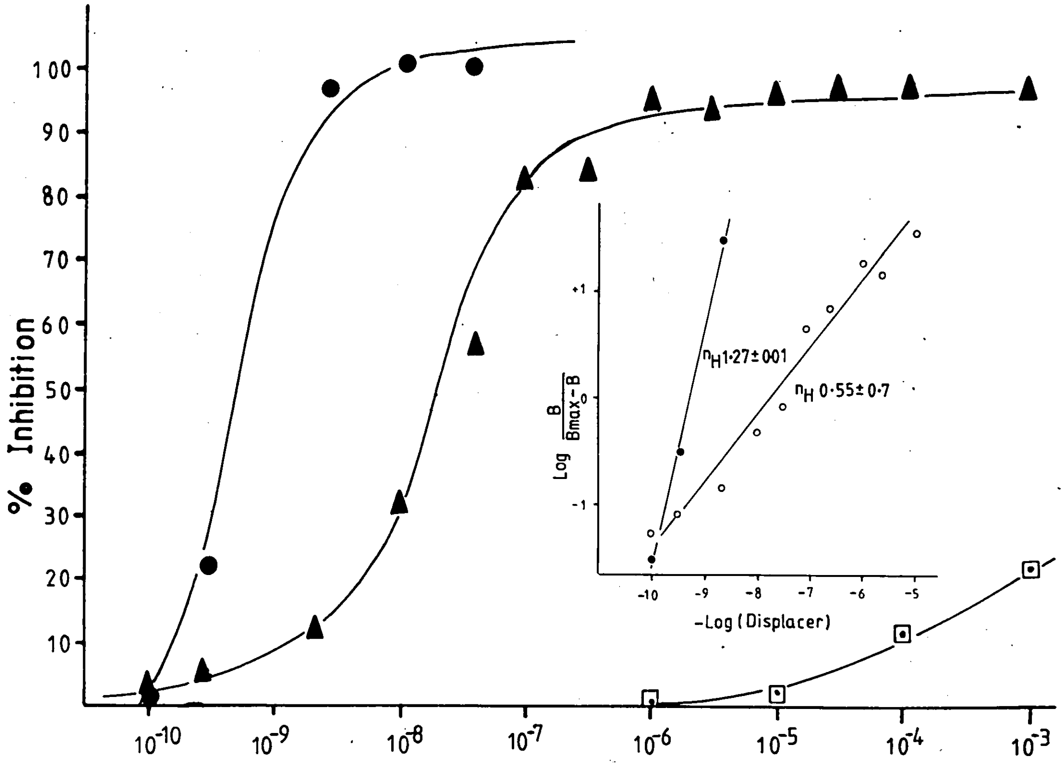
Figure 17. *Competitive inhibition of ^{3}[H] l QNB binding (1.6 nM free ^{3}[H] ligand concentration) at 25 °C for 30 minutes in Na-K phosphate buffer (pH 7.4) to chick brain tissue slice receptor by varying concentrations on N-methy scopolamine (NMS)* —▲—▲—▲—▲— *, atropine sulphate (Atr.)* —●—●—●—●— *and d-tubocurarine* —⊡—⊡—⊡— *. Each data point repesents the mean of 6 determinations from 2 brains. The results are expressed as a percentage of the concentration of ^{3}[H] l QNB binding in the absence of competing antagonist. Inset: Hill plot of the competitive inhibition data of figure 17. The Hill coefficient N_h was found to be 1.27±0.01 and 0.55±0.7 for atropine sulphate and N-methyl scopolamine respectively. The SEM was found to be no greater than 6% for each data point.*

Figures 16 and 17.

16 Inhibition of ^{3}[H] α Bungarotoxin binding.



17 Inhibition of ^{3}[H] *l* Quinuclidinyl benzilate binding.



of $[^3H]$ *l* QNB binding) of $6 \cdot 10^{-10}$ M. The inhibition curve for NMS on the other hand, occurs over a wider range of concentrations and is a little more flattened than that shown by atropine. The ID_{50} for NMS in competition with $[^3H]$ *l* QNB binding is $3 \cdot 10^{-8}$M. The maximum percentage inhibition of total $[^3H]$ *l* QNB binding was 99% and 97% for atropine and NMS respectively, on the other hand, even at concentrations that might be expected to saturate MAChR $1 \cdot 10^{-3}$M, D-tubocarine displaced only 19% of $[^3H]$ *l* QNB binding sites.

When the data of figure 17 are replotted according to Hill (see figure 17a) the calculated Hill coefficients for atropine and NMS were determined to be 1·27 and 0·55 respectively. The discrepancy between these two values is difficult to explain. The Hill value for NMS differs sufficiently from 1 to suggest, as elsewhere in this report, that muscarinic antagonist binding distinguishes more than one binding site (see discussion).

3.7 Protein calibration of chick brain tissue slices.

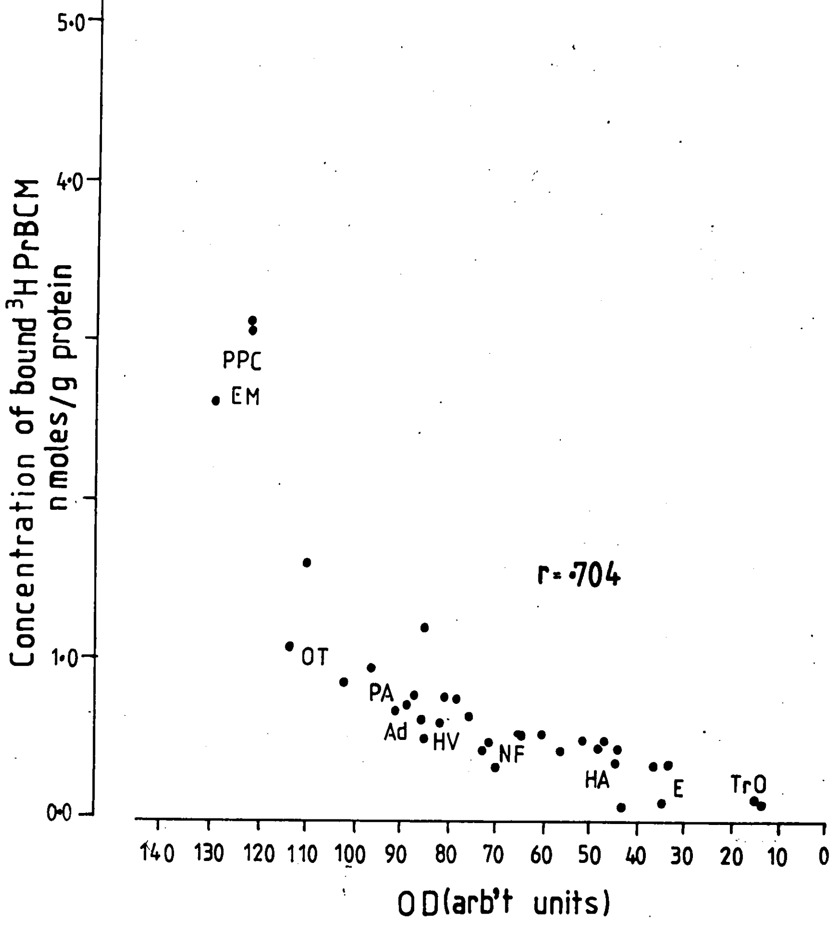
In order to determine the protein content of adult chick brain (4 weeks post hatch) tissue slices of light microscope autoradiograms and tissue sections used for $[^3H]$ MAChR ligand binding kinetic studies, a protein section area calibration curve was constructed (see figure 19). Chick brain section areas of a known and constant section thickness (20 pm) were measured with the aid of a Videoplan Image Analyser (Kontron, West Germany). The brain tissue slice circumferance was traced using a light pen; and the data was fed to the Videoplan computer which calculated the surface area.

Figure 18. *Concentration of specifically bound ^{3}[H] PrBCM to microdissected regions of autoradiographed 5 week post hatch chick brain tissue sections versus the optical density (OD) of silver grains overlying those same regions of the receptor labelled autoradiographed tissue section. Each data point is the mean of a variable number of determinations (from 3 to 12) from 1 brain, the SEM was no found to be no greater than 18%for each data point.*

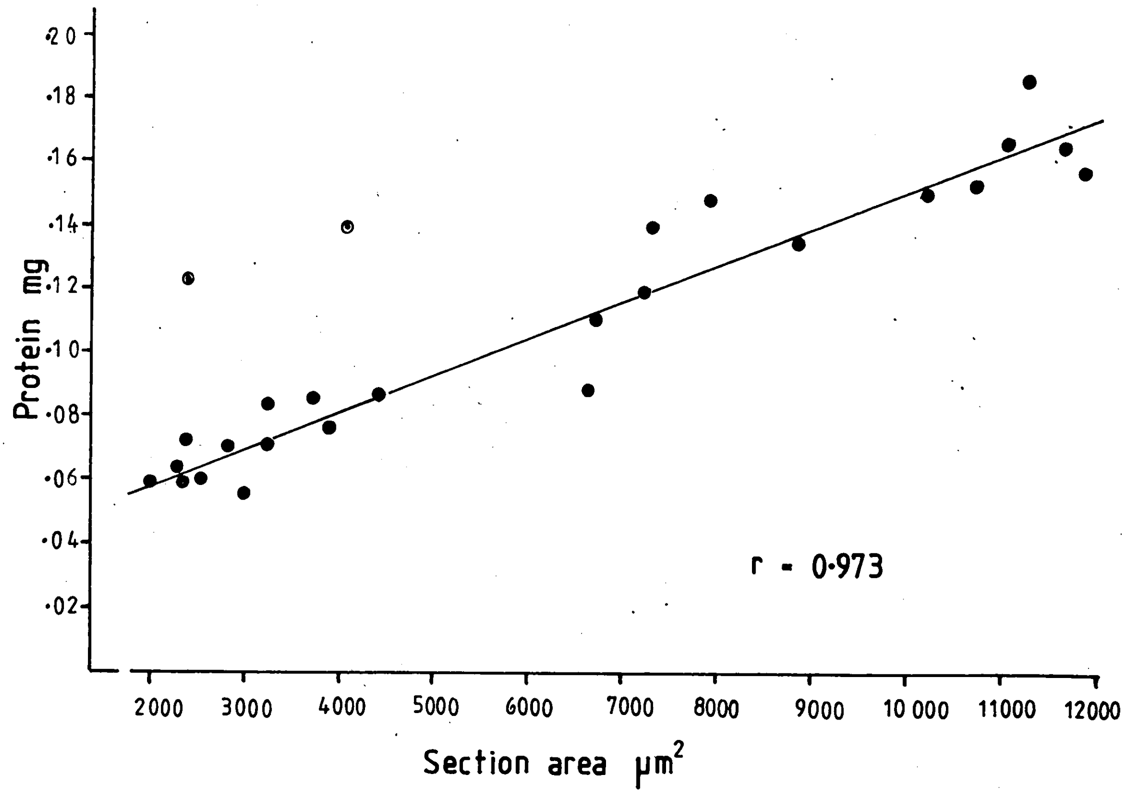
Figure 19. *Protein concentration (determined after Lowry et. al., 1951) versus the section area of chick brain tissue slices. Section ares were measured using a Video Image Analyser (Kontron, West Germany). Each data point is 1 determination. The results are expressed as mg protein versus μm^2 of the regional area dissected . The sample correlation coefficient $r = 0.973$.*

Figures 18 and 19.

18 Regional concentration of bound ^{3}[H] ligand v optical density of autoradiogram silver grain density.



19 Calibration: Protein v tissue slice section area.



The sections were then assayed for protein content (see methods) and the data points plotted against section area. The sample correlation coefficient was calculated to be 0•973, indicating a near perfect linear relationship between the sample values, protein and tissue area. Estimates of protein content of micro-dissected brain regions of ^{3}[H] PrBCM labelled chick brain autoradiograms, shown below, were calculated from this calibration curve by measuring the area of the brain region to be dissected and reading protein content from the calibration curve.

3.8 Calibration of tissue slice area and concentration of bound ^{3}[H] antagonist.

Figure 19 shows the binding of ^{3}[H] PrBCM at a free ligand concentration of 2•5nM to cryostat cut tissue sections of chick brain as a function of section thickness at a sectioning temperature (closed circles) of -20°C. These data demonstrate that ^{3}[H] PrBCM binding (DPM) is proportional to 2µm increments in tissue section thickness, assuming that the area of the tissue section remains constant . Furthermore, the data of figure 19 demonstrate that ^{3}[H] PrBCM gains access to MAChR below the surface of the tissue section and is not simply binding to receptor sites on the surface. The proportional increase in ^{3}[H] PrBCM binding shows that the increments suggested by the cryostat thickness gauge are linear, although it is not possible to say whether each section is precisely of the thickness indicated.

The variation (S.E.M.) in tissue slice section thickness was found not to exceed 11.4%, as given by the concentration of bound ^{3}[H] ligand. It may be seen that a number of open circle data points showing DPM values of far greater variance, are included in figure 19 for a given tissue section thickness, cut immediately after warm air had been blown onto the surface of the brain, and after the brain had been sprayed with coolant. These data show that section thickness is temperature sensitive and suggest a possible cause for any variance in receptor ligand binding values of later studies. For kinetic analysis each section was cut at a precise time interval to allow warm air moving into the body of the cryostat at the time of removing the previous section to dissipate and for the temperature of the cryostat and brain to equalise.

3.9 Quantitation of ^{3}[H] antagonist labelled autoradiograms and comparison of optical density versus direct count measurements.

Figures 21 and 22 show optical density traces recorded from several different levels in a rostrocaudal plane of an ^{3}[H] PrBCM labelled parasagittal autoradiogram tissue section of lateral aspects of a 4 week post hatch chick brain. The traces show that there is a marked difference in regional silver grain density across the brain. The highest specific optical density recorded over the mesencephalic lentiform nucleus (LM) is 28.4 times greater than the density of non specific silver grains. The lowest specific to non specific ratio, apart from that recorded over fibre tracts, is that shown here for the ectostriatal core of the chick forebrain, where specific silver grain densities are just 2.3 times that of non specific values.

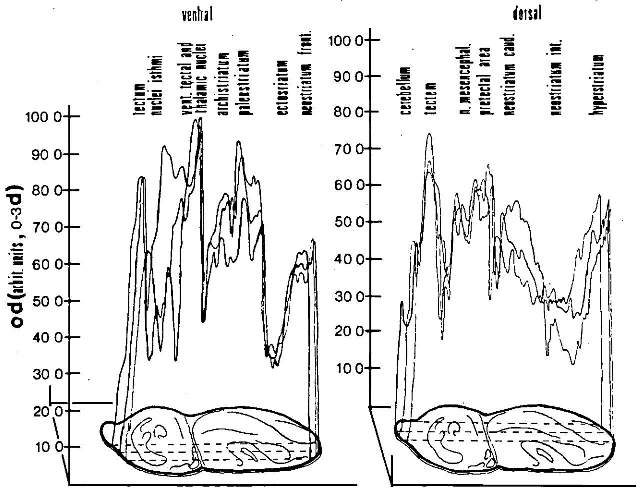
Figure 20 shows aligned and composite density traces from a parasagittal autoradiogram of a 48 hour post hatch chick brain labelled by ^{3}[H] PrBCM.

Figure 20. *A series of overlaid optical density traces of regional silver grain densities of an $^3[H]$ PrBCM labelled autoradiographed tissue section of a 48 hour post hatch chick brain. The traces on the left of the figure are from ventral aspects of the parasagittal brain section (cut at approx. the stereotaxic coordinate Lateral 4.00), and on the right dorsal aspects.*

Figure 21 and 22. *A series of* single *optical density traces of regional silver grain densities of 2 autoradiographed tissue sections of the 4 week post hatch chick brain. Optical density units are arbitrary (cm's). Along the abscissa are given stereotaxic section area coordinates. The traces taken from a tissue section lateral to that represented by the optical density traces of figure 22.*

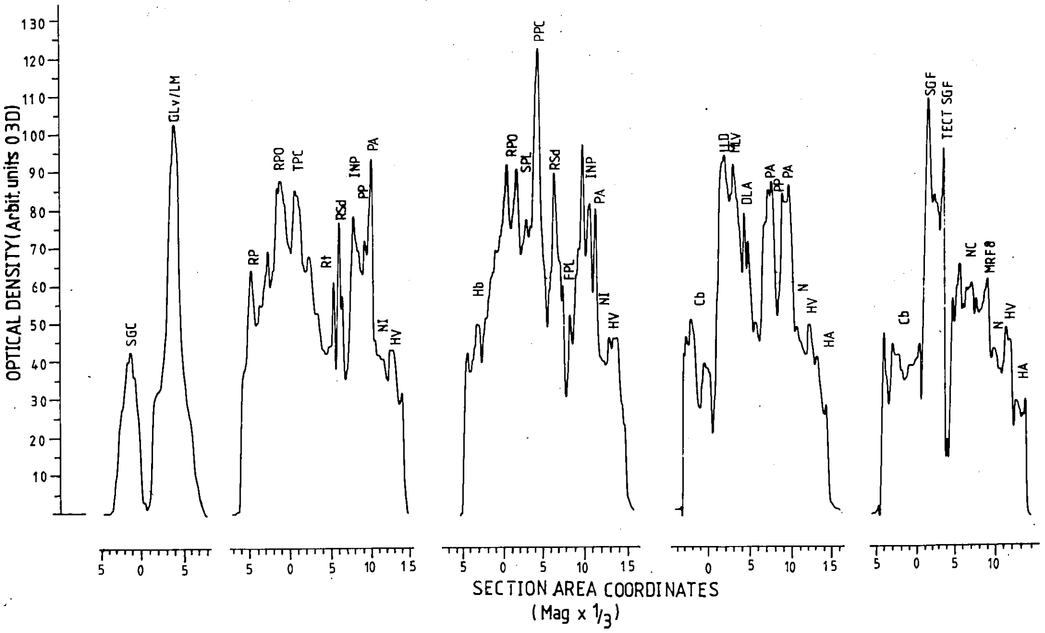
Figures 20 to 22.

20

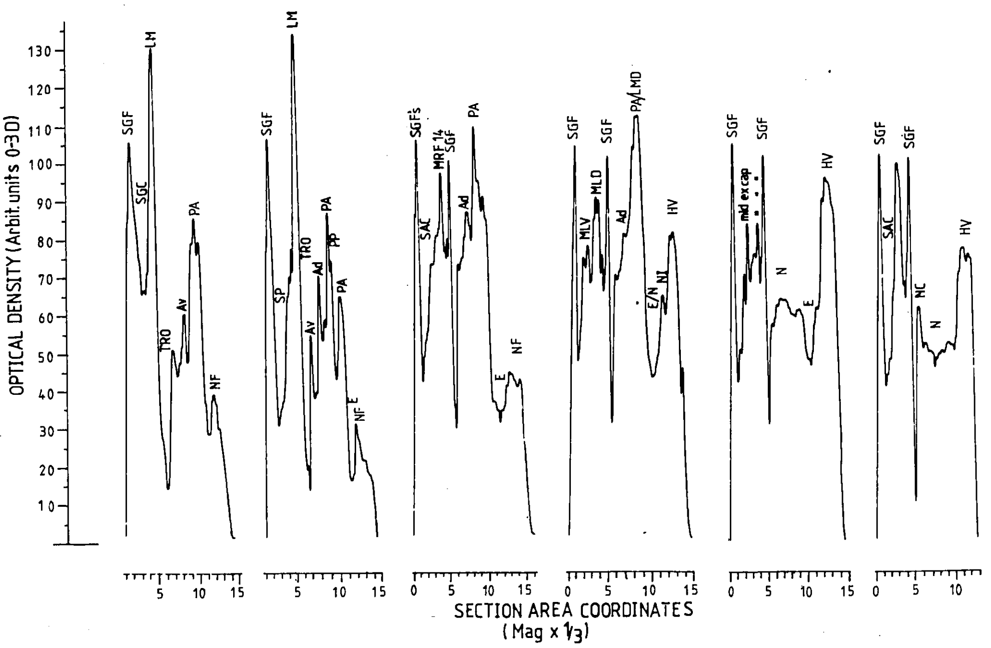


21

Optical density traces of ^{3}[H] ligand labelled autoradiograms



22



The difference in arbitrary density units between the optical density traces of the 48 hour old chick brain and those of the 4 week post hatch brain does not necessarily reflect any 'real' difference in ^{3}[H] antagonist binding, but a difference in exposure times to the overlying nuclear photographic emulsion.

In addition to photomicrographic and densitometric analysis of regional MAChR concentration, autoradiographic tissue sections, labelled by ^{3}[H] PrBCM for the adult chick, were microdissected into specific brain regions and the concentration of bound ^{3}[H] ligand determined by scintillation counting (see methods).

Figure 18 shows the concentration of specifically bound ^{3}[H] PrBCM (p moles/ mg brain protein) plotted against optical densities for the same brain regions. The two methods of quantitation show a very good correlation with a coefficient r of 0.704. It is only over regions showing the highest concentrations of bound ^{3}[H] ligand, eg. GLV and PPC, that the correlation between density and direct counts of specifically bound ^{3}[H] PrBCM deviates substantially from linearity. It is possible that such deviations are the result of technical limitations in sensitivity of the densitometer.

3.10 Direct count quantitation of ^{3}[H] PrBCM and ^{3}[H] α BTX binding in select areas of the chick brain.

The figure photomicrographs of this study, showing autoradiographic localisation of ^{3}[H] antagonist labelled cholinergic receptor, suggest that there are marked differences in the concentrations of MAChR between the major subdivisions of the chick brain, ie. telencephalon, diencephalon, mesencephalon etc. However, the data of Table 4 show that the concentration of ^{3}[H] PrBCM binding sites at all of these major subdivisions is approximately equivalent. Only the cerebellum and to a lesser extent the medulla oblongata show below average concentrations of muscarinic receptor. The ratios of ^{3}H PrBCM binding concentrations for the chick forebrain, optic lobe, midbrain, hindbrain and cerebellum compared to whole brain values were calculated to be 1.07, 0.91, 1.17, 1.77 and 2.6 respectively.

However, the concentration of specifically bound muscarinic ligand within these major subdivisions was found to vary markedly. For example, the ratio of ^{3}[H] PrBCM concentrations in the hyperstriatum ventrale (HV) and paleostriatum augmentatum (PA) of the chick forebrain with respect to binding in the ectostriatum were found to be 6.5 and 8.6 respectively. In the midbrain variation between regions was even greater; the ratio of binding between the geniculate (GLv) and optic tract (TrO) for example was calculated to be 62.7.

In contrast, the concentration of ^{3}[H] α BTX binding sites between the forebrain, optic lobe and midbrain was found to vary substantially; the highest concentrations recorded from the optic lobe (40±13 pmoles/g) and anterior midbrain (160±25 pmoles/g), and lowest in the forebrain (15±2 pmoles/g) and cerebellum (19±3 pmoles/g). The concentration of ^{3}[H] α BTX binding sites throughout the chick brain was found to be at least an order of magnitude lower than that observed for ^{3}[H] PrBCM binding concentrations (see Table 4). However, it must be borne in mind that ^{3}[H] α BTX binding was determined from the brains of chicks aged two weeks post hatch, while the data for ^{3}[H] PrBCM binding is taken from 4 weeks post hatch chick brains.

3.11 Antagonist binding properties and autoradiographic procedure..

Two autoradiographic procedures have been used in the 'visualization' of ^{3}[H] receptor ligand distribution in this report; wet emulsion coating and apposition autoradiography (see Methods and Rogers 1967 for review).

Preliminary studies established that chick brain tissue sections labelled for MAChR *in vitro* by ^{3}[H] *l* QNB could not be wet emulsion coated (WEC) or subjected to post labelling fixing schedules. Following development of WEC - ^{3}[H] QNB labelled autoradiogram chick brain tissue sections, it was found that ^{3}[H] *l* QNB dissociated from the receptor binding site and diffused through the emulsion to accumulate in high concentrations around the circumference of the tissue section ('halo' artifact). Silver grains within the photographic emulsion over the tissue section itself were uniformly and lightly distributed across the whole section.

In order to try and prevent this diffusion artifact, tissue sections were postfixed in Carnoy's fluid following *in vitro* receptor labelling by ^{3}[H] *l* QNB. On development of autoradiograms there was a complete absence of silver grains. However, concentrations of specifically bound ^{3}[H] *l* QNB to chick brain slices prior to fixing in Carnoy's were found to be high, and it was only after fixing that these concentrations of bound ^{3}[H] *l* QNB were lost from the tissue section with a concomitant increase in the number of counts in the washing buffer. ^{3}[H] *l* QNB is a reversibly binding antagonist, and Carnoys fixing probably causes considerable conformational changes in the synaptic membrane proteins, in any case sufficient to cause dissociation of ^{3}[H] *l* QNB from the receptor. Taking the above factors into consideration, ^{3}[H] *l* QNB distribution was eventually demonstrated free of diffusion or dissociation artifacts by apposition autoradiography which does not require post fixing or coating of the tissue section with wet, warm emulsion.

Apposition autoradiographic methods, particularly when using ultra sensitive tritium film, resulted in silver grain patterns of specifically bound ^{3}[H] *l* QNB to chick brain tissue sections which were of exceptionally high contrast and consequently easily photographed (see figures 39, 41 to 70). However, the form and density of silver grains were found not to accurately reflect the known concentration differences in specifically bound ^{3}[H] ligand between brain regions following optical density recordings, and although the pattern of silver grain distribution gave a very clear idea of the regions most dense in MAChR, the resolution of the silver grain image was poor. The reason for this lies in the properties of the photographic emulsion. Exposure of LKB tritium film to β particle emissions results in a'stellar like system' of silver grains which cover an area well in excess of the silver grains in Ilford G5 and L4 nuclear emulsions following tritium exposure.

The procedural problems encountered in the visualization of ^{3}[H] *l* QNB binding in the chick brain were not encountered in the irreversibly bound antagonist ^{3}[H] PrBCM. Tissue sections labelled by ^{3}[H] PrBCM could be both post fixed in Carnoy's and wet emulsion coated. The developed autoradiograms showed silver grain patterns of exceptionally high resolution and markedly different densities between brain regions (see figure 29). On the other hand, there was some evidence for silver grain image artifacts.

3.12 Artifacts of autoradiographic proceedure.

Obviously, artifactual silver grain patterns must be recognised before any meaningful interpretation of autoradiograms can be made in terms of describing receptor distribution and density.

A number of artifacts resulting from wet coating autoradiographic proceedure have been observed in ^{3}[H] PrBCM and ^{3}[H] α BTX labelled tissue sections. With respect to ^{3}[H] PrBCM binding, wet emulsion coating resulted in pressure and diffusion artifacts. Pressure artifacts are easily recognised as streams of silver grains extending away from the tissue sections. The properties of the nuclear emulsion (L4 and G5) are such that tension and torsion of the emulsion, as it flows along the surface edge of the tissue section, results in silver grain patterns. Diffusion artifacts are not always so easily recognised.

In this report a very good example of a diffusion artifact is apparent over dorsal anterior regions of parasaggital sections of the chick forebrain, labelled by ^{3}[H] PrBCM and wet emulsion coated. There is a loss of specifically bound ^{3}[H] PrBCM, particularly over the hyperstriatum accessorium (HA) which, if it had not been for the comparative results of apposition autoradiography,would have been interpreted as a region devoid of muscarinic receptor (see figure 24). In point of fact as consequence of the viscosity or temperature of the photographic emulsion as it flowed over the tissue section, during wet emulsion coating, particularly over regions most exposed to the full flow of the emulsion, specifically bound molecules of ^{3}[H] PrBCM probably dissociated and diffused away from their original (specific) receptor binding sites.

I, at one time, interpreted another ligand dissociation artifact as a specific pattern of MAChR arranged as columns of receptor over dorsal aspects of the forebrain roof (see figure 24). I now know that such an interpretation was totally erroneous, but this example illustrates the importance of confirming an autoradiographic observation by an alternative methodology. This particular artifact was patterned because of differential drying of the tissue section from most dorsal aspects first, during freeze thaw mounting of the section (see methods), since it was always dorsal aspects of both parasagittal and frontal sections which first came in contact with the glass slide. Compare for instance the pattern of silver grain shown in figures 24A,B of an earlier autoradiogram series of a 48 hour old chick brain with autoradiograms of a later date, showing ^{3}[H] PrBCM distribution in the adult chick brain (see figures 32-34)

3.13 Exposure periods for light microscope autoradiography.

All the photomicrographs of this report showing ^{3}[H] PrBCM and ^{3}[H] α BTX binding to chick brain tissue slices are taken from autoradiograms, the photographic emulsion of which has been exposed to tritium β particle emissions for periods up to 105 days . For light microscope autoradiography such extensive exposure times are unusual (see Rogers 1967). The reason was to blacken the overlying photographic emulsion sufficiently heavily to permit light field photomicrographs of silver grain patterns and density of high resolution and strong contrast. Quite surprisingly, there was no loss of resolution over regions of high silver grain density, and, in addition, optical density measurements suggest no differences in the ratio of high to low silver grain densities between autoradiograms exposed for 30 days and those exposed for 100 days.

3.14 Muscarinic receptor distribution in the post hatch chick brain.

Chick brain anatomy and morphology is, in general, well characterised (see Introduction, section 1.6). However, during the course of this study, it became equally apparent that established anatomical description for certain regions of the chick brain, for example the hyperstriatum, basal ventral forebrain wall , medial and ventral midbrain and medulla, remain poorly defined. During the following description of muscarinic receptor distribution, it will be shown that in most instances the localisation of receptor fields conforms with established anatomical and morphological description of the chick brain. However, in some cases receptor field localisation and morphological division do not match. It could be the case that, in some instances, established anatomical description may be incorrect, and that muscarinic receptor distribution may reveal anatomical divisions and boundaries hitherto unrecognised by past studies employing more established histochemical

Figures 23 and 24. A dark field photomicrograph (A) and projected drawing (B) of the pattern and density of silver grains of an autoradiographed parasagittal section (≡ L 4·75 (23) and L 3·25 (24) of the 24 hou post hatch chick brain in vitro receptor labelled by ^{3}[PrBCM. The abscissa and ordinate represent approximate stereotaxic coordinates. Scale bar = 1mm It should be noted that these photomicrographs of autoradiogram sections have not been counter stained, the image represents the distribution of silve grains only which, in turn, represent the pattern and distribution of ^{3}[H] PrBC labelled muscarinic receptor. The lack of silver grains over dorsal aspects of the telencephalon and optic lobe is very probably an artifact of sectioning a freeze thaw mounting procedure (see text).

Figure 25. A light field photomicrograph (A) and projected drawing (B) of the pattern and density of silver grains of an autoradiographed frontal section across the caudal forebrain and anterior midbrain (≡ Anterior (A 6·75). Note that the ventral surface of the brain is compacted, resulting from freezing the brain base first on dry ice.

Figure 23.

Muscarinic receptor distribution. 24 hours post hatch

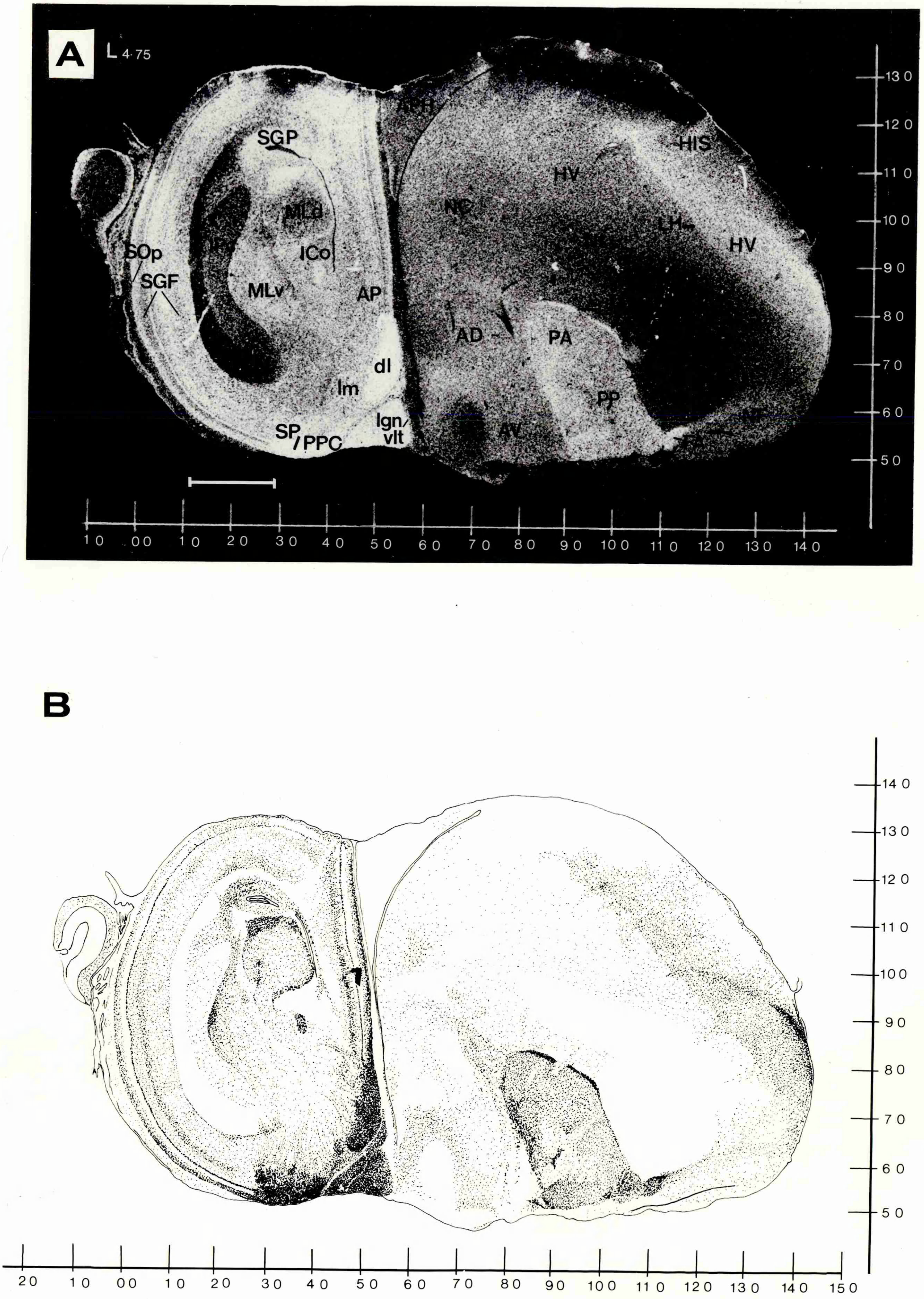
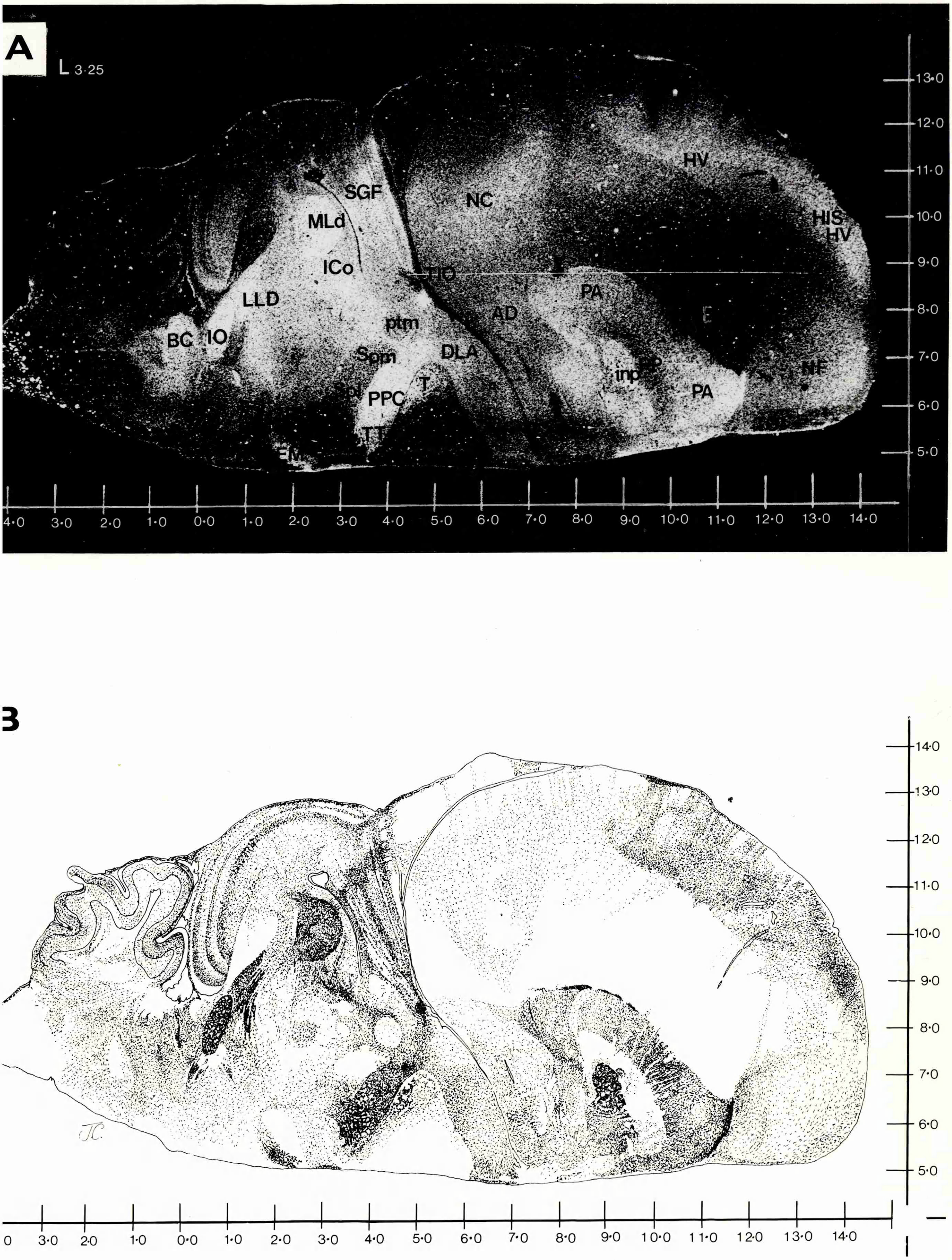

A
L 4.75
SGP
APH
HIS
HV
NC
MLd
LH
HV
SOp
ICo
SGF
MLv
AP
AD
PA
dl
lm
PP
lgn/vlt
SP/PPC
AV
B
10 00 10 20 30 40 50 60 70 80 90 100 110 120 130 140
130 120 110 100 90 80 70 60 50
20 10 00 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150
140 130 120 110 100 90 80 70 60 50

Figure 24.


A
L 3·25
HV
SGF
NC
HIS
MLd
HV
ICo
TIO
PA
LLD
AD
E
BC
IO
ptm
Spm
DLA
inp
NF
Spl
T
PPC
PA
TT
EM
13·0
12·0
11·0
10·0
9·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
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B
14·0
13·0
12·0
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8·0
7·0
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JC
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0·0
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3·0
4·0
5·0
6·0
7·0
8·0
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11·0
12·0
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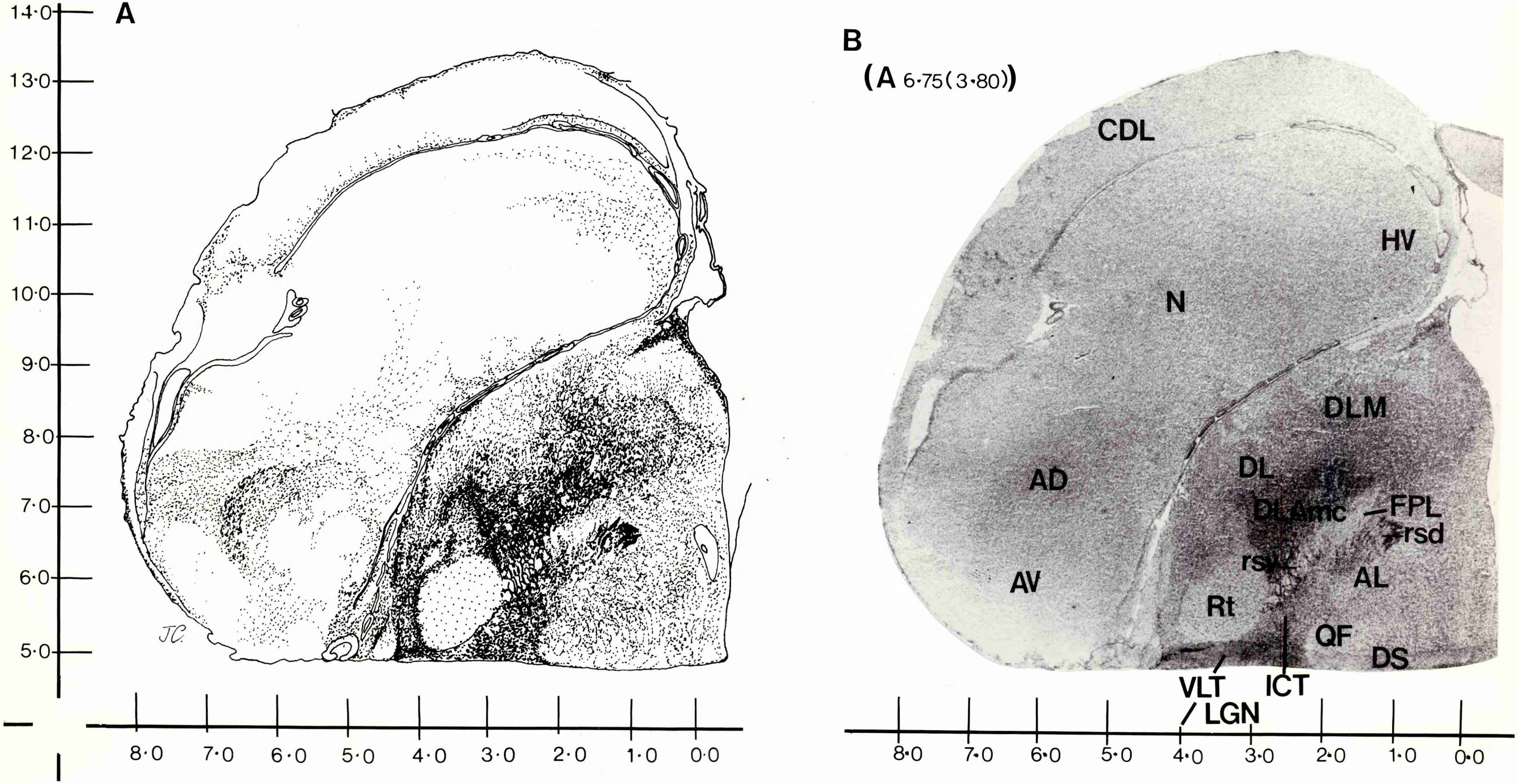
14·0

Figure 25.


A
14·0
13·0
12·0
11·0
10·0
9·0
8·0
7·0
6·0
5·0
JC
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0
B
(A 6·75(3·80))
CDL
HV
N
DLM
DL
AD
DLAmc
FPL
rsd
rsv
AL
AV
Rt
QF
DS
VLT
ICT
LGN
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0·0

stains. I have indicated some of these anatomically mismatched muscarinic receptor fields as numbered MRF's. I do not mean to suggest that these receptor fields denote morphologically and functionally distinct brain localities, but merely for the moment, to draw the reader's attention to their presence.

The following description of muscarinic receptor distribution is for the 5 week post hatch chick brain. MAChR distribution is shown by *in vitro* ^{3}[H] antagonist labelling (see section 2.2) of over 2000 tissue sections, cut from four 5 week post hatch chick brains, and subjected to light microscope autoradiography. While the following description is of silver grain distribution and density, I have shown elsewhere in this study (see section 2.11) that silver grain density of ^{3}[H] antagonist labelled autoradiograms corresponds closely with the concentration of specifically bound receptor antagonist.

Identification of chick brain cholinergic receptor locations in this study is largely based on the collated descriptive anatomical studies of Ariens Kappers (1967) and the stereotaxic atlases of Tienhoven and Juhasz (1962) for the chick brain and Karten and Hodos (1967) for the pigeon brain. Identification of chick brain anatomy was also made by reference to the finding of numerous anatomical, morphological and topographic studies of selected regions of the chick and other avian brain. In particular the studies of Cajal (1911), Le Vail and Cowan (1971), Hunt and Webster (1975), and Angaut and Reperant (1976) for the optic tectum, Meier et al. (1974) for the dorsal thalamus, Cowen et al.(1961), Benowitz and Karten (1976), Crossland and Uchwat (1979) for visual pathways, Karten and Dubbeldam (1973) for the paleostriatum, Krayniak and Siegel (1978) for septal structures and Zeier and Karten (1971) for the archistriatum, amongst others. In addition, anatomical structures were identified in my own series of histologically stained chick brain sections, using cresyl echt violet, Toluidine blue, Pyronine Y and Silver albumose staining techniques.

3.14.1 Telencephalon.

Olfactory lobe and Tuberculum olfactorium.

The olfactory lobe of the chick brain is characterised by generally low densities of muscarinic cholinergic receptor (MAChR), (low density $>$ 100 pmoles/g protein and $<$ 400 pmoles/g protein). The glomerular cell layer (GLomL) is very low in MAChR ($\leq$ 100 pmoles/g protein). The external granule cell layer (EGrL) and mitral cell layer (MitL) are populated by marginally higher than low muscarinic receptor densities (see figures 37 and 38 at the intercept of coordinates Anterior (A) 14-16 and the Horizontal (H) 6). Only the inner granule cell layer (IGrL) has densities of muscarinic receptor which are bordering on 'moderate' (ie. 400 pmoles/g protein).

At the point of entry of the olfactory nerve to the olfactory bulb, there is a moderately dense but poorly defined muscarinic receptor field (moderate density $>$ 400 pmoles/g protein and $<$ 750 pmoles/g protein) which does not correspond with any previously described morphological structure of the chick olfactory bulb. Caudally, the low to moderate density receptor field of the IGrL is continuous with the moderate muscarinic receptor densities of the area praepyriformis (Rose, 1914) which is probably homologous, at least in part, with the tuberculum olfactorium (TO). The adjective 'continuous' is used in the present context to distinguish between two adjacent silver grain (muscarinic receptor) fields which obviously correspond to two morphologically distinct localities, but which are not sharply delineated at their juncture by silver grain distribution or density.

MAChR density in the TO steadily increases caudally to reach high (> 750 pmoles/g protein and < 1100 pmoles/g protein) to very high muscarinic receptor density (> 1100 pmoles/g protein), particularly where the TO adjoins the paleostriatum augmentatum (PA) and lobus parolfactorius (LPO) (see figures 37 and 38, A 12-15, H 6 and figures 44-50, Lateral (L) 0-3, H 6). On a basis of silver grain distribution, it is very difficult to discern the limits of the TO MAChR field. Rostrally, for example, the TO MAChR field is continuous with a moderate to dense muscarinic receptor field, designated in figures 44 and 45, L 1-5, H 6, as muscarinic receptor field 15 (MRF 15), which underlies a region identified as the nucleus basalis (BAS). Similar to MRF 15, the TO MAChR field is continuous with three other receptor fields, MRF 5 and MRF 6 (see figures 43-45, coordinates L 0-1, H 6-7) and anteroventromedially with MRF 4 (see figure 49, L 0, H 5-6). MRF 4 lies within the area of the nucleus accumbens (AC) which according to Ariëns Kappers (1967) is an extension of the paleostriatum around the ventral wall of the ventricle (see below).

Paleostriatum.

The paleostriatal complex (PC) of the chick telencephalon consists of four major subdivisions, the paleostriatum augmentatum (PA), situated dorsally and ventrally to the paleostriatum primitivum (PP) which largely encloses the nucleus interpeduncularis (INP). There is some question as to whether the fourth division, the lobus parolfactorius (LPO), is a morphologically/anatomically distinct region from the PA as suggested, amongst others, by Karten and Dubbeldam (1970). The LPO is situated medially and anteriorly to the PA and PP. The PA, LPO and INP are dense to very dense in muscarinic cholinergic receptor. The PP on the other hand is low to very low in MAChR density (see figures 23-33 and 40-48). All established anatomical subdivisions of the PC in addition to others which, to my knowledge, have not been described before, are distinguishable solely on the basis of silver grain density and distribution (ie. MAChR density and distribution).

Dorsally and anterodorsally, the high density MAChR field of the PA and LPO ends abruptly along the lamina medullaris dorsalis (LMD) (see figures 26-38, 43-53 and 72A). The LMD is the most dense MAChR field of the chick telencephalon. The LMD MAChR field does not interface all dorsal, anterior and lateral aspects of the PC from the adjoining and overlying neostriatum, but is seen to be a thin sheet (lamina) of irregular thickness which is punctured to a greater or lesser extent, depending on the region , by numerous fibre bundles recognised through their absence of silver grains (ie. MAChR) and seen as striations across and through dorsoanterior aspects of the PA MAChR field (see figures 30-32: A10-12, H 8-10 and figure 45: L 4-6, H 8-10). Dorsally, dorsomedially and anteromedially, the MAChR field of the LMD , where enclosing the LPO, is continuous and rarely punctured by traversing fibres (see figures 35-38: A 10-13, H 8-10 and figures 50-53: L 1-5, H 8-11). However dorsoanteriorly, where the PA adjoins the ectostriatum (E), the LMD is absent or at least unlabelled at this juncture for muscarinic receptor (see figures 29-31: A 10-12, H 8-10 and figures 45: L 3-5, H 7-10).

The density of fibre bundles of the Tractus fronto-thalamicus et thalamo frontalis (FT) and, if distinct, Tractus thalamostriaticus (TTS) is the greatest at the juncture between the PA and E than for any other between the PC and overlying neostriatum.

Anteroventrally, the LMD MAChR field projects rostrally beneath the ectostriatum (E) as an upward projecting horn of receptor (see figure 28: A 11, H 5-6 and figures 27-29: A 11, H 7). and figures 45, L1-5, H 7-8) . More medially, the LMD MAChR field broadens and is seen to lie almost perpendicular to the basal wall of the chick telencephalon (see figures 29-31: A 11-12, H 6-8). At this point the LMD is separating the anteroventral limits of the PC from the Tractus fronto archistrialis (FA) and more medially from the nucleus basalis (BAS) (see Zeier and Karten, 1980). The LMD MAChR field limits most posterolateral aspects of the PC (see figures 27: A 8, H 6-9), but not posteromedially, where the LMD is conspicuously absent from the juncture between the dorsoposteromedial PC and dorsal archistriatum (Ad).

Muscarinic receptor density in the PA is not uniform. This is particularly evident from the silver grain patterns of $[^3H]$ PrBCM labelled parasagittal autoradiogram chick brain sections (see figures 23 , 72A and figures 27-36). In the PA, as elsewhere in the chick brain, MAChR distribution and density seem to be intimately related to the form and number of pervading fibres. In the dorsoposteromedial PA there are distinctly lighter patches of muscarinic receptor density which appear to correspond with areas of convergence and/or divergence of fibre bundles between which MAChR density is uniformly high (see figure 72 a). However, the dorsoanterior PA is also heavily pervaded by fibre bundles and yet, unlike the dorsoposteromedial MAChR field of the PA , the density of receptor is greatest over regions in immediate contact with these pervading fibres. The difference between the two regions is that in the former the fibres are probably not converging/diverging as described for the dorsopostero-medial PA. The reason for higher MAChR densities along the fibre tract margins (see figure 23 A and B and figures 72 A and 74 B) may be reflecting a greater number of collateral and axonal varicosity synaptic contacts with dendrites or neurons intrinsic to the PA (see Discussion).

The ventromedial MAChR field of the PA is also differentiated by higher density patches of receptor (see figure 72 a) but, unlike dorsal and dorsoposterior regions of the PA, there are very few pervading fibre tracts . The variation in muscarinic receptor density in the ventromedial PA is perhaps related to the distribution of olfactory inputs to the PA/ LPO via the TO (see earlier).

There is some evidence from the present findings to suggest that MAChR distribution distinguishes an interfacing lamina, similar to the LMD, between the anteroventral PA and paleostriatum primitivum (PP). The dense MAChR field of the PA ends abruptly at the PP juncture, but most often the line of demarcation is jagged because of traversing fibres. However occasionally, particularly more medially, the demarcation zone becomes smoother and, at points between traversing fibres, receptor density is noticeably higher than for the adjoining PP or PA (see figures 34 and 35: A 9-11, H 8-10 and figure 51). This proposed PA/PP interfacing lamina is most clearly seen between the ventral PA MAChR field and the overlying PP.

The paleostriatum primitivum (PP) is very low in MAChR density, mainly because of the presence of numerous closely packed fibre bundles which are unlabelled for receptor. Areas between these fibre bundles are populated by low and low to moderate densities of MAChR (see figures 29-36 and 72 A). There clearly is a close association between the PP and the Fasciculus prosencephali lateralis (FPL) (see figure 34: A 9-10, H 7), the fibre bundles of which converge from or diverge into the PP on route to or from other regions of the telencephalon. Centrally placed between the PP and FPL is the nucleus interpeduncularis (INP) which is populated by very high densities of MAChR (see figures 29-35: A 9-10, H 6-9, figures 49-51: L 3-5, H 6-9 and figure 72 A). The muscarinic receptor field of the INP is continuous with that of the PA laterally and LPO medially. Similar to the dorsoanterior receptor field of the PA and PP the INP is heavily pervaded by fibre bundles and, again similar to the PA , the density of silver grains is highest over regions in immediate contact with these fibres. As described for the PA interface with the neostriatum and that between the PP and PA , there is a band of very high density MAChR enclosing the INP dorsally and dorsoanteriorly from the PP (see figure 24 a and b).

The MAChR field of the LPO, unlike that of the PA, is uniformly very dense in muscarinic receptor (see figures 37-40: A 11-14, H 5-9, and figures 47-53, L 1-4, H 6-11). Further, it is not differentiated by unlabelled pervading fibre bundles. Anteriorly, the LPO MAChR field appears to extend along the ventral margin of the chick telencephalon beyond the LPO's hitherto recognised limits (see MRF 5, 6 and 15 of figures 42-45: L 0-5, H 6). These MAChR fields do not, to my knowledge, correspond to any established anatomical or morphological division described for this region of the chick forebrain. Anteromedially, the LPO MAChR field is also continuous beneath the most ventralward extension of the forebrain ventricle. with an equally dense receptor field corresponding in position to the accumbens (AC) (see figures 43-50: L 0-1, H 6).

Archistriatum.

The archistriatum is a large heterogeneous nuclear mass in the caudal ventrolateral portion of the chick forebrain. In parasagittal plane, over posterolateral aspects of the chick forebrain, the archistriatum is characterised by a moderate to high density MAChR field which corresponds to the archistriatum intermedium dorsale (Aid) of Zeier and Karten (1980) (see figures 22, 26-28: A 6-9, H 8-10). The Aid MAChR field is sickle shaped in parasagittal plane. In a caudal to rostral direction the Aid begins as a relatively deep receptor field which narrows anteroventrally to end along the most lateral and dorsal aspects of the PC (see figure 26: A 6-9, H 6-9 and figure 48: L 7, H 7-8). At this juncture between the Aid and dorsolateral PC (LPO), the two high density muscarinic receptor fields are continuous. Anteriorly, the Aid MAChR field is not sharply demarcated from the overlying and adjoining neostriatum, but continues anteriorly as a diffuse, moderately dense receptor field, shown by figure 26 as MRF 5, which probably corresponds to the archisstriatum anterior (Aa). Dorsoposteriorly, the Aid is sharply demarcated from the overlying neostriatum caudale (Nc) by the lamina archistrialis dorsalis (LAD) which, similar to the LMD of the PC, is populated by high densities of muscarinic receptor (see figure 26: A 6-7, H 8-9 and figures 54-57: L 6-9, H 6-9).

MAChR density and distribution posteroventrally to the Aid is extremely complex and difficult to decipher, mainly because of the numerous pervading fibres of the anterior commissure(CA), tractus occipitomesencephalicus (OM), and tractus fronto-archistrialis (FA) (see figures

54-59: L 4-9, H 5-9). Ventromediolaterally, the archistriatum is characterised by two areas of low MAChR density which are surrounded on all sides by very high densities of receptor (see figure 51: L 4-8, H 5-7). The division of the archistriatum, as distinguished by MAChR distribution and density, do not correspond with those described by Zeier and Karten (1980) for the pigeon archistriatum.

Neostriatum.

The neostriatum is the largest of all basal telencephalic nuclei. It begins slightly caudal to the frontal pole of the chick hemisphere, designated here the neostriatum frontale (NF), and extends to the caudal pole of the basal part of this telencephalic division, the neostriatum caudale (NC). The NF is in general populated by uniformly low densities of muscarinic receptor. However, differentiated against the low MAChR densities of the NF is a 'wisp'-like, moderately dense receptor field emanating from ventral aspects of the LMD and gradually fading into the lower receptor densities of dorsal aspects of the NF.(see figures 23-34, A 13-16, H 6-9 and MRF 13 in figures 27 and 28). The neostriatum intermedium (NI), situated between the dorsal PC and lamina hyperstriatica (LH) of the hyperstriatum is also populated by low densities of muscarinic receptor (see figures 22,23 and 45-47: L 4-9, H 8-12). However more medially, in particular where the NI adjoins the telencephalic ventricle, muscarinic receptor density is moderate (see figures 48-50: L 1-2, H 10-12). Posteromedially, there is a discrete high density receptor field corresponding in position to the capsule interna occipitatis (CIO), designated MRF 8 in figures 49-53: L 3-5, H 11.

The NC, similar to other aspects of the neostriatum, is largely populated by low and low to moderate densities of MAChR with little differentiation of receptor density pattern. Caudally however, the NC is characterised by an extensive, moderate muscarinic receptor field (MRF 32) which lies dorsocaudally to the LPO and extends in a dorsolateral direction to fade into the lower receptor density field of the surrounding neostriatum (see figure 54-58: L 2-6, H 9-12). MRF 32 of the chick neostriatum may correspond to the magnocellular nucleus of the anterior neostriatum, as described recently for the zebra finch brain (Ryan and Arnold, 1981). Situated dorsomedially to MRF 32 of the NC and lying ventrally to the medial hyperstriatum ventrale is a well defined region of the NC which is populated by very low densities of muscarinic receptor, a region which corresponds to Field L (Rose 1914) (see figures 54-58: L 0-5, H 10-13).

Hyperstriatum.

The hyperstriatum begins very near to the frontal pole of the hemisphere and extends caudally over the tractus occipitalis and neostriatum. The subdivisions of the hyperstriatum have always proved difficult to distinguish using more classic histochemical stains , but fortunately for this study, MAChR distribution appears to delineate unambiguously all major hyperstriatal subdivisions.

The hyperstriatum accessorium (HA), situated immediately beneath the dorsocaudal roof of the chick hemisphere, is moderately dense in MAChR (see figures 41-48: L 0-3, H 10-15) in those sections labelled for receptor by the antagonist $[^3H]$ *l* QNB. However, those chick brain sections labelled by $[^3H]$ PrBCM show the HA to be low to very low in MAChR (see figures 23-25, 32-37: A 10-15, H 10-14.) and 41 to 50: L 0-3, H 10-14).

The hyperstriatum of the chick is the only brain region to show different relative densities of muscarinic receptor, depending upon the receptor label used, eg. [^{3}H] *l* QNB or [^{3}H] PrBCM. In [^{3}H] *l* QNB labelled autoradiogram sections MAChR density is not uniform in the HA, but is more dense over extreme mediodorsal limits of the hemisphere roof (see figure 42-45: L 0-2, H 11-14). MAChR density gradually falls, ventrolaterally, to reach very low densities at the juncture of the HA to the hyperstriatum intercalatus (HIS) which may alternatively be the hyperstriatum dorsale (HD), or both. The HIS/HD is very low in muscarinic receptor, and, on the basis of MAChR distribution, there is no evidence for an intervening lamina between the HA and HIS (see figures 42-45: L 1-4, H 11-13). The HD/HIS does not extend to the most medial limits of the hemisphere, but is separated from the medial hemisphere wall by a high density muscarinic receptor field (MRF 2)which projects dorsally from medial aspects of either the hyperstriatum dorsale (HD) or hyperstriatum ventrale ventrodorsale (HVvd) to ventromedial aspects of the HA MAChR field (see figures 42-48: L 0-1, H 9-10). The MAChR field of the HA is also differentiated, posteromedially, by a complex pattern of high muscarinic density (MRF 1), the complexity reflecting the convergence or divergence of fibres of the tractus septomesencephalicus (TSM). In fact, MRF 1 may correspond to an aspect of the area entorhinalis (Rose, 1914), alternatively the hippocampus pars dorsalis (see figures 47-49: L 0-2, H 14-15).

The HD/HVvd is populated throughout by uniformly very high densities of muscarinic cholinergic receptor in those sections labelled by [^{3}H] *l* QNB (see figures 41-50: L 1-6, H 9-15). Rostrally, the HD/HVvd MAChR field extends across the forebrain as a *lamina* of uniform thickness from the extreme dorsolateral margin of the hemisphere to the medial ventricle. At the juncture of the HD/HVvd to the HD/HIS, there is no sharply delineated boundary. However, between the HD/HVvd and hyperstriatum ventrale (HV) or hyperstriatum ventrale ventroventrale (HVvv), there is a well defined demarcating lamina of very high muscarinic receptor density (see figures 42-48: L 1-6, H 9-14). However, in those sections labelled by $[^3H]$ PrBCM (see figures 37 and 38: A 12-15, H 7-12) this dividing layer is seen to be very low in muscarinic receptor density. This difference is not a reflection of section plane, since the $[^3H]$ l QNB labelled parasagittal section of figure 72 similarly reveals a very high density MAChR lamina between the HD/HVvd and HV/HVvv.

Identification of hyperstriatal divisions is based on the pigeon brain stereotaxic atlas of Karten and Hodos (1967). Zeier and Karten (1971) have published evidence which shows the HIS (figure 2 of that report) to lie lateroventrally to the HD at stereotaxic planes anterior to 10.00 and 11.00 and to partly enclose the HD at anterior 12.00. However, in the atlas of Karten and Hodos (1967) the HIS is shown to lie dorsomedially to the HD. If the HIS lies latero-ventrally to the HD, then lateral aspects of the region identified as HD/HVvd may correspond to the HIS, in which case the 'HIS' is dense in muscarinic receptor. On the other hand, the complexity of dorsal hyperstriatal subdivisions, as shown by Zeier and Karten (1971), is not reflected by muscarinic receptor distribution as shown here for the chick brain. Youngren and Phillips (1978) in their 3 day post hatch chick brain atlas do not show the HIS at all, and van Tienhoven and Juhusz (1962) suggest that the HD and HIS are one and the same region in the chick. It is my impression that the low density receptor field between the HA and HV/HVvvd may represent both the HIS and HD.

The hyperstriatum ventrale is the largest hyperstriatal division and is populated by very high densities of MAChR. In frontal plane, the HV is 'wedge' shaped, being broadest along the dorsolateral margin of the forebrain hemisphere. Anterolaterally the HV MAChR field extends to the dorsolateral limits of the hemisphere (see figures 41-47: L 1-9, H 10-14). More caudally however, the most lateroventral aspects of the HV are separated from the hemisphere roof by the area corticoidea (see below). Ventrally, the very dense HV MAChR field is separated from the underlying neostriatum by the lamina hyperstriatica (LH). Depending upon the plane and level of tissue section, the LH is sometimes seen to be even more dense in MAChR than adjoining regions of the HV (see figure 24 A: A 8-13, H 9-13 and figures 26-34: A 9-14, H 9-13).

Posteromedially, the HV MAChR field contracts to occupy a position along the medial hemisphere ventricle, where the HV dips ventrally towards the septum (see figures 50-57: L 1-3, H 9-13). This aspect of the HV has been designated the medial hyperstriatum ventrale (MHV) (Bateson et al., 1978). The MHV is populated by even higher densities of MAChR than other aspects of the HV. Ventromedially, the very dense MAChR of the MHV apparently ends at the juncture to the medial hemisphere ventricle. It is curious that the equally high MAChR densities of the dorsal septum, on the ventral side of the hemisphere ventricle from the MHV, gives the impression of morphological continuity between the MHV and septum (see figures 52 and 53: L 0-1, H 9-10). A very similar transventricle juxtaposition of very high MAChR densities is found between the ventrodorsal limits of the MHV-HV and a discrete receptor field situated within the area corticoidea dorsolateralis/medialis (CDL/M) (see figures 48-51; L 3-6, H 13-15). This HV-CDL apposition is even more curious when viewed in parasagittal plane (see figures 24, and **32-38** : A 8-11, H 12-15), where the main body of the HV MAChR field may be seen to end dorsomedially, well short of dorsal aspects of the hemisphere ventricle. However, extending from the dorsal aspects of the HV is a 'whisp' like, moderate to dense MAChR field (MRF 21, in figures 32 and 33: A 9-10, H 13-15) which extends to that point of the forebrain ventricle which is immediate opposite the very dense MAChR field described above for the CDL. Although in parasagittal plane the hemisphere ventricle is seen to very clearly separate MRF 21 from the CDL, the hemisphere ventricle does not separate dorsolateral aspects of the MHV-HV from all 'cortical' areas of the chick forebrain. Between levels, anterior 7.5 and 9, the very dense MAChR field of the HV fades into a subcortical area which is moderately dense in muscarinic receptor (see figures 45-48; L 4-7, H 12-14). This region corresponds to the dorsolateral surface area component to ectostriatum (**Kappers et al,** 1967), but in addition,to dorsal aspects of the area tempero-parieto-occipitalis and the region of divergence or convergence of fibres of the tractus archistriatalis dorsalis (DA). More caudally, the chick cortex becomes thinner and is once more separated from the moderately dense muscarinic receptor field of the lateral forebrain by the hemisphere ventricle (see figures 49-54: L 4-9, H 7-13).

Area corticoidea and hippocampus.

Uncertainty as to the subdivision of the avian dorsal hyperstriatum (see hyperstriatum) is carried over to that region of the forebrain hemisphere which lies dorsal and medial to the hemisphere ventricle, a region corresponding to the area entorhinalis (Rose 1914; van Tienhoven and Juhász, 1962), subiculum (Craigie, 1930; 1932), hippocampus pars dorsalis , and hippocampus (Karten and Hodos, 1967; amongst others). Areas of the chick hippocampus (Hp) are not only difficult to distinguish from dorsomedial aspects of the HA, but and in addition, both the Hp and HA are difficult to distinguish, dorsally and dorsolaterally, from the area corticoidea (C). Here I will describe MAChR distribution in those areas of the HP and C which lie caudal to the plane of section, anterior 12•00.

At the point where the fibres of the tractus septomesencephalicus (TSM) converge to or diverge from the main body of the TSM, there are two very high density MAChR fields, described and designated earlier as MRF's 1 and 2 (see figures 45-50: L 0-3, H 11-15). Both of these MRF's appear to be intimately related to the separating fibres of the TSM and are clearly distinct from other aspects of the HA, which are only moderately dense in cholinergic receptor (see above). However, both MRF 1 and MRF 2 are continuous with a generally moderately dense receptor field which extends as a thin lamina along most of the dorsal and lateral circumference of the chick forebrain. A lamina which corresponds to the hemisphere cortical layer, described in the chick stereotaxic atlas of van Tienhoven and Juhász (1962). In fact, MRF 1 is seen to take up a position which is dorsomedial, anteriorly, but lateral caudally. During this positional transition, the discreet character of this very high density receptor field remains unchanged.(see figures 49-54: L 0-7, H 10-15). MRF 2 on the other hand becomes intermingled and indistinguishable from the now high muscarinic receptor densities of the HA (see figure 48: L 0-3, H 13-15).

. It is of interest to note that in frontal sections, caudal to anterior 8·5, muscarinic receptor distribution does not distinguish any region corresponding to the HIS. Laterally to the high MAChR densities of the HA, muscarinic receptor density decreases, but still differentiates a region which is best described as 'subcortical', lying between the moderately dense circumventing cortical layer, and medially, the very high densities of the HV. This subcortical region, similar to the HA, appears to be differentiated by columns of higher receptor density which, more caudolaterally, fade into the low to moderate cholinergic receptor densities of the neostriatum (but see section 3.12)

As MRF 1 takes up a position more lateral to the hippocampus, dorsomedial aspects of the Hp, which at this level is generally very low in muscarinic receptor density (see figures 48-52: L 0-2, H 12-15), are differentiated by a further moderately dense receptor field, designated MRF 9 in figures 50-54, which probably corresponds to the nucleus parahippocampalis pars linearis (PHL) (Benowitz and Karten, 1976). Ventromedially, between the hemisphere medial wall and the hemisphere ventricle, the hippocampus is populated by moderate to low receptor densities. However, it is 'significant' to note that MAChR distribution in this region differentiates a *laminated* structure to the HP, which appears to be the result of the moderately dense receptor field, reported above to lie along the extreme medial and dorsal hemisphere margin, folding in upon itself along the wall of the hemisphere ventricle (see figures 57-60: L 0-3, H 12-15). The area parahippocampus (APH), lying between MRF 1 of the chick cortex and the hippocampus, is generally very low in muscarinic receptor density.

Septum.

Frontal ^{3}[H] *l* QNB labelled autoradiogram sections of the chick hemisphere, at a section level anterior 12·5, distinguish a very high density muscarinic receptor field, designated MRF 4 in figures 44-48: L 0-3, H 6-7. MRF 4 corresponds to the most rostral limits of the chick septum and is seen to extend dorsally along the medial hemisphere wall towards the hippocampus. More caudally, MRF 4 is restricted to ventromedial aspects of the forebrain hemisphere; at this level, i.e. anterior 11·00 to 12·00, MRF 4 corresponds in position to the nucleus of the diagonal band of Broca (NB) (see figures 47-49: L 0-3, H 6-7 and figures 38-40: A 11-12, H 6).

The septum, dorsal to the NB, is differentiated into a very high density receptor field, dorsally, and a generally low to moderate, heterogeneous field of muscarinic receptor over ventral aspects. Rostrally, the very dense MAChR field of the dorsal septum runs ventrally along the edge of the hemisphere ventricle (see figure 49: L 1-2, H 6-10). More caudally, the very high receptor densities of the dorsal septal field run down the *medial* hemisphere wall towards the NB and nucleus preopticus dorsolateralis (Kuhlenbeck, 1937). It may be that this field of dense muscarinic receptor in the dorsal septum corresponds to both the lateral and medial septal nuclei (Karten and Hodos, 1967, amongst others), although there is no evidence on the basis of receptor density or distribution to indicate a point of demarcation between lateral and medial aspects of this nucleus. The low to moderate muscarinic receptor densities in ventral aspects of the septum correspond to aspects of the nucleus commissuralis septi (CoS), commissura pallii (CPa) and tractus cortico-habenularis et cortico-septalis (CHCS), although none of these morphological features are distinguished by MAChR distribution. The main body of the TSM, on the other hand, is clearly visible running along the medial edge of septal nuclei (see figures 48-52: L 0-1, H 6-9).

3.14.2 Diencephalon and Mesencephalon.

Thalamus.

The nuclear and interstitial cell masses of the thalamus are in general populated by high densities of muscarinic receptor. One nucleus in particular, the nucleus rotundus (Rt), dominates the anterior midbrain by its size, but in addition and as an exception to the above generalisation, by its almost complete absence of muscarinic receptor (see figures 23,24 and 72: A 6-7, H 6-9, and figures 25,58-63 and 72: L 2-3, H 6-9). The marked difference in muscarinic receptor density between the Rt and adjoining thalamic regions highlights the discrete character of this nucleus, particularly laterally.

In sharp contrast to the near absence of receptor over most of the Rt, there is an extreme, very high density receptor field over dorsolateral aspects of the Rt. This very dense MAChR receptor field clearly lies *within* the main body of the Rt (see figures 32-34: A 5, H 7-8 and, in particular, figure 72 B), but, more medially, is continuous with high MAChR densities corresponding to the nucleus triangularis (T) (see figures 60-62: L 2, H 8). As observed for other chick brain regions of exceptionally high

MAChR density, the densely populated MAChR field of the dorsal Rt is reticulate in pattern, punctured by areas devoid of receptor which correspond to large calibre fibres or bundles.

Dorsolateral to the Fasiculus prosencephali lateralis (FPL)and rostral to the Rt, is a heterogeneous MAChR population corresponding to the nucleus dorsolateralis anterior thalami, pars lateralis (DLL) and pars magnocellularis (DLAmc) (see figures 32-34: A 6-7, H 7-9, and figures 55-58: L 2-4, H 6-9). The density of MAChR in the DLL is generally high, but the DLAmc is populated by even higher muscarinic receptor densities, in particular where adjoining the main body of the FPL and rostral Rt. The very high MAChR densities of the DLAmc are differentiated as a network of dense receptor which continues ventrally, to pervade, in a similarly intricate pattern, the fibre bundles of the FPL . This FPL invaginating MAChR field corresponds to the nucleus reticularis thalami, pars dorsalis (RSd) and is seen to be continuous, ventral to the FPL, with another dense network of MAChR corresponding to the ventral component of the reticularis thalami (RSv) (see figures 34, 35: A 8, H 6-8; figures 55-59: L 2-3, H 6-7, but particularly see figure 25, L 2-3, H 6-7).

Muscarinic receptor distribution in the dorsomedial thalamus is generally moderate to high in density but, compared to ventral thalamic regions, is relatively undifferentiated. The nucleus dorsolateralis anterior thalami (DLA), which lies medial to the DLL, is populated by high densities of MAChR, particularly where adjoining the main body of the Tractus fronto-thalamicus et thalamo frontalis (see figures 34-37: A 7-9, H 9-10; and figures 57-59: L 2-3, H 7-9). More medially still, the high MAChR densities of the DLA are continuous with a moderate density muscarinic receptor field corresponding to the nucleus dorsolateralis posterior thalami (DLP)and nucleus dorsomedialis posterior thalami (DMP),which together are seen as a diffuse MAChR field enclosing the nucleus dorsointermedius posterior thalami (DIP) which is low in muscarinic receptor density (see figures 60-61: L 0-2, H 9-10). Dorsal to the DLL, DLA and DLM, is the nucleus superficialis parvocellularis (SPC),alternatively the nucleus tractus septomesencephali, which lies immediately beneath the dorsal roof of the thalamus and is populated by high densities of MAChR (see figures 33 and 34: A 7-9, H 8-9 and figures 57-61: L 0-4, H 8-10). Dorsolaterally, the MAChR field of the SPC encloses the main body of the TSM, which is devoid of MAChR. Immediately dorsal to the TSM is an extremely high density spot of MAChR which lies at the interface of the SPC to the dorsal extension of the lemniscal nuclei (LM); this receptor field has been designated in figure 32: A 5, H 9 and figure 60: L 3-4, H 9 as MRF 21. A similarly very high density spot of MAChR is seen within the SPC, but more dorsomedially to MRF 21, which has been designated MRF 26 (see figure 36: A 8, H 9). Dorsomedial to the SPC,the nucleus habenularis lateralis (HL) and subhabenular nucleus (SHM) are populated by moderate to high densities of muscarinic cholinergic receptor. The stria medullaris (SMe), enclosed by the HL and SHM, is seen to be devoid of receptor (see figures 57-60: L 0-2, H 10-11). Immediately dorsal to the SMe is an extreme high density MAChR field, MRF 32 in figure 60: L 0-1 H 11, which corresponds to the nucleus habenularis medialis (HM).

Immediately ventral and extending slightly rostral to the Rt is a heterogeneous field of moderate to high density MAChR corresponding to the nucleus ventrolateralis thalami (VLT). Receptor density in the VLT is highest in regions immediately adjoining the main body of the FPL (see figures 34 and 35: A 6-9, H 6-7). Anteriorly, the MAChR field of the VLT is continuous along the extreme ventral margin of the FPL, with the equally high densities of receptor reported earlier to populate extreme ventrocaudal aspects of the telencephalic basal ganglia, a region corresponding to the tuberculum olfactorium (TO). Mediodorsally, the higher densities of MAChR over dorsal aspects of the VLT, similar to the DLAmc, are continuous with the very high density network of MAChR reported earlier to correspond to the RSv (see figures 34 and 35: A 7-8, H 6-7 and figures 55-57: L 2-3, H 5-6). More caudomedially, the MAChR field of the VLT extends dorsally between medial aspects of the Rt and lateral aspects of the tracts ansa lenticularis (AL), occipitomesencephalicus (OM) and quintofrontalis (QF), to occupy a position which corresponds to the nucleus intercalatus thalami (ICT) and nucleus posteroventralis thalami (PV) (Kuhlenbeck). MAChR density in the ICT is moderate, but similar to the PV , is not homogeneous. Aspects of the ICT, which are juxtaposed to the AL,QF and OM, are populated by extremely high densities of muscarinic receptor, a region which is heavily pervaded by fibres of every calibre and which more correctly corresponds to the nucleus subrotundus (SRt) (see figure 60: L 1-2, H 6-9). The very high density SRt MAChR field extends dorsomedially to take up a position ventral to the nucleus ovoidalis (OV); but it is separated from the OV by a thin band, which is free of MAChR, identified as the tractus ovoidalis (TV). Some of the fibre bundles pervading dorsal aspects of the SRt MAChR field are probably constituents of the TV, in addition to fibre bundles of the OM (see figure 37: A 6-7, H 8-9 and figures 60-62: L 0-2, H 7-9).

The nucleus ovoidalis (OV) is highly characteristically labelled for MAChR, in that, similar to a number of other relay nuclei of the chick midbrain, the OV is populated by very high densities of muscarinic receptor around the circumference of the nucleus, particularly dorsomedially, while central aspects are almost completely devoid of muscarinic cholinergic receptor (see figure 60; L 1, H 8-9).

Pretectal nuclear masses.

On the line between the mesencephalon and diencephalon (ie. sulcus limitans) are a group of nuclei which are generally considered pretectal, since they appear to be concerned, to a very considerable extent, in the interrelation of tectal with diencephalic and other centres of the avian brain (cf. Kappers, 1967). These nuclei are the nucleus pretectalis (PT), the nucleus principalis precommissuralis (PPC), the nucleus spiriformis pars lateralis (SpL) and pars medialis (SpM) and the nucleus subpretectalis (SP). With these nuclei belong the lenticular mass of gray, consisting of the nucleus lentiformis mesencephali, pars magnocellularis (LMmc) and pars parvocellularis (LMpC), which are thought to be continuous with the gray of the tectum (cf. Kappers, 1967).

The nucleus principalis precommissuralis (PPC) (see below) lies between the Rt and the tectal gray, and throughout its area is one of the most dense muscarinic receptor fields of the chick brain (see figure 31; A 4-7, H 6-8 and figure 60: L 4, H 6-8). The limits of the PPC nuclear mass have never been fully explained; for example, the stereotaxic atlas of Karten and Hodos (1967) draws no line of demarcation between dorsocaudal aspects of the PPC and the nucleus DLA. However, the present findings reveal that the very high muscarinic receptor densities of the PPC end as a bulbous projection centred within fibre tracts of the tractus septomesencephalicus pars dorsalis, tractus septomesencephalicus pars tectalis and tractus thalamo-frontalis lateralis; this dorsoanterior PPC projection is shown in figures 60-62: L 3-4, H 8-9 as MRF 24. At this level, PPC-MRF 24 is comparatively undifferentiated by pervading fibre bundles. However, as the MAChR field of the PPC moves ventrocaudally around the lateral circumference of the Rt, the number of pervading fibres, recognised by their absence of silver grains, increases to reach a maximum, where the PPC takes up a position dorsocaudal to the Rt (see figures 31-37: A 4-6, H 6-8 and figures 60-64: L 1-4, H 5-9). In general, dorsal aspects of the PPC are less heavily pervaded by fibre bundles and are more dense in muscarinic receptor than ventrolateral aspects. Laterocaudoventrally, the MAChR field of the PPC is pervaded by fibres of the tractus tectothalamicus (TT), which runs between the nucleus subpretectalis (SP)and Rt. Dorsocaudal areas of the PPC MAChR field, on the other hand, are pervaded by fibres of the tractus occipitomesencephalicus (OM) and tractus habenulo-interpeduncularis (HIP) (see figures 35 and 36: A 5, H 7-8 and figure 75: L 1-2, H 7-9). Ventrolaterally, the MAChR field of the PPC is continuous with one of three very high density muscarinic receptor fields along the diencephalon-ventromedial tectal margin,the nucleus superficialis or, alternatively, the nucleus geniculatis lateralis, pars dorsalis principalis (GLdp) (see figures 31 and 32: A 5-6, H 6-7 and figures 62-64: L 4, H 6).

Dorsocaudal to dorsal aspects of the PPC is the nucleus spiriformis lateralis (SpL), which is populated by moderate densities of MAChR (see figure 32: A 3-5, H 7-8). The SpL appears to be intimately associated with the PPC, and the interface between the SpL and PPC is populated by very high densities of cholinergic muscarinic receptor. The nucleus spiriformis medialis (SpM) is low to moderately dense in MAChR (see figures 33 and 34; A 4-5, H 8-9). Situated dorsal to the PPC, T, and SpL and enclosed by diffuse and moderately dense muscarinic receptor fields of the area pretectalis (AP) and pretectalis diffusus (PD), is the nucleus pretectalis (PT) which is circular in shape and highly characteristically labelled for MAChR. Similar to MAChR distribution in the OV (see earlier), the PT is populated by moderate to high densities of receptor around its circumference, with a slightly broader receptor field rostrally. However, central aspects of the PT are very low in MAChR (see figures 23, 31-33; A 4-5, H 8-9 and figures 63 and 64: L 4, H 7-9).

Geniculate, lemniscal (syncephalic), ectomamillary nuclei and tectal gray.

Lying along the ventral margin of the midbrain to the optic lobe are three very high density muscarinic receptor fields, corresponding to the nucleus lentiformis mesencephalicus (LM), the nucleus geniculatis lateralis ventralis, pars dorsalis principalis (GLdp) and nucleus geniculatis lateralis ventralis, pars ventralis (GLv) (nomenclature of Karten and Hodos, 1967). The LM may be better known by the nomenclature of Rendal (1924) as the nucleus superficialis synencephali, or as described by Cowan, Adamson and Powell (1961) as either tectal gray or the nucleus externus.

Rostrally, the LM MAChR field lies alongside the lateral edge of the Rt (see figure 59: L 4-5, H 6). More caudally, ie. anterior 5.00, the LM MAChR field extends from the edge of the tractus isthmo opticus (TIO), dorsally, to the optic tract (TrO), ventrally (see figures 60-62 and figure 76: L 4-5, H 6-9). At this level, the LM is not uniformly populated by very high densities of muscarinic receptor, but is differentiated into medial and lateral high density receptor fields, which may correspond to the LM divisions pars magnocellularis (LMmc) and pars parvocellularis (LMpc) and which are separated by a central area populated by lower densities of receptor, pervaded by fibres arising from the main body of the TrO (see figure 76: L 4-5, H 6-9). Laterally, the very high MAChR densities of the LM are continuous with and arise within the tectal lamina, stratum grisea et fibrosum (SGF) (see figures 17, 29: A 4-6, H 6-8 and figures 59-62). Rostrally, ie. anterior 5·00, the LM MAChR field completely interfaces the tectal laminae from other diencephalic nuclei. At this level, the LM is separated from the PPC/GLdp MAChR field by a fibre tract which extends from the TrO, ventrally, to the TIO, dorsally. Caudal to anterior 5·00, the LM MAChR field rapidly contracts to a circular and still very dense MAChR field, lying lateral to the GLdp and adjoining ventromedial limits of optic tectum (see figures 30-34: A 4-5, H 5-6 and compare with figures 62-66: L 4-5, H 5). In this position the LM MAChR field corresponds to the nucleus ectomamillaris (EM) (nomenclature Karten and Hodos, 1962) or nucleus externus (Cowan, Adamson and Powell, 1962). The present findings suggest that on a basis of MAChR distribution, the LM and EM are the same nucleus. The very dense MAChR field of the EM gradually declines in size, until its lateroventral position between the ventromedial tectum and more medial aspects of the midbrain is taken up by the branchium colliculi superioris (BCS), which is completely devoid of muscarinic receptor ^{3}H antagonist binding sites (see figures 32-35: A 3-4, H 6 and figures 64-69: L 3-4, H 6-7). It is of interest to note that the EM is completely distinct from the nucleus of the basal optic root (BOR) (see figures 35 and 36: A 4, H 5 and see below).

Situated medial to the LM-EM MAChR field, but separated from by an area devoid of receptor, is the GLdp. The GLdp is differentiated into a ventral component which is populated by extreme very high densities of muscarinic receptor, and a dorsal component which is moderately dense in MAChR (see figure 32, 33, 72 and 74 B: A 4-5, H 6, and figures 61-65:L 3, H 5-6). The moderate to high density MAChR field of the dorsal component of the GLdp is continuous with the very high density of muscarinic receptor in the PPC (see above and figure 72 B). Similar to other high density cholinergic receptor fields, reported here for the chick brain, the GLdp, particularly dorsal aspects, are heavily populated by fibres.

Situated medial to the GLdp and extending beyond this nucleus, both rostrally and caudally, is the nucleus geniculatis lateralis ventralis (GLv) which, similar to the GLdp, is characterised by extreme very high densities of muscarinic receptor, ventrally, while dorsal aspects of the GLv are populated by slightly lower densities of receptor. Rostromedially, dorsal aspects of the GLv extend to the nucleus preopticus anterior (POA) (see figures 37: A 8-9, H 5-6), where the receptor field of the GLv is seen to be continuous and indistinguishable from that of the VLT and ICT. Caudomedially, ie. anterior 6•0, lateral 2.5, dorsal aspects of the GLv are massively invaded by fibres of the basal optic root (TrEM) (see figures 35 and 36: A 5-6, H 5-6). The TrEM, at this level, is split into three or four fibre tracts between which are bands of very high muscarinic receptor density, extending from the VLT to most caudal aspects of the GLv (see figure 35).

Central nuclei and fibre tracts of the optic lobe.

Areas central to the optic lobe and immediately medial to the optic ventricle are dominated be the nucleus mesencephalicus lateralis, pars dorsalis (MLd) (see figures 22, 23, 27-33,71,72 and particularly 75: A 5-7, H 10-12, and figures 65-68: L 4-6, H 9-12). Muscarinic receptor density in the MLd is not uniform and distinguishes an underlying morphological complexity which, hitherto, has not been fully recognised. The circumference of the MLd, apart from a small break caudoventrally, is populated by extreme, very high densities of MAChR (see figures 29-30 and 65). However, towards the centre of this nucleus, muscarinic receptor density rapidly declines. The MLd core is completely devoid of cholinergic receptor. The encloseing MAChR field (MLd ex cap) is broadest and most dense over dorsal and ventral aspects of this nucleus (see figures 29-30: A 3-5, H 11). The caudoventral gap in the MLd ex cap is 'guarded', so to speak, by two very high density circles of MAChR. The MAChR field of the MLd ex.cap., at a section level lateral 5•0, is continuous with the equally high muscarinic receptor densities populating the substantia grisea et periventricularis (SGP). The SGP, lateral to lateral 5•0, completely encloses and adjoins the optic ventricle (see figures 27-28 and 66-68: L 4-7, H 6-12).

Medially, the MLd is enclosed by the nucleus intercollicularis (ICo) which is populated by a comparatively homogeneous, moderate to high density muscarinic receptor field (see figures 29-34, 65-66 and figure 75). The ICo is continuous and, on a basis of MAChR density and distribution, indistinguishable, medially, from moderate to high densities of MAChR populating the substantia grisea centralis (GCt). The ICo is shown by the atlas of Tienhoven and Juhász (1962) to be part of the tractus cerebellaris (Groebbels) of the chick brain. Within the ICO, situated medioventrally and ventrally to the MLd, are two discreet, very high density MAChR fields, shown in figure 65 as MRF 25 and MRF 14 respectively. MRF 14 extends along the ventral margin of the ICo to an area immediately beneath the tractus isthmo opticus (TIO) to form a complex MAChR field of very high density which corresponds in position to the radix mesencephalicus nervi trigemini (RxVM) (see figures 65-68: L 1-5, H 8-9). MRF 14 corresponds to no previously recognised morphological division of the ICo. Immediately caudoventromedial to, and extending along the entire caudomedial limits of the ICo is an area completely devoid of muscarinic receptor which corresponds to the tractus tectospinalis of Tienhoven and Juhász (1962), after Papez (1929).

Situated caudoventromedially to the tractus tectospinalis and MLd, is the nucleus mesencephalicus lateralis, pars ventralis (MLv), also known as the formatio reticularis lateralis mesencephali. Figures 27-31; A 2-4, H 6-12 show the MLv to be populated by moderate densities of MAChR. Over lateral aspects of the optic lobe, the MLv MAChR field completely encloses the optic ventricle and, over caudal aspects, is seen to be continuous with the extreme, very high density MAChR field of the substantia grisea et fibrosa periventricularis (SGP). More medially, ie. lateral 4·00, the moderate density MAChR field of the MLv sweeps ventrally towards the very high MAChR field of the LM (see figure 29; A 2-3, H 6-12). More medial to lateral 4·0, the MLv is characterised by a moderate to high density MAChR field, situated dorsocaudal to the MLd and shown in figure 31: A 2-3, H 11 as MRF 20. This MLv muscarinic receptor field sweeps ventrocaudally towards a complex of very high density muscarinic receptor fields which correspond to the lemniscus lateralis (LL), nucleus lemnisci lateralis, pars dorsalis (Groebbels) (LLd) and nucleus isthmo opticus (IO) (see figures 23 and 30-33: A 1-3, H 8-9, and figure 70: L 3, H 8-10).

Caudal to the MLv, the nuclei isthmi, pars parvocellularis (Ipc) and pars magnocellularis (Imc) are populated by very low densities of muscarinic receptor (see figures 22,27,28, and 71: A 3-4, H 7-11, and figures 69-70: L 4-7, H 6-9).

Laminae of the optic tectum.

The optic tectum of the chick is, by and large, populated by high densities of MAChR (see figures 75 and 88). The most external layer, the stratum opticum (SOp) or sublayer I (nomenclature of Cajal, 1891) is a continuation of the optic tract (TrO) which, in the post hatch chick brain, is completely devoid of muscarinic receptor antagonist binding sites. Sublayers II a and II b of the stratum grisea et fibrosum (SGF) or sublayers 2 and 3 (nomenclature of Cowan et al., 1961) are populated by uniformly high densities of MAChR. Occasionally, running through all the laminae of the SGF, there are fibres of large calibre which are visible by their absence of MAChR. Sublayers II e and II d are low to moderately dense in MAChR. Sublayer II f, alternatively sublayer 7, is populated by extreme, very high densities of muscarinic receptor and, in contrast to MAChR distribution between other laminae of the optic tectum, the MAChR densities of II f are sharply demarcated. Sublayer II e and II g are moderately dense in muscarinic cholinergic receptor. Sublayer II b and II of the SGF are very dense in MAChR. Dorsocaudally, the very high MAChR densities of II h and II i are clearly distinguishable from the lower moderate muscarinic receptor densities populating the stratum grisea centrale (SGC) (see figure 71). However, ventrocaudally, the high receptor densities of IIh and IIi are indistinguishable and continuous with the MAChR of the SGC. Muscarinic receptor in the caudal SGC are low to moderate in density and heavily pervaded by large calibre fibre bundles. The stratum album centrale (SAC) is almost completely devoid of muscarinic receptor. It should be noted that the MAChR fields of all tectal laminae are continuous, ventrally, with the extreme, very high density muscarinic receptor field of the LM-EM (see figure 72 B).

Tegmental areas of the mesencephalon.

The mediocaudal mesencephalon of the chick brain is populated by low to low to moderate densities of muscarinic receptor. The distribution of MAChR in these regions is diffuse and, compared to rostral mesencephalic structures, undifferentiated. Throughout this region, the low to moderate density MAChR fields are heavily pervaded by fibre bundles (see figures 35-40: A 1-5, H 4-9, and figures 65-70: L 1-4, H 6-9). The distribution of MAChR in tegmental aspects of the mesencephalic reticular nucleus (MLv) has already been touched upon. More medially, the moderate MAChR densities of the MLv are continuous with slightly higher than moderate MAChR densities corresponding to the formatio reticularis medialis mesencephali (FRM). The FRM, in turn, is indistinguishable on a basis of MAChR distribution and density from the locus ceruleus (LoC) (see figures 37 and 38: A 4-6, H 7-9). The moderate to high MAChR densities of the FRM and LoC are not sharply delineated from adjoining caudomedial MAChR fields of the mesencephalon. The nucleus ruber (Ru), in contrast to the diffuse MAChR fields of the mediocaudal mesencephalon, is populated by very high MAChR densities over dorsomedial aspects, but low muscarinic receptor densities, ventrolaterally (see figures 66: L 1-2, H 8).

Situated along the dorsomedial roof of the caudal midbrain and seen as a clear extension of the moderate to high density MAChR field of the ICo, is the substantia grisea centralis (GCt) (see figure 36: A 1-6, H 6-8). The high MAChR densities of the GCt continue in a caudal direction as far as the nucleus vestibularis medialis (VeM) and includes the nucleus immediately adjoining the VeM rostrally, the nucleus tegmenti dorsalis (TD) (Gudden). Caudally, the GCt runs into the fasiculus longtitudinalis medialis (FLM). During the transission of the GCt into the FLM, MAChR density rapidly declines, particularly over central aspects of the GCt, until central aspects of the FLM are completely devoid of MAChR (see figure 38: A 0-3, H 4-5 and figure 72 C). Rostral to the FML, the MAChR field of the GCt completely encloses the nucleus of Edinger-Westphal (EW) which is also devoid of receptor (see figure 37: A 3-4, H 8-9 and figures 67-68: L)-1, H 9-10). Immediately ventral and slightly caudal to the EW, there is an extremely complex field of very high density MAChR which corresponds to the nuclei nervi occulomotori, pars dorsalis (OMd), pars ventralis (OMv), nucleus nervi trochlearis (nIV) and mediocaudal aspects of the locus ceruleus (LoC) (see figures 37-40: A 2-4, H 7-9, but particularly figure 72 C). The OMv appears to be populated by lower densities of MAChR than the OMd, but this is probably a reflection of a greater number of unlabelled fibre bundles pervading the OMv. Ventral to the occulomotor complex and sharply distinguished against the low to moderate densities of adjoining posterior mesencephalic regions, there is an area almost completely devoid of MAChR, corresponding to the axons of the nervus occulomotorius (N III) and decussatio brachiorum conjunctivorum (DBC). Immediately lateral to the N III is a population of moderate to high density MAChR, roughly circular in outline and relatively undifferentiated by fibre bundles, which corresponds in form and position to the nucleus tegmenti pedunculo-pontinus, pars compacta (TPc)

(see figures 35 and 36: A 2-3, H 6-8). The nucleus sensorius principalis nervi trigemini (PrV), situated caudomedial to the IO- LLD complex (see above), is populated by high densities of muscarinic receptor (see figures 32-33: A 0 - P 1, H 7-8).

Hypothalamus.

The lateral hypothalamus (LHy) is populated by uniformly moderate densities of muscarinic receptor (see figure 37: A 4-7, H 5-6). More medial and posterior aspects of the hypothalamus (PLH) are low to moderately dense in MAChR, as is the area hypothalami posterius (AHP) which is differentiated only by the fibres of the infundibulum (IN). The area ventralis (Tsai) (AVT), which lies immediately caudal to the IN, is populated by moderate to high densities of muscarinic receptor (see figures 39-40: A 4·0, H 5-6).

Medulla oblongata or hindbrain.

Medial aspects of the hindbrain, situated ventral to the FLM, are taken up, almost exclusively, by the nuclei of the reticular system, ie. the nucleus reticularis pontis oralis (RPO), nucleus reticularis pontis caudalis, pars gigantocellularis (RPgc)and nucleus reticularis pontis caudalis (RP) (see figures 33-38: P 0-4 and A 0-3, H 3-6). The reticular system (RT) of the chick is in general populated by moderate densities of MAChR. The RPO is populated by moderate to high densities of receptor and largely encloses the lower MAChR field of the RPgc and RT . More medially, the RT is populated by patches of high receptor density (see figure 38, MRF 30) which, however may be an artifact of autoradiographic proceedure. Caudoventral to the RP ,the nucleus paragigantocellularis lateralis (PCL)and olivary nucleus (OI) are populated by moderate to high densities of muscarinic cholinergic receptor . Dorsal and medial to the PCL, the nucleus et tractus descendens nervi trigemini (TTD) is almost devoid of MAChR (see figures 35-38), as is the funiculus ventralis (FV).

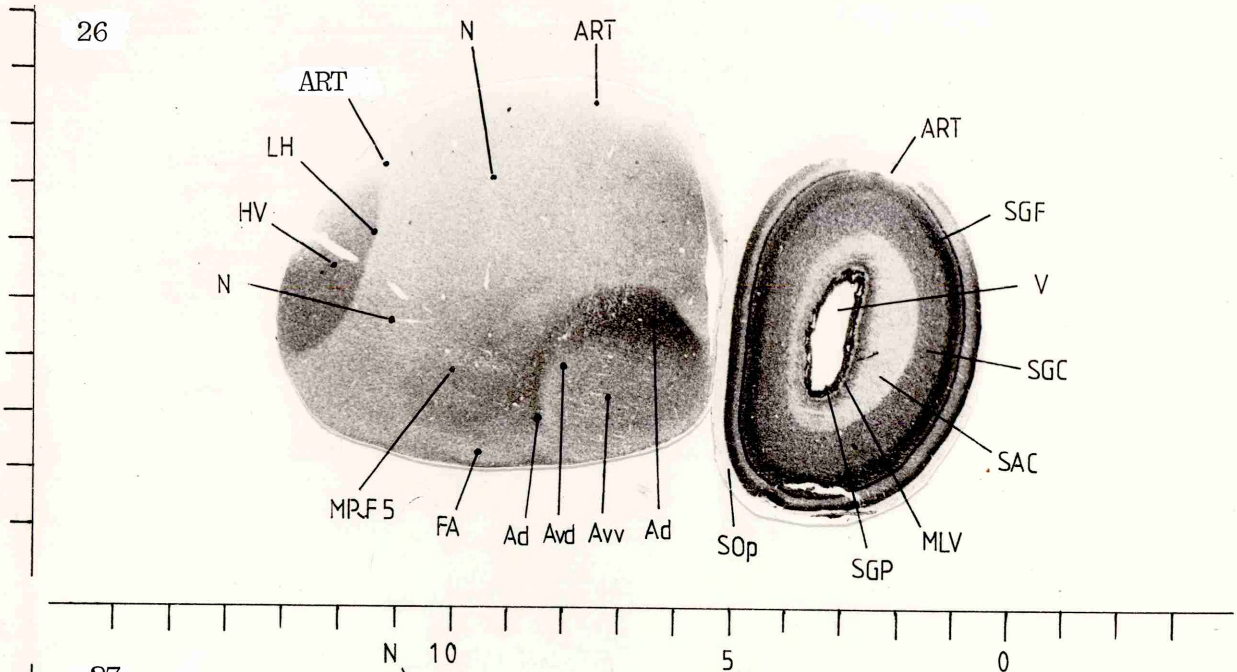
Situated dorsal to the TTD and FV is a generally very high density complex of MAChR corresponding to the column nuclei, nucleus vestibularis medialis (VeM), nucleus intermedius (IM), nucleus solitarius (S), nucleus motorius dorsalis nervi vagi (nX)and nucleus nervi hypoglossi (nXII) (see figures 35- 38: P 2-5, H 6-8). The complexity of patterning and distribution of MAChR over these nervi-nuclei is such as to prohibit a detailed description here; suffice to say that very high densities of muscarinic receptor are patterned as a network, the MAChR densities interwinding between areas devoid of receptor which probably correspond to bundles of descending fibres.

Figures 26 to 38 . *A series of light field photomicrographs of parasagittal autoradiographed brain sections (10 µm thick) of the 5 week post hatch chick brain, showing the distribution and density of silver grains reflecting the distribution and regional relative concentrations of* $[^3H]$ *PrBCM labelled MUSCARINIC CHOLINERGIC RECEPTOR. Ordinate and abscissa are approximate stereotaxic coordinates. All the sections shown are from one brain, and each section from 26 to 38 is medial to the preceeding section. Some of the sections appear generally darker or lighter than others, as a result of a combination of factors including differences in autoradiogram development times (not exposure), changes in the 'strength of photographic developer during development, and differences in photographic reproduction of these figure photomicrographs. As for figures 23 to 25, the sections shown in figures 26 to 38 have not been counter stained, the image is of silver grain distribution and regional density only. These sections have been 'wet emulsion coated (see methods).*

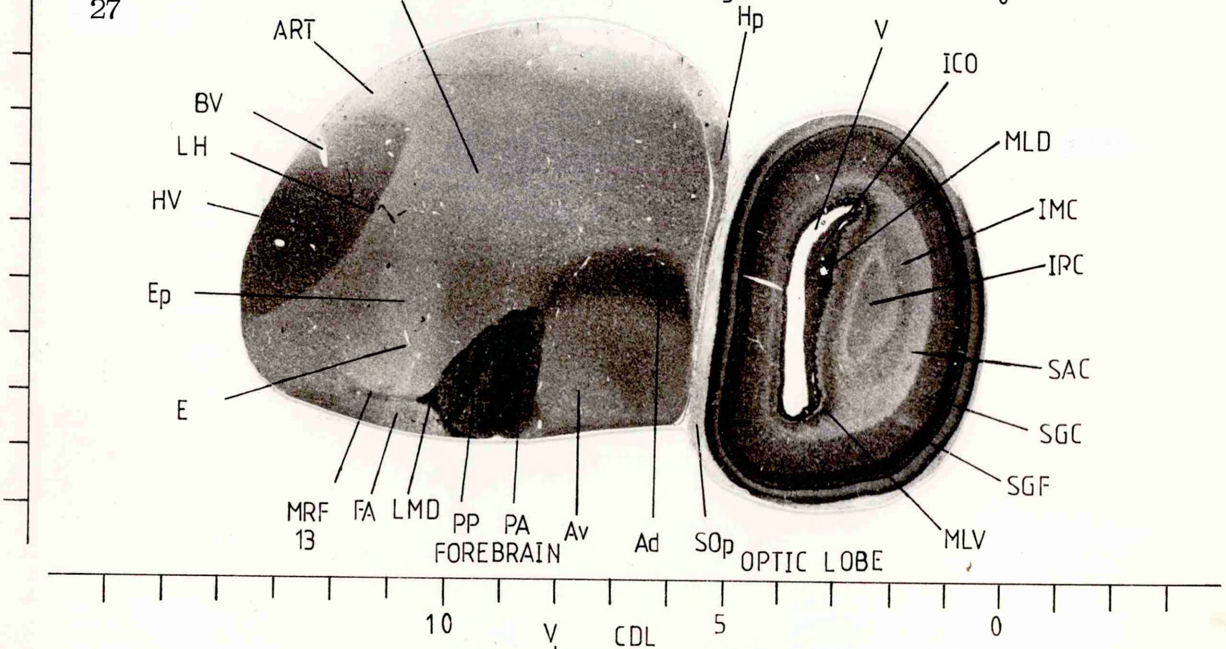
Figures 26 to 28

MUSCARINIC RECEPTOR DISTRIBUTION: 5 WEEK POST HATCH
LIGAND ^{3}[H] PrBCM.

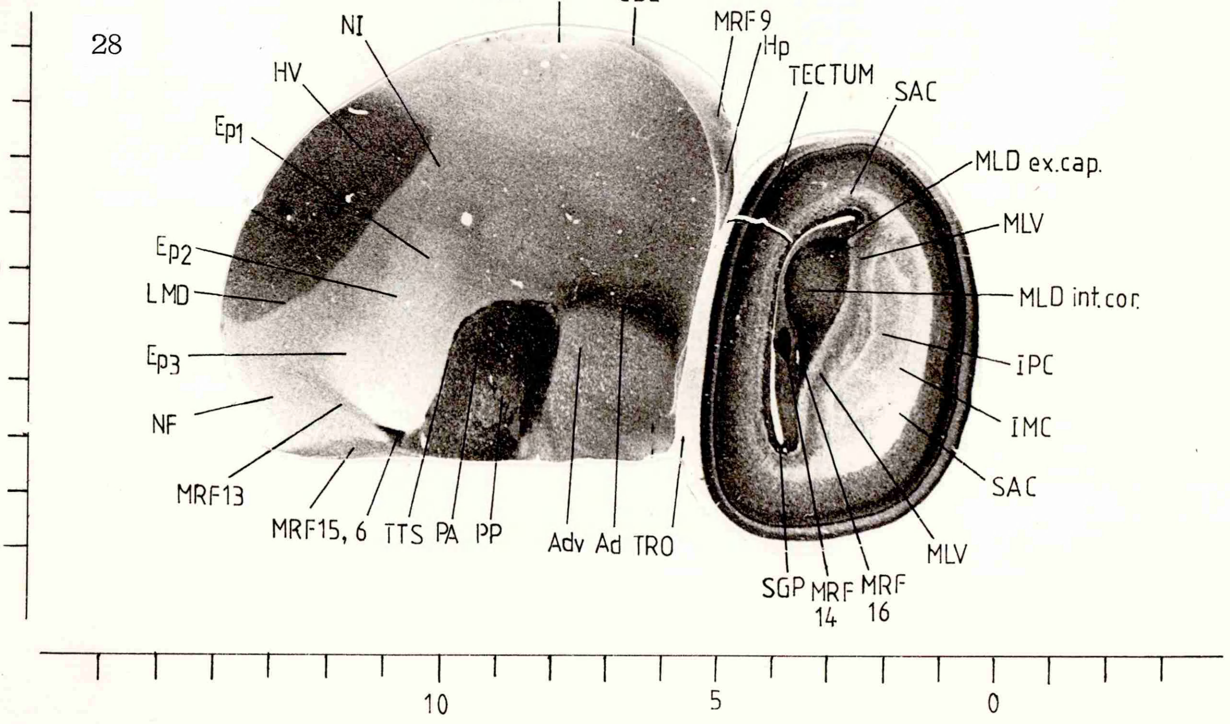
26


N
ART
ART
LH
HV
N
ART
SGF
V
SGC
SAC
MLV
SGP
SOp
MRF 5
FA
Ad
Avd
Avv
Ad
10
5
10
5
0

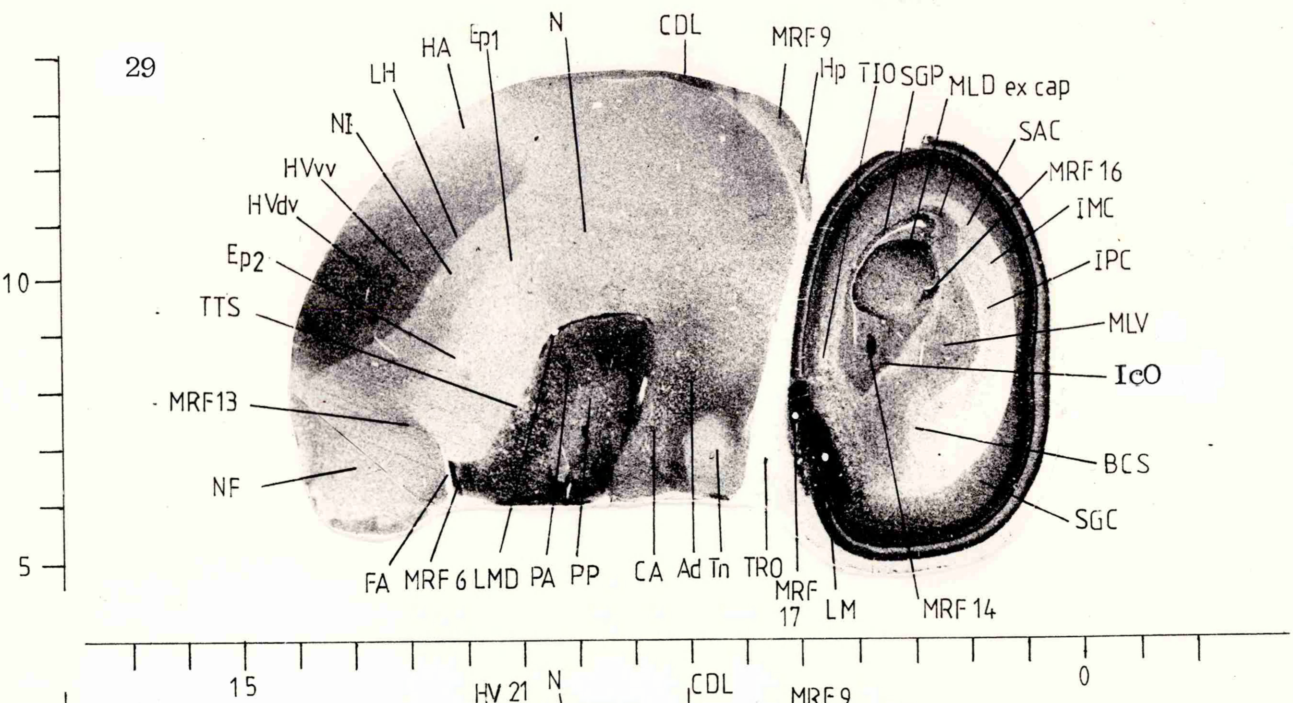
27


N
Hp
V
ICO
ART
BV
LH
HV
MLD
IMC
IPC
Ep
SAC
E
SGC
SGF
MRF 13
FA
LMD
PP
PA
Av
FOREBRAIN
Ad
SOp
OPTIC LOBE
MLV
10
5
10
5
0

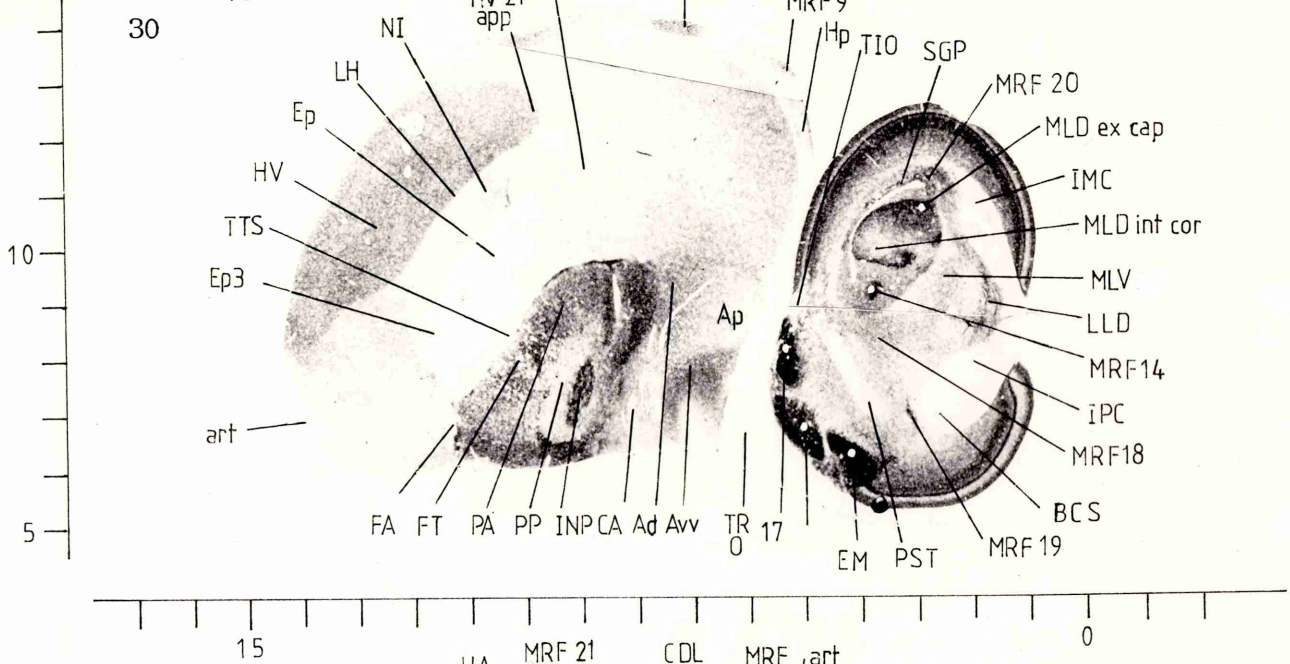
28


V
CDL
NI
MRF 9
Hp
HV
TECTUM
SAC
Ep1
MLD ex.cap.
MLV
Ep2
LMD
MLD int.cor.
Ep3
IPC
NF
IMC
MRF13
SAC
MRF15, 6
TTS
PA
PP
Adv
Ad
TRO
MLV
SGP
MRF 14
MRF 16
10
5
10
5
0

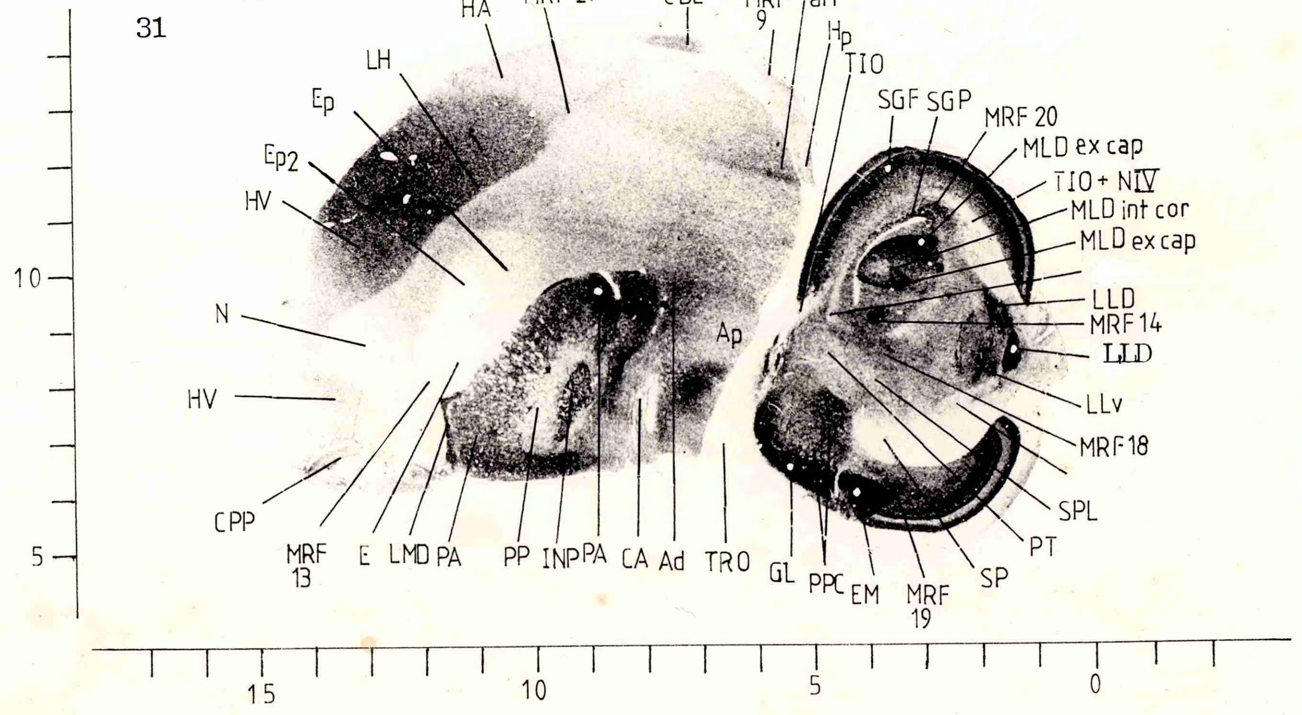
29


N
CDL
Ep1
HA
LH
NI
HVvv
HVdv
Ep2
TTS
MRF13
NF
FA
MRF 6
LMD
PA
PP
CA
Ad
Tn
TRO
MRF 17
LM
MRF 14
MRF 9
Hp
TIO
SGP
MLD ex cap
SAC
MRF 16
IMC
IPC
MLV
IcO
BCS
SGC
10
5
15
0

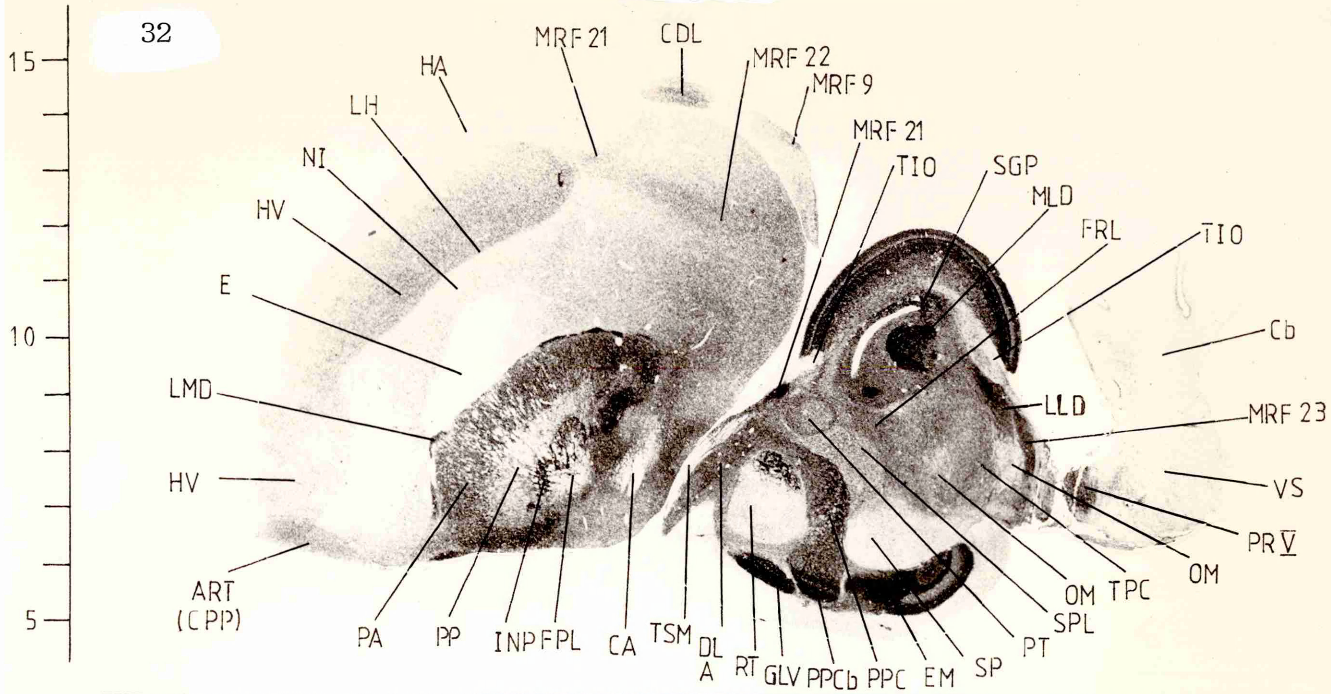
30


HV 21 app
N
CDL
NI
LH
Ep
HV
TTS
Ep3
art
FA
FT
PA
PP
INP
CA
Ad
Avv
Ap
TR O
17
EM
PST
MRF 9
Hp
TIO
SGP
MRF 20
MLD ex cap
IMC
MLD int cor
MLV
LLD
MRF14
IPC
MRF18
BCS
MRF 19
10
5
15
0

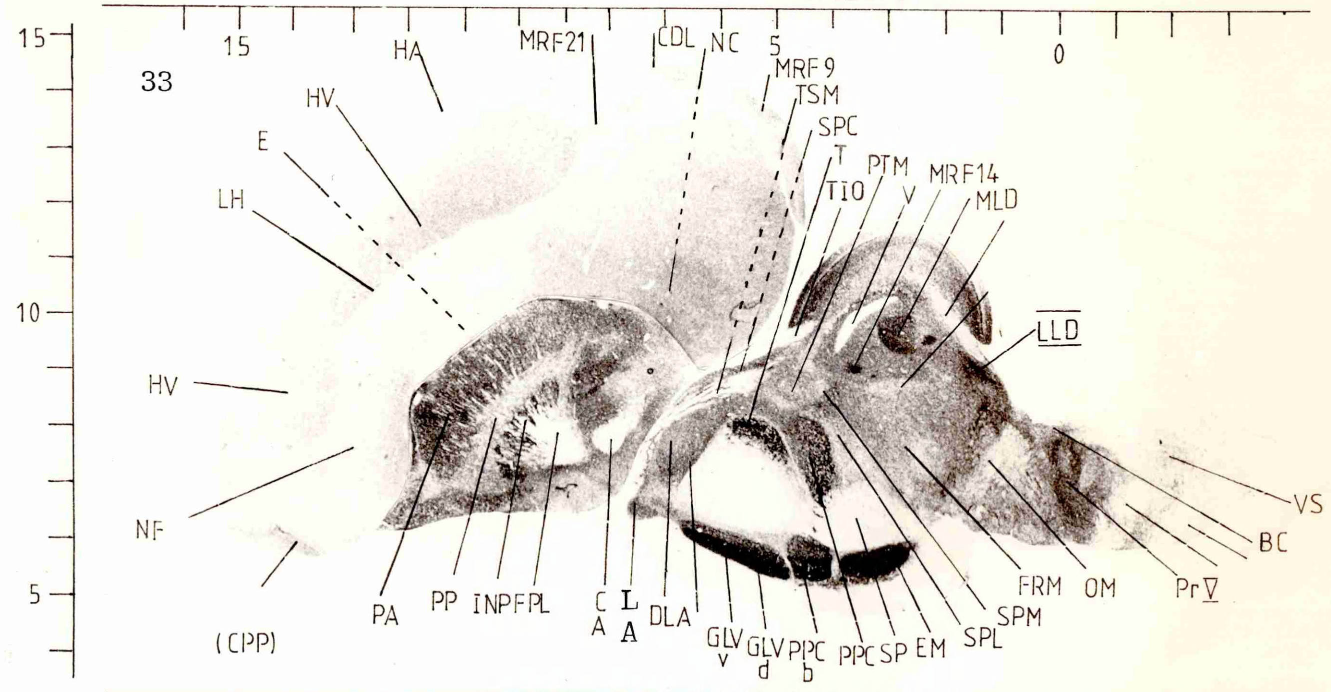
31


HA
MRF 21
CDL
MRF 9
art
Hp
TIO
LH
Ep
Ep2
HV
N
HV
CPP
MRF 13
E
LMD
PA
PP
INP
PA
CA
Ad
Ap
TRO
GL
PPC
EM
MRF 19
SGF
SGP
MRF 20
MLD ex cap
TIO + NIV
MLD int cor
MLD ex cap
LLD
MRF 14
LLD
LLv
MRF18
SPL
PT
SP
10
5
15
10
5
0

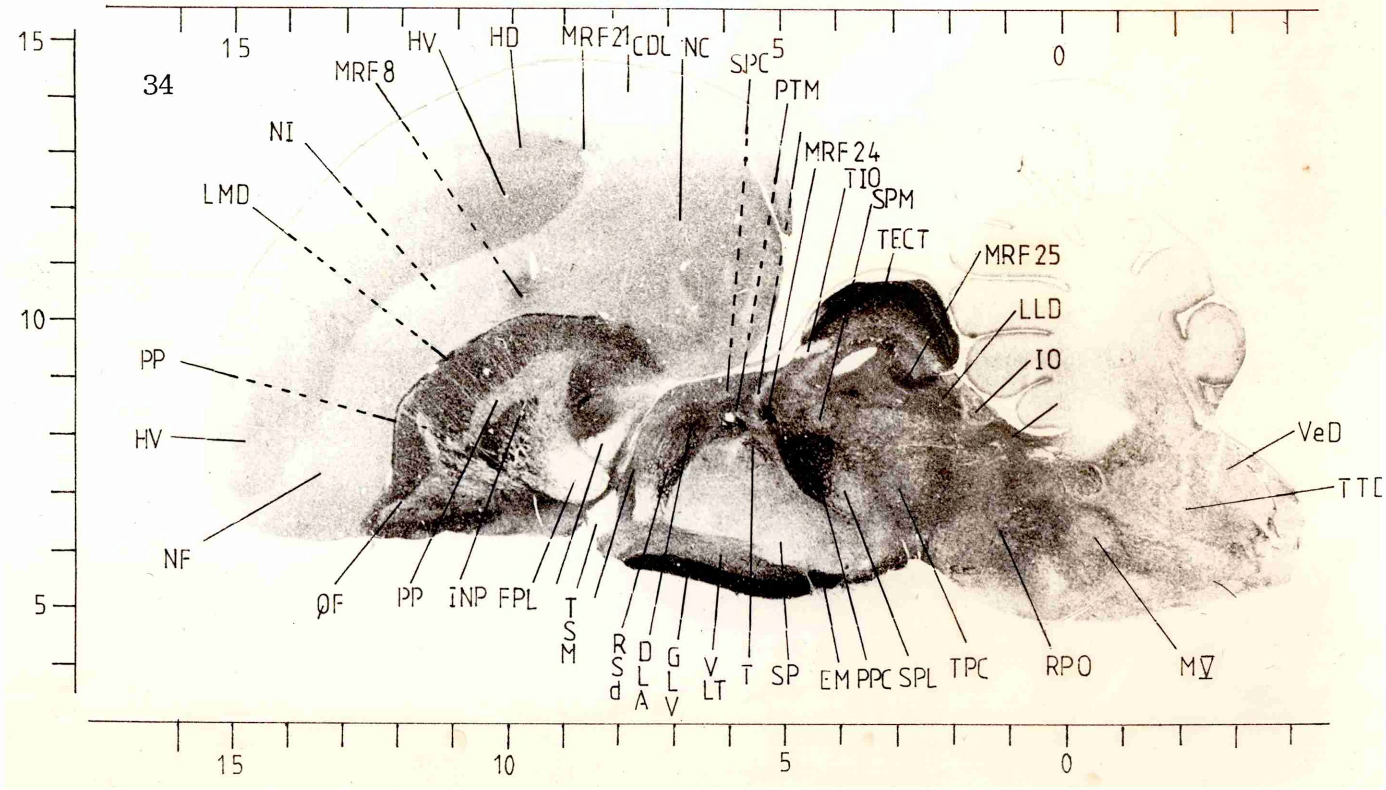
32


15
10
5
HA
MRF 21
CDL
MRF 22
MRF 9
LH
NI
MRF 21
TIO
SGP
MLD
FRL
HV
E
TIO
Cb
LMD
LLD
MRF 23
HV
VS
PR V̄
ART
(CPP)
PA
PP
INP
FPL
CA
TSM
DL A
RT
GLV
PPCb
PPC
EM
SP
PT
SPL
OM
TPC
OM

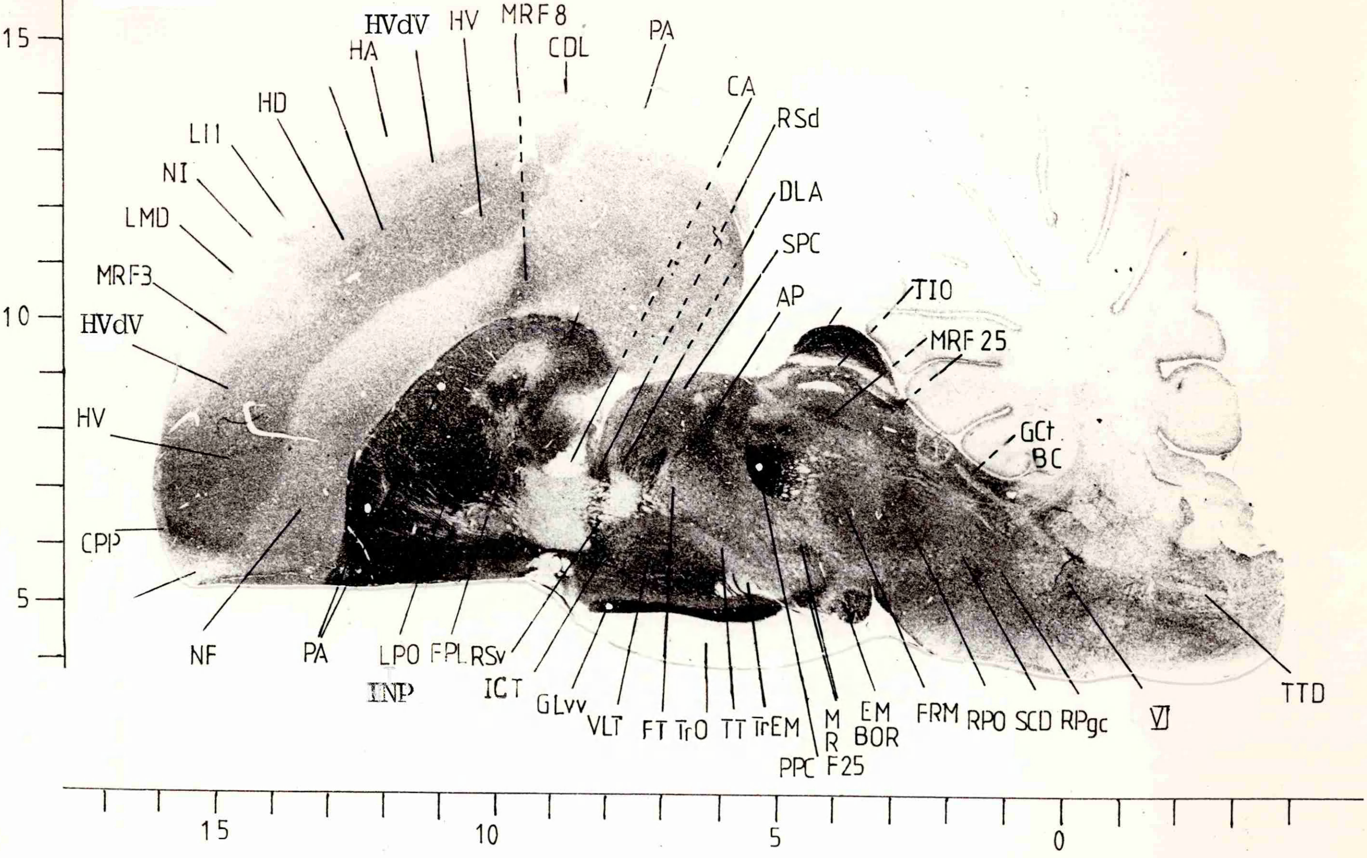
33


15
5
0
15
10
5
HA
MRF21
CDL
NC
MRF 9
TSM
HV
SPC
T
E
PTM
TIO
MRF14
LH
V
MLD
LLD
HV
NF
VS
BC
PA
PP
INP
FPL
C A
L A
DLA
GLV v
GLV d
PPC b
PPC
SP
EM
SPL
SPM
FRM
OM
Pr V̄
(CPP)

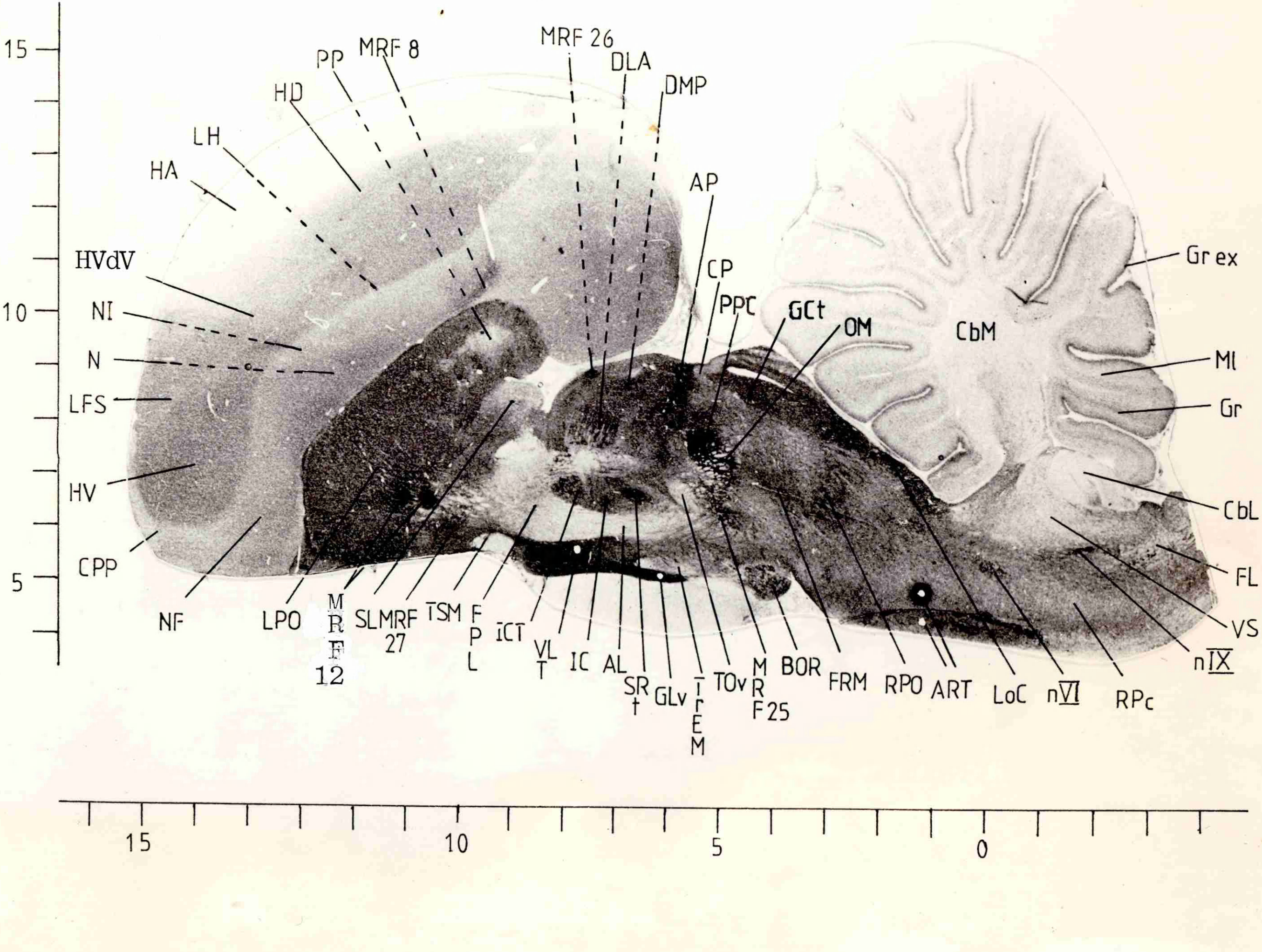
34


15
5
0
15
10
5
HV
HD
MRF21
CDL
NC
SPC
MRF 8
PTM
NI
MRF 24
TIO
LMD
SPM
TECT
MRF 25
LLD
IO
PP
HV
VeD
TTC
NF
OF
PP
INP
FPL
TSM
RSd
DLA
GLV
VLT
T
SP
EM
PPC
SPL
TPC
RPO
MV̄
15
10
5
0

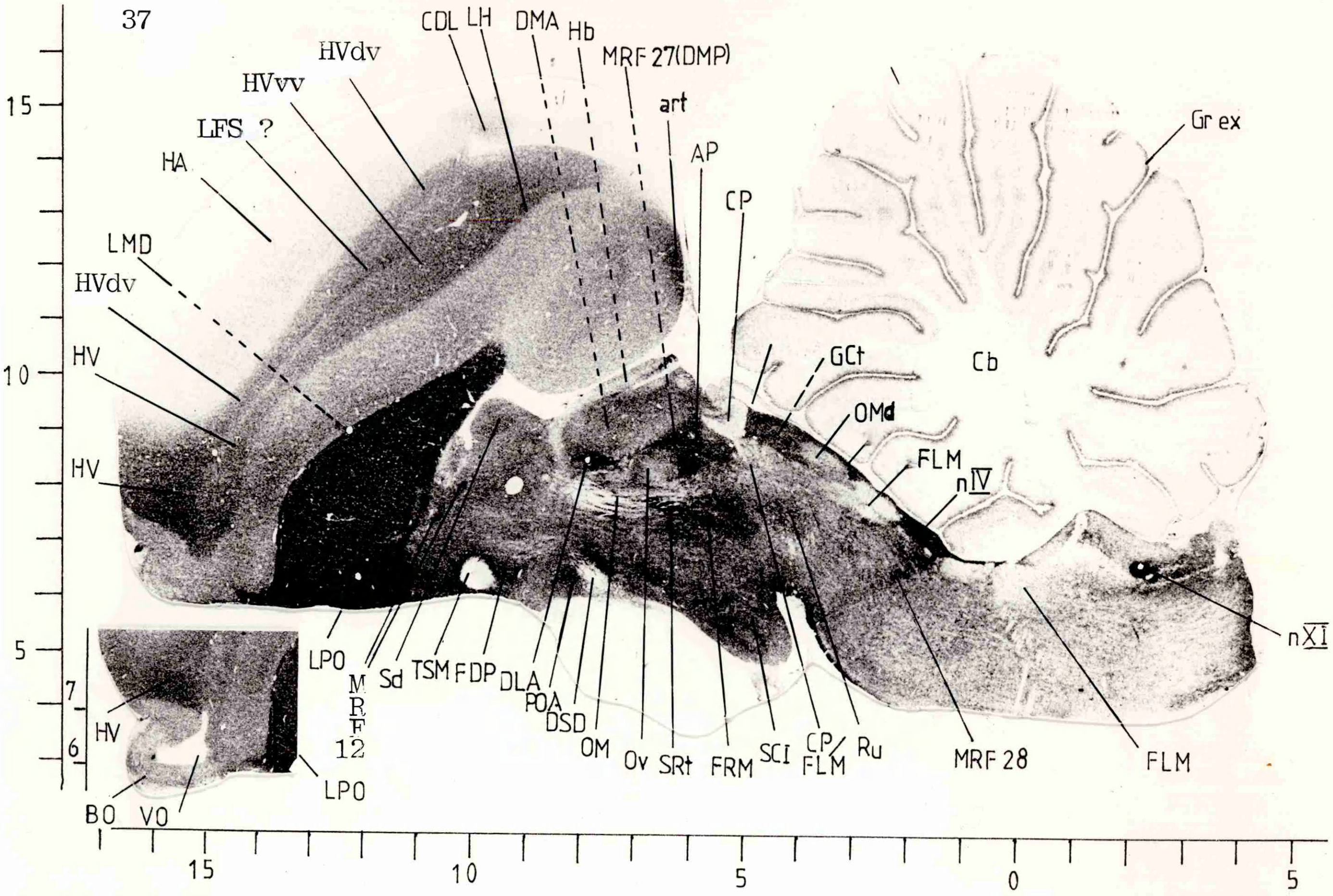
35


HVdV
HV
MRF 8
CDL
PA
HA
HD
LI1
NI
LMD
MRF3
HVdV
HV
CPP
CA
RSd
DLA
SPC
AP
TIO
MRF 25
GCt
BC
NF
PA
LPO
FPL
RSv
INP
ICT
GLvv
VLT
FT
TrO
TT
TrEM
M R F25
PPC
EM
BOR
FRM
RPO
SCD
RPgc
$\underline{\overline{VI}}$
TTD
15
10
5
15
10
5
0

36


PP
MRF 8
HD
LH
HA
HVdV
NI
N
LFS
HV
CPP
MRF 26
DLA
DMP
AP
CP
PPC
GCt
OM
CbM
Gr ex
Ml
Gr
CbL
FL
VS
n$\underline{\overline{IX}}$
NF
LPO
M R F 12
SL
MRF 27
TSM
F P L
ICT
VL T
IC
AL
SR t
GLv
Tr E M
TOv
M R F25
BOR
FRM
RPO
ART
LoC
n$\underline{\overline{VI}}$
RPc
15
10
5
15
10
5
0

37


15
10
5
7
6
CDL
LH
DMA
Hb
MRF 27(DMP)
HVdv
HVvv
LFS ?
HA
art
AP
CP
Gr ex
LMD
HVdv
HV
HV
GCt
Cb
OMd
FLM
nIV
nXI
LPO
M
R
F
12
Sd
TSM
FDP
DLA
POA
DSD
OM
Ov
SRt
FRM
SCI
CP
FLM
Ru
MRF 28
FLM
HV
BO
VO
LPO
15
10
5
0
5

38

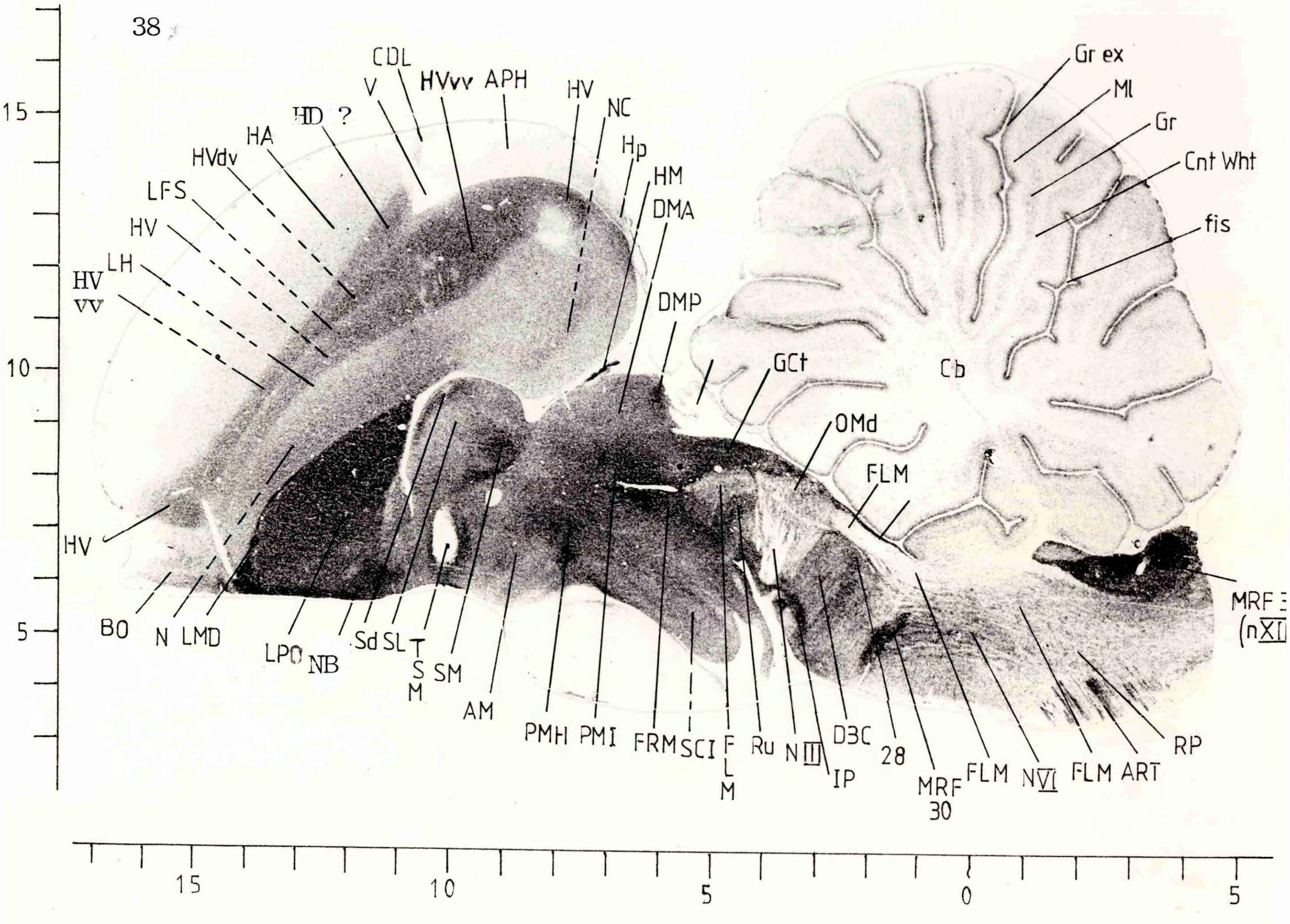
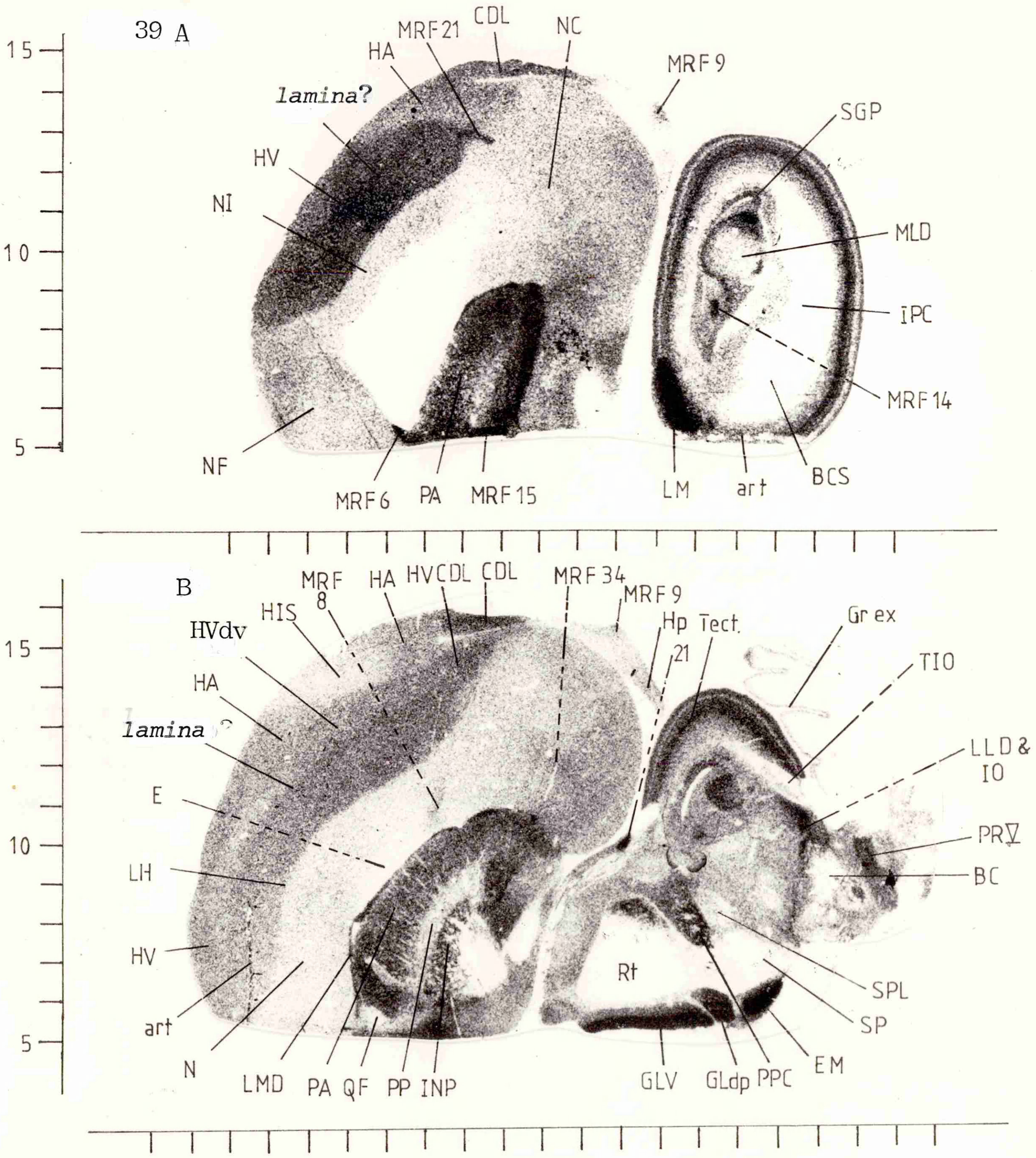

15
10
5
CDL
V
HVvv
APH
HD ?
HA
HVdv
LFS
HV
LH
HV
vv
HV
NC
Hp
HM
DMA
DMP
GCt
Cb
OMd
FLM
Gr ex
Ml
Gr
Cnt Wht
fis
MRF 3
(nXI
HV
BO
N
LMD
LPO
NB
Sd
SL
T
S
M
SM
AM
PMH
PMI
FRM
SCI
F
L
M
Ru
NIII
IP
D3C
28
MRF
30
FLM
NVI
FLM
ART
RP
15
10
5
0
5

Figure 39A and B. Light field photomicrographs of the pattern of silver gr
and regional density in LKB tritium film exposed and app
to parasagittal brain sections of the 5 week post hatch chick brain labelled
^{3}[H] Quinuclidinyl benzilate to Muscarinic Cholinergic Receptor.

Figure 40A and B. Light field photomicrographs of the pattern of
silver grains and regional density in Wet emulsio
coated frontal autoradiographed brain sections of the 5 weeks post
hatch chick brain labelled by ^{3}[H] Propylbenzilyl choline mustard to
Muscarin Cholinergic Receptor.


39 A
15
10
5
lamina?
HA
MRF 21
CDL
NC
MRF 9
SGP
HV
NI
MLD
IPC
MRF 14
NF
MRF 6
PA
MRF 15
LM
art
BCS
B
15
10
5
MRF 8
HA
HVCDL
CDL
MRF 34
MRF 9
HIS
HVdv
Hp
Tect.
21
Gr ex
TIO
HA
lamina?
LLD & IO
E
PR V
LH
BC
HV
art
N
LMD
PA
QF
PP
INP
Rt
SPL
SP
GLV
GLdp
PPC
EM

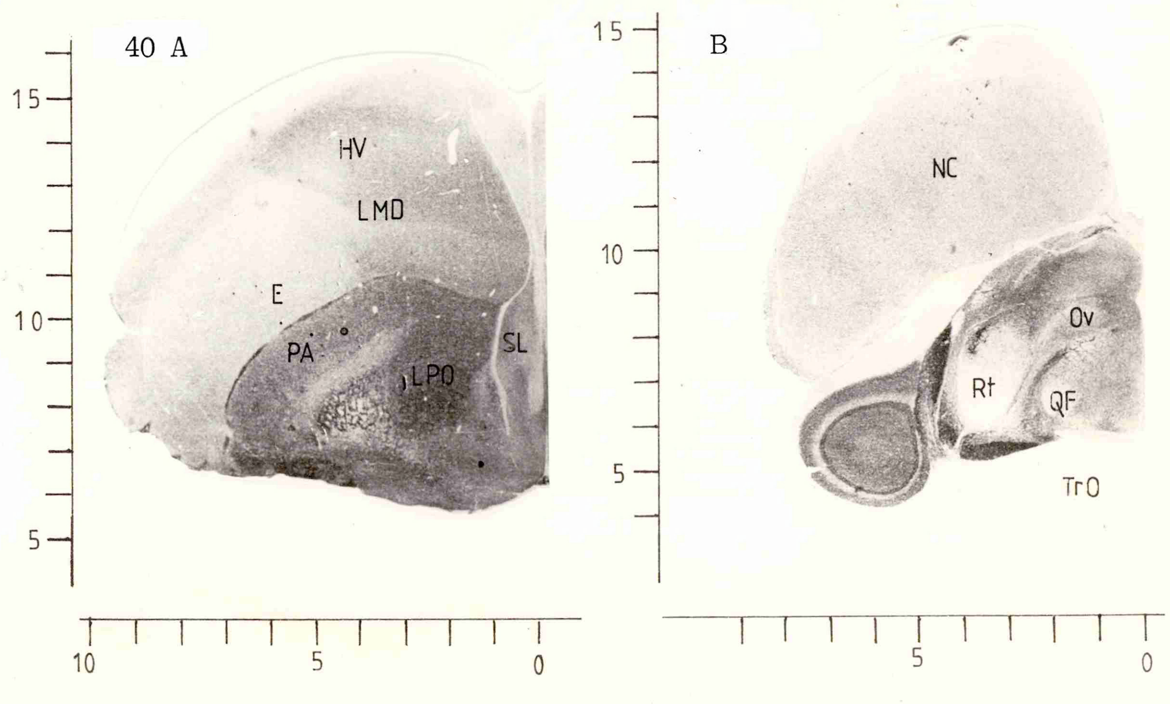

40 A
15
10
5
HV
LMD
E
PA
LPO
SL
10
5
0
B
15
10
5
NC
Ov
Rt
QF
TrO
5
0

Figure 41 to 70. *A series of photomicrographs of the pattern of silver grain distribution and density in LKB tritium film exposed and apposed to frontal brain sections of the 5 week post hatch chick brain labelled by* ^{3}[H] *l* *Quinuclidinyl benzilate* *to MUSCARINIC CHOLINERGIC RECEPTOR. As in figures 39 A and B, figures 41 to 70 are photographs of silver grain density alone,with no undelying tissue section. The contrast of these sections is considerably more marked than those labelled by* ^{3}[H] *PrBCM and 'wet emulsion coated'. This is primarily the result of the greater 'sensitivity' of LKB tritium film. However it should be noted that silver grain density in the hyperstriatum ventrale (HV) is considerably greater (apparently) in* ^{3}[H] *l QNB labelled LKB tritium film exposed sections than for* ^{3}*H PrBCM labelled and 'wet emulsion 'coated autoradiograms.*

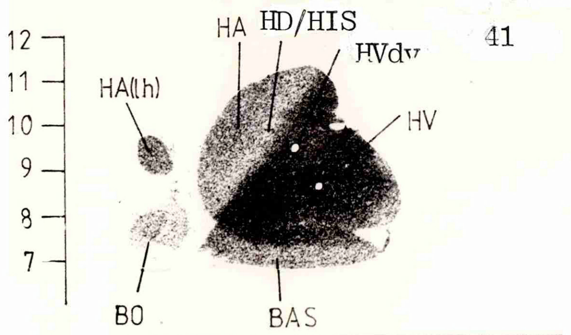
Figure 71 A and B. *Light field photomicrographs of parasagittal autoradiographed sections across the optic lobe of the 5 week post hatch chick brain showing the diastribution of* ^{3}[H] *PrBCM labelled MUSCARINIC RECEPTOR. Figure 71 B is medial to 71 A.The lighter densities of silver grains towards dorsal aspects of the optic lobe is probably an artifact of proceedure. Wet emulsion coated. Scale bar = 1 mm .*

Figures 41 to 70

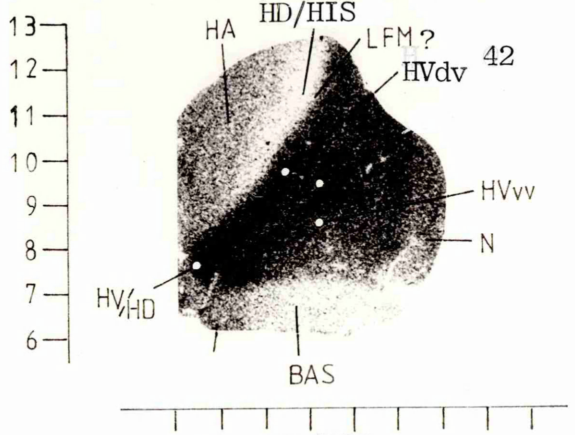
MUSCARINIC RECEPTOR DISTRIBUTION : 5 WEEKS POST HATCH:

FRONTAL: LIGAND ^{3}H 1 QNB

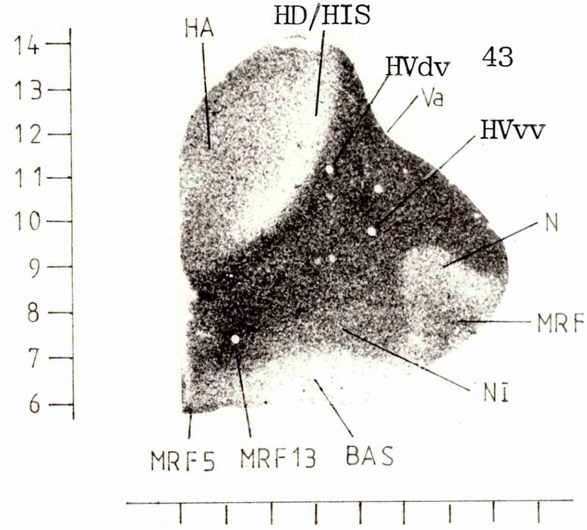
41


HA
HD/HIS
HVdv
HA(lh)
HV
BO
BAS
12
11
10
9
8
7

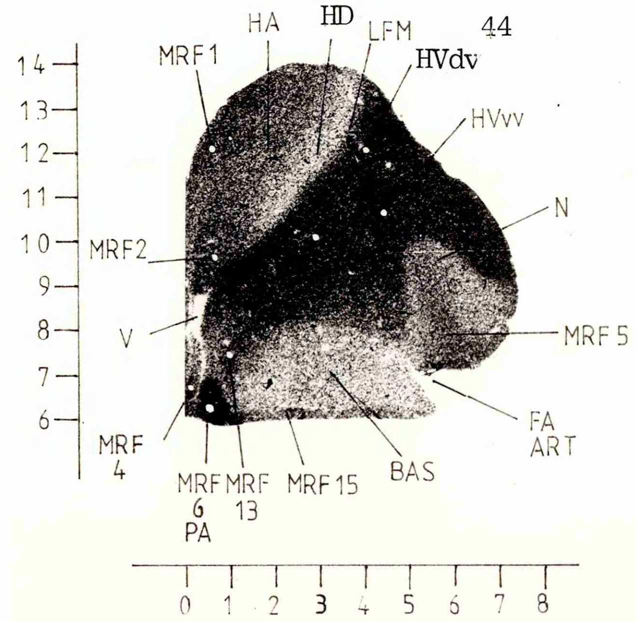
42


HA
HD/HIS
LFM ?
HVdv
HVvv
N
HV/HD
BAS
13
12
11
10
9
8
7
6

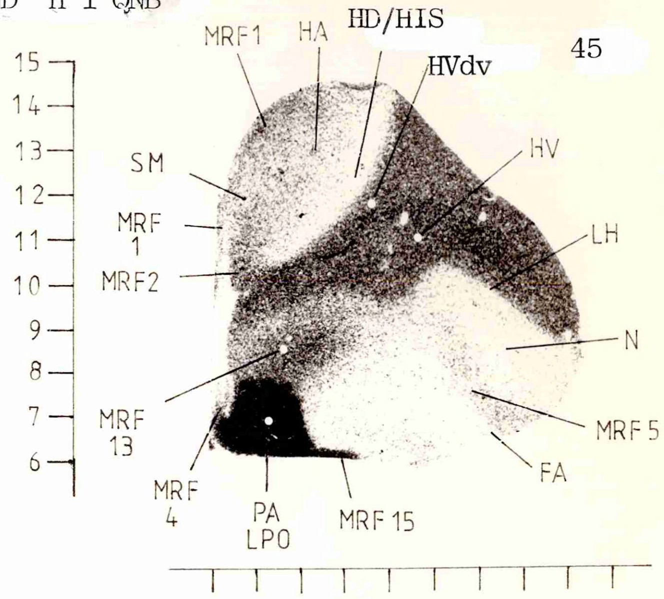
43


HA
HD/HIS
HVdv
Va
HVvv
N
MRF 5
NI
MRF5
MRF13
BAS
14
13
12
11
10
9
8
7
6

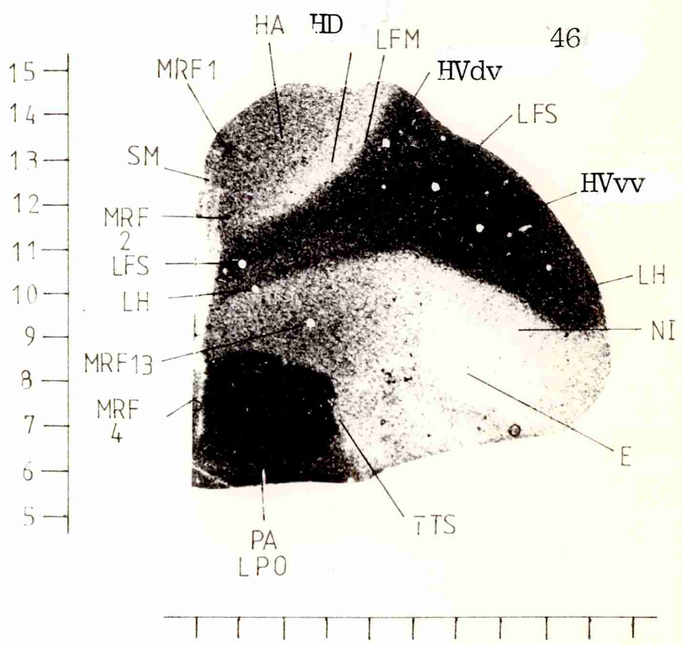
44


HA
HD
LFM
HVdv
MRF1
HVvv
N
MRF2
V
MRF 5
FA ART
MRF 4
MRF 6 PA
MRF 13
MRF15
BAS
14
13
12
11
10
9
8
7
6
0
1
2
3
4
5
6
7
8

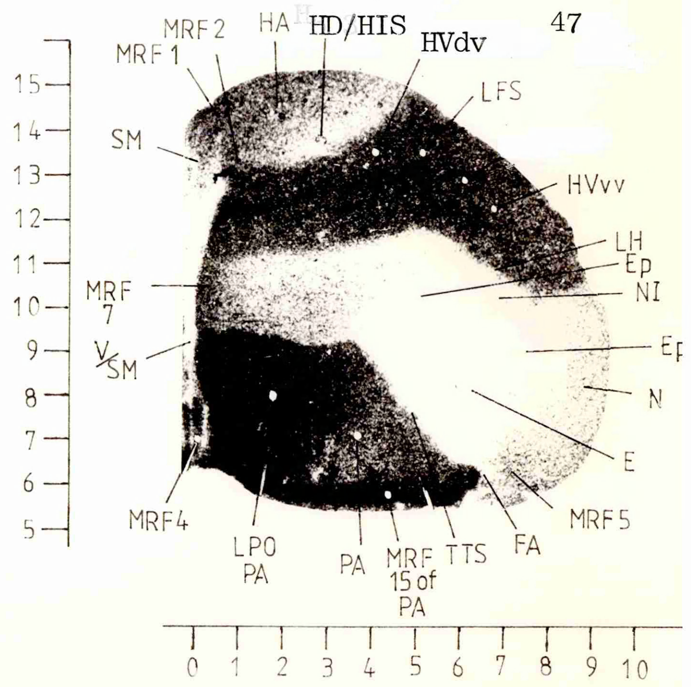
45

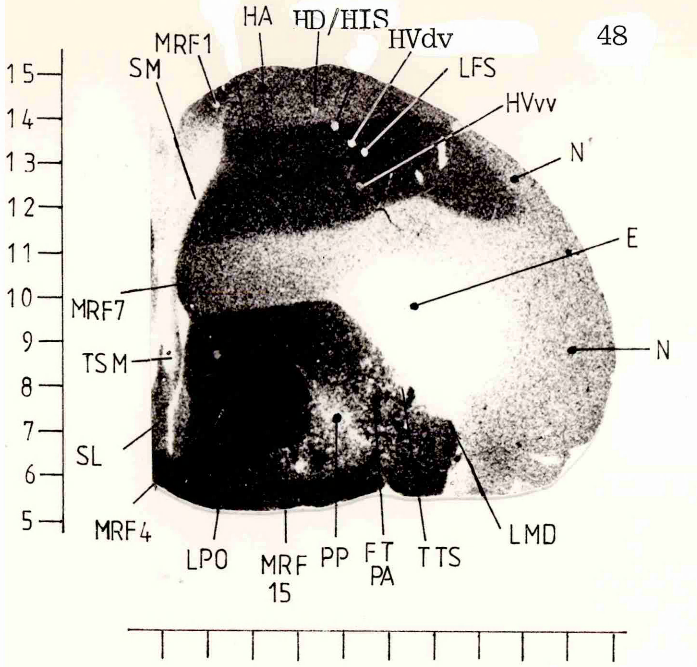

MRF1
HA
HD/HIS
HVdv
SM
HV
MRF 1
LH
MRF2
N
MRF 13
MRF 5
FA
MRF 4
PA LPO
MRF 15
15
14
13
12
11
10
9
8
7
6

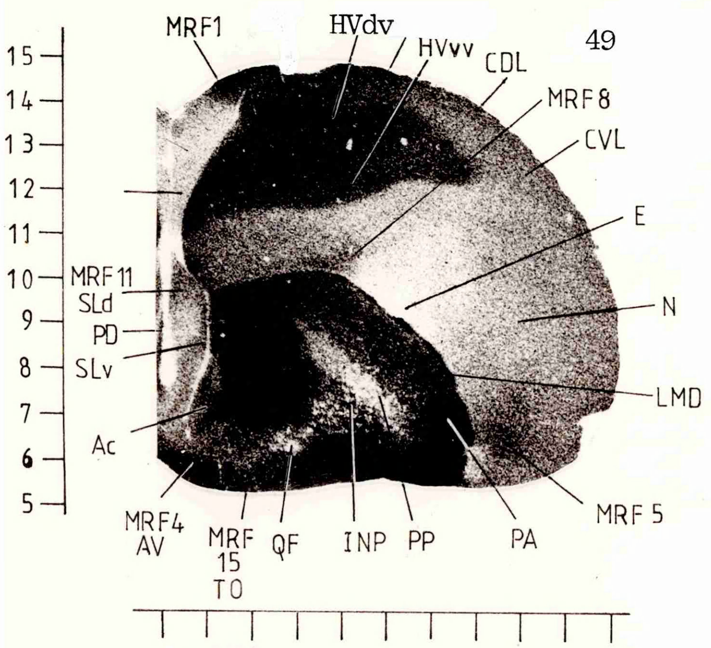
46

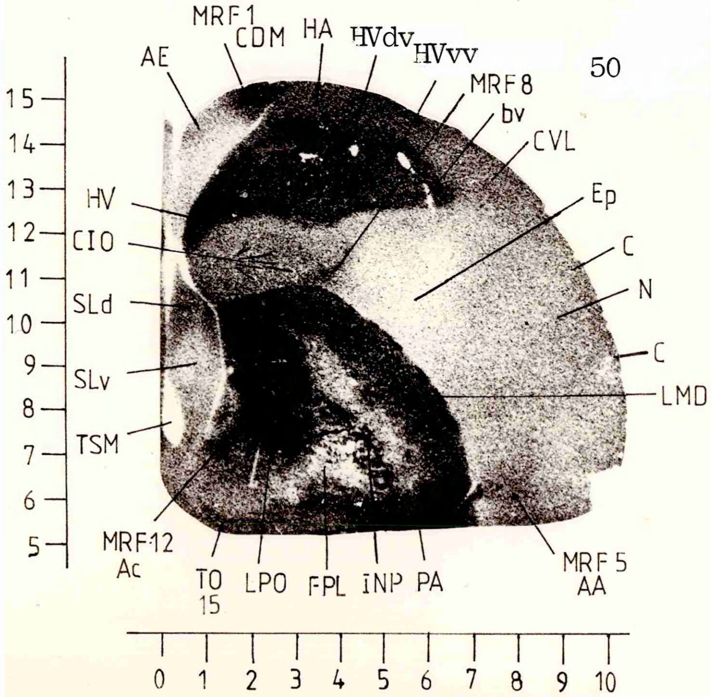

HA
HD
LFM
HVdv
MRF1
LFS
SM
HVvv
MRF 2
LFS
LH
LH
NI
MRF13
MRF 4
E
TTS
PA LPO
15
14
13
12
11
10
9
8
7
6
5

47


MRF 2
MRF 1
HA
HD/HIS
HVdv
LFS
SM
HVvv
LH
Ep
NI
MRF 7
Ep
V/SM
N
E
MRF4
LPO PA
PA
MRF 15 of PA
TTS
FA
MRF5
15
14
13
12
11
10
9
8
7
6
5
0
1
2
3
4
5
6
7
8
9
10


48
HA
HD/HIS
HVdv
MRF1
SM
LFS
HVvv
N
E
N
MRF7
TSM
SL
MRF4
LPO
MRF 15
PP
FT PA
TTS
LMD
15
14
13
12
11
10
9
8
7
6
5


49
MRF1
HVdv
HVvv
CDL
MRF8
CVL
E
N
LMD
MRF11
SLd
PD
SLv
Ac
MRF4 AV
MRF 15 TO
QF
INP
PP
PA
MRF 5
15
14
13
12
11
10
9
8
7
6
5


50
MRF1
AE
CDM
HA
HVdv
HVvv
MRF8
bv
CVL
Ep
C
N
C
LMD
HV
CIO
SLd
SLv
TSM
MRF12 Ac
TO 15
LPO
FPL
INP
PA
MRF 5 AA
15
14
13
12
11
10
9
8
7
6
5
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1
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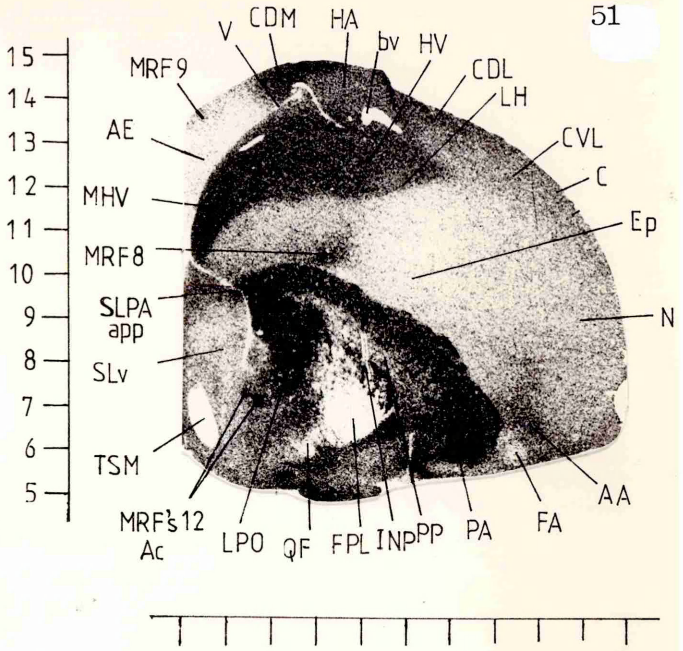
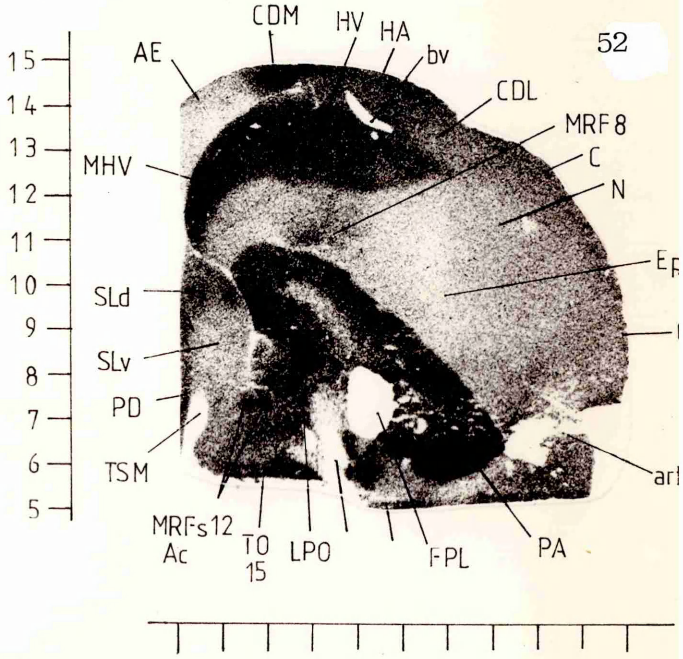
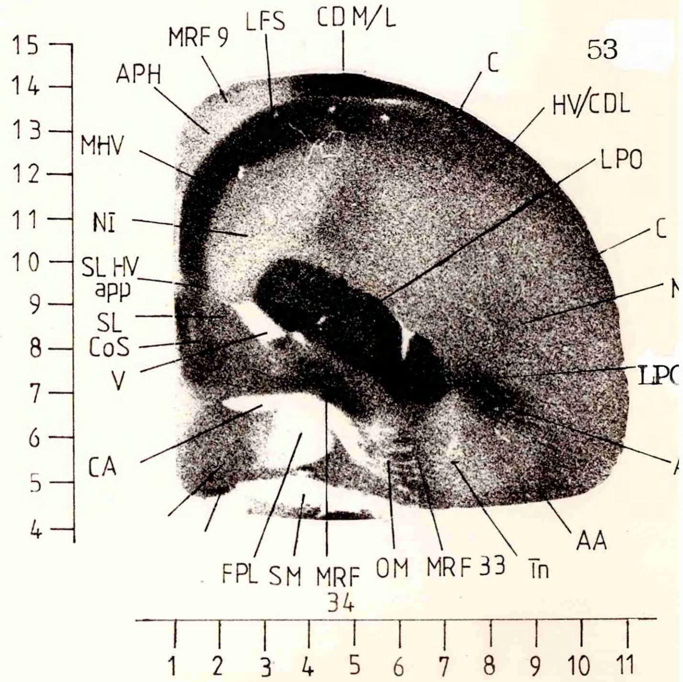
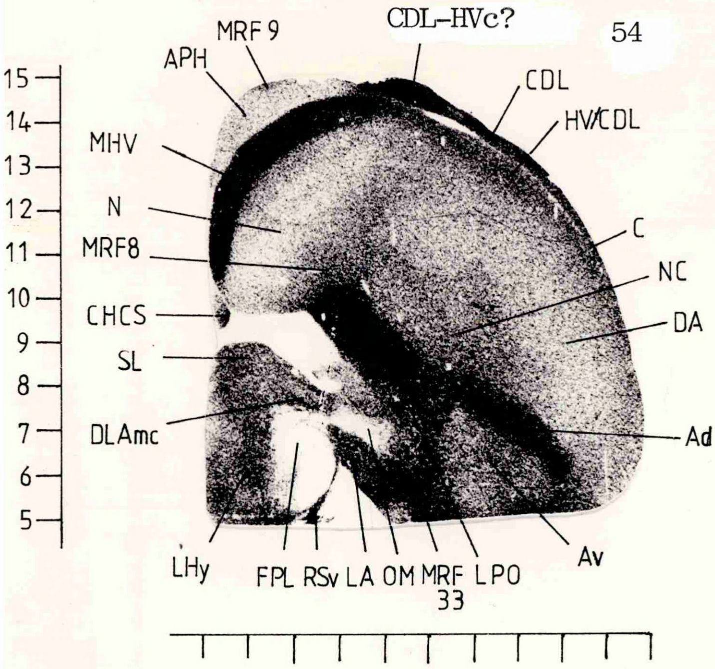
4
5
6
7
8
9
10


51
V
CDM
HA
bv
HV
CDL
LH
MRF9
AE
CVL
C
MHV
Ep
MRF8
SLPA app
N
SLv
TSM
MRF's 12 Ac
LPO
QF
FPL
INP
PP
PA
FA
AA
15
14
13
12
11
10
9
8
7
6
5


52
CDM
HV
HA
bv
AE
CDL
MRF8
C
MHV
N
Ep
SLd
SLv
PD
TSM
art
MRFs12 Ac
TO 15
LPO
FPL
PA
15
14
13
12
11
10
9
8
7
6
5

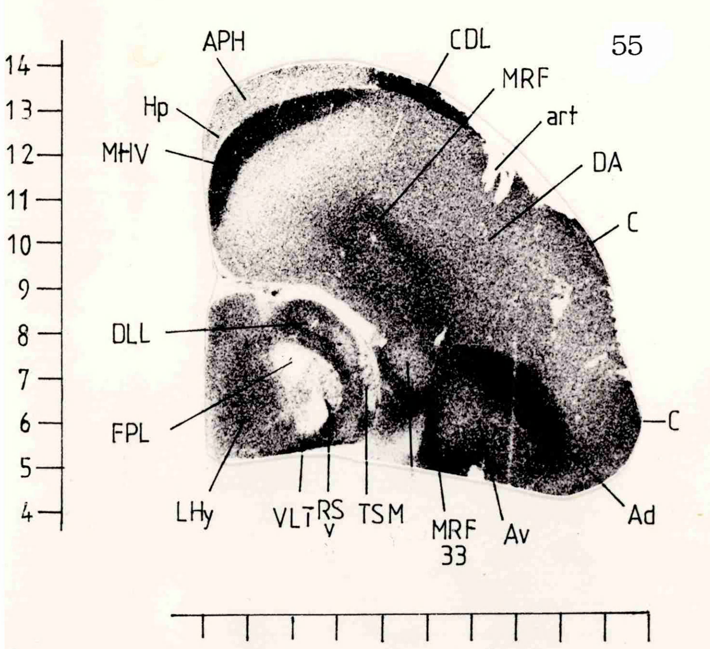

53
MRF 9
LFS
CDM/L
APH
C
HV/CDL
MHV
LPO
NI
C
SL HV app
SL
CoS
V
LPO
CA
AA
FPL
SM
MRF 34
OM
MRF 33
Tn
15
14
13
12
11
10
9
8
7
6
5
4
1
2
3
4
5
6
7
8
9
10
11

54



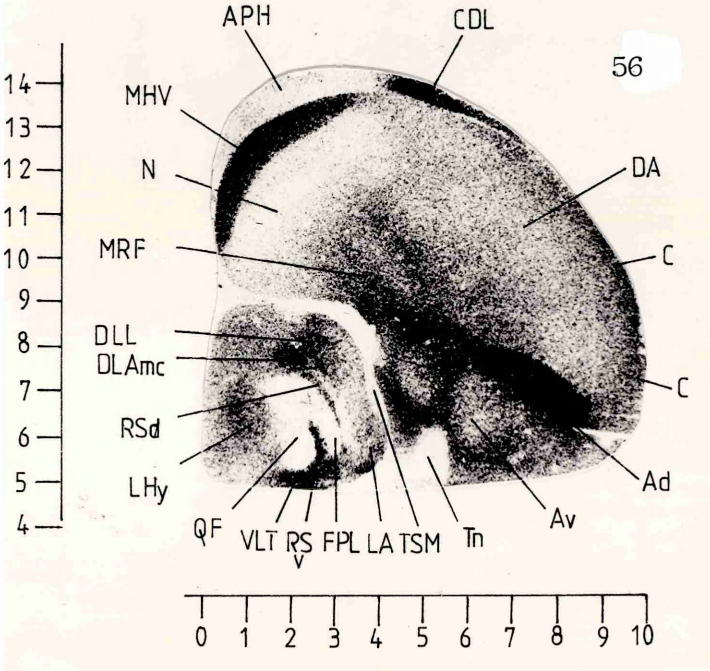
CDL–HVc?
MRF 9
APH
CDL
HV/CDL
MHV
N
MRF8
C
NC
CHCS
DA
SL
DLAmc
Ad
LHy
FPL
RSv
LA
OM
MRF 33
LPO
Av
15
14
13
12
11
10
9
8
7
6
5

55



APH
CDL
MRF
art
Hp
MHV
DA
C
DLL
FPL
C
LHy
VLT
RS v
TSM
MRF 33
Av
Ad
14
13
12
11
10
9
8
7
6
5
4

56



APH
CDL
MHV
N
DA
MRF
C
DLL
DLAmc
C
RSd
LHy
QF
VLT
RS v
FPL
LA
TSM
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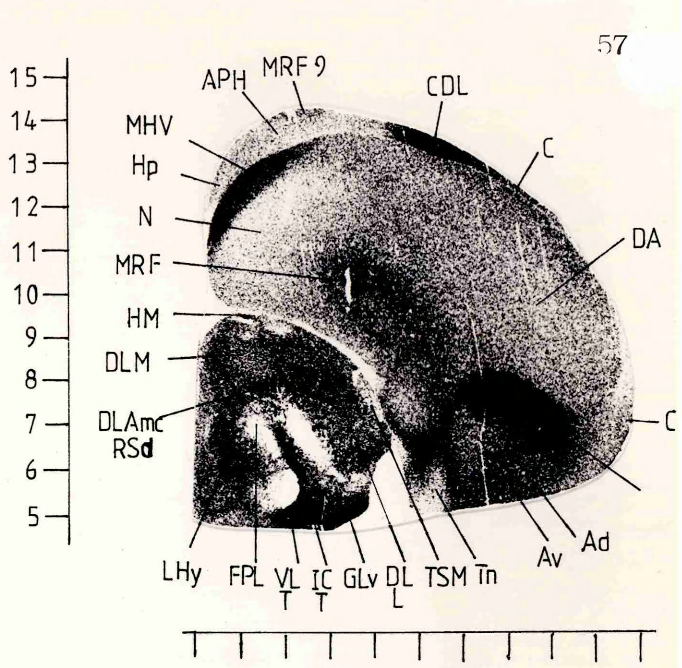

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MRF 9
CDL
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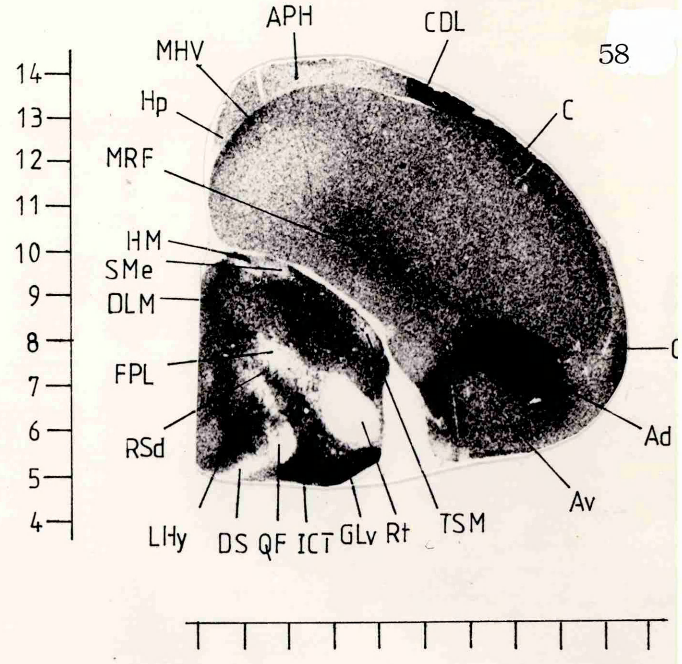
58



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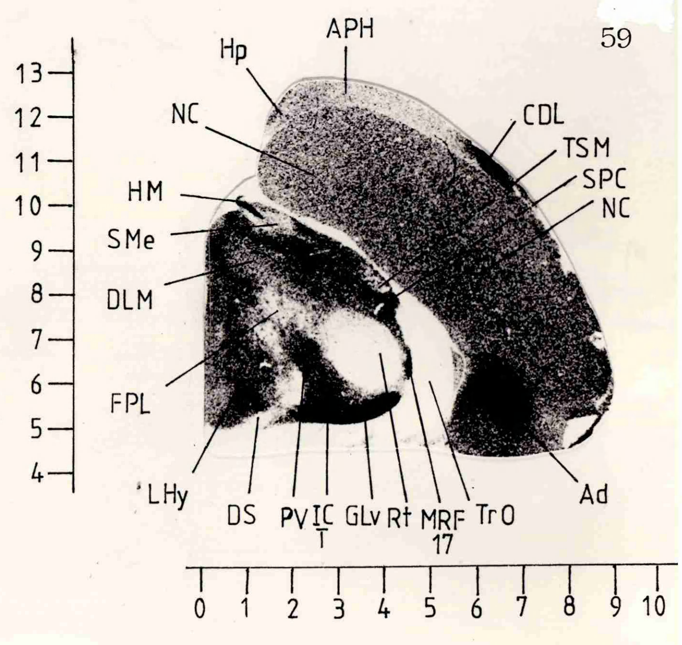

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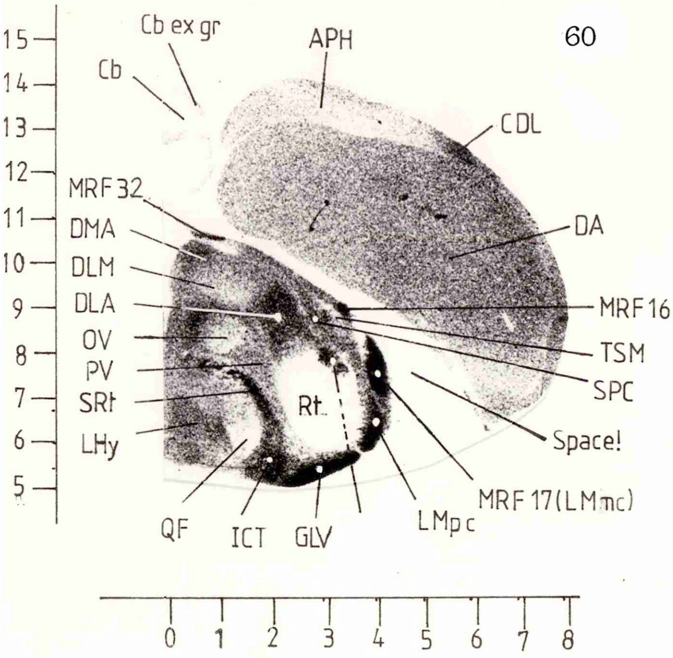
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Cb ex gr
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MRF 17 (LMmc)
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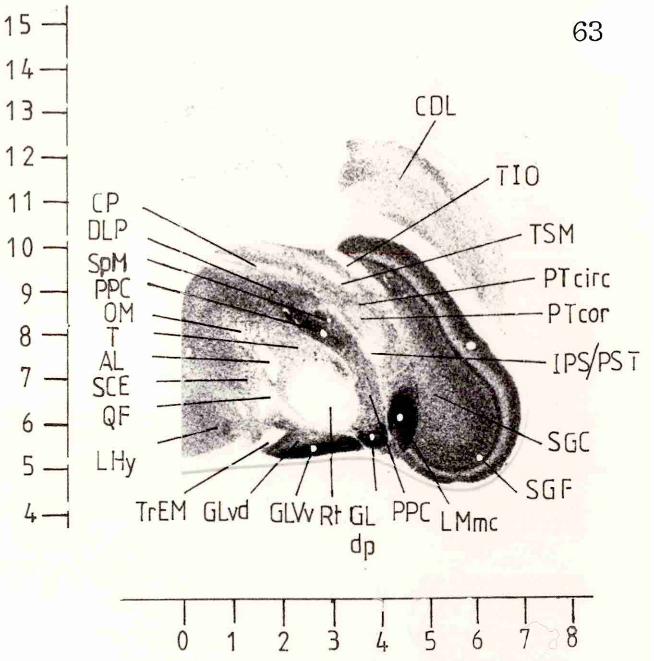
63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PPC
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SCE
QF
LHy
SGC
SGF
TrEM
GLvd
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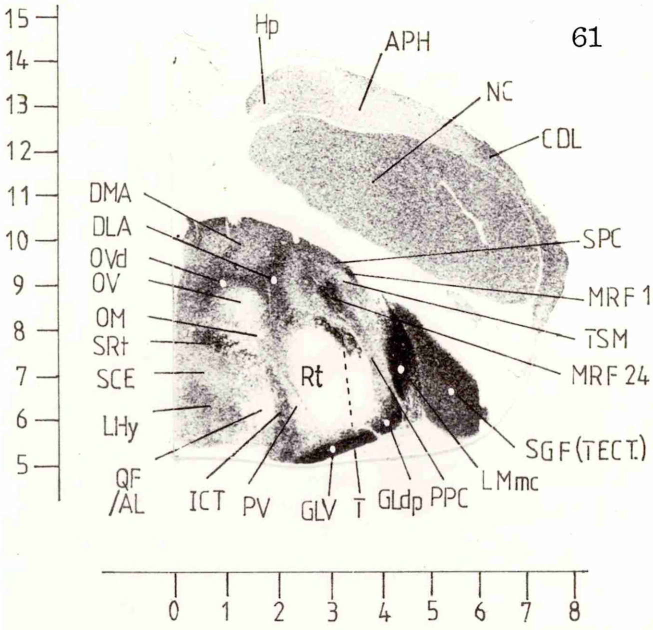
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SCE
LHy
SPC
MRF 16
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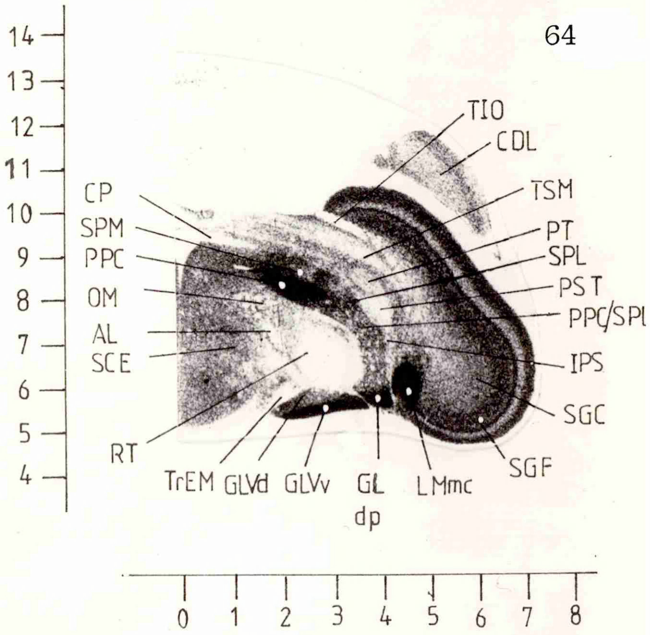
64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SPL
PST
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IPS
SGC
SGF
CP
SPM
PPC
OM
AL
SCE
RT
TrEM
GLVd
GLVv
GL
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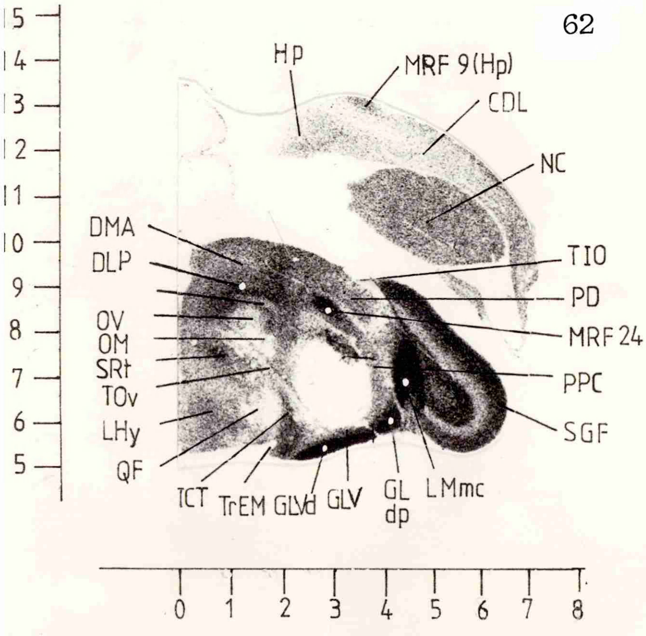
62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5
Hp
MRF 9 (Hp)
CDL
NC
DMA
DLP
OV
OM
SRt
TOv
LHy
QF
TIO
PD
MRF 24
PPC
SGF
ICT
TrEM
GLVd
GLV
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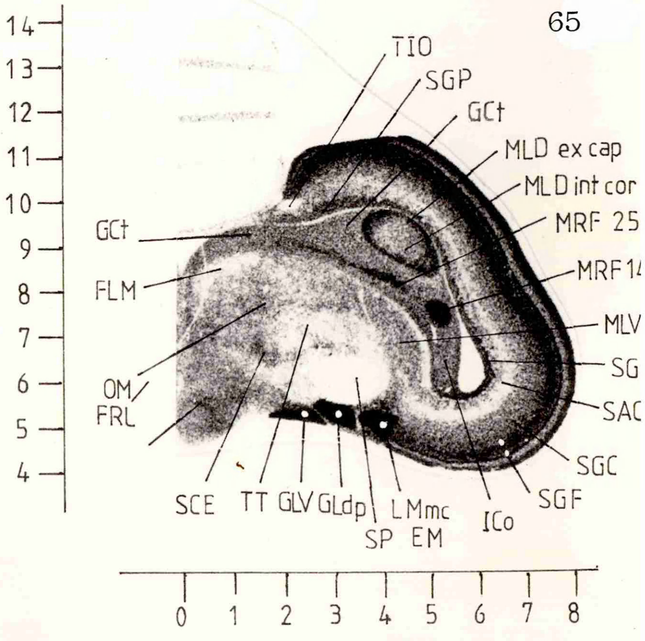
14
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6
5
4
TIO
SGP
GCt
MLD ex cap
MLD int cor
MRF 25
MRF 1
MLV
SG
SAC
SGC
SGF
GCt
FLM
OM/
FRL
SCE
TT
GLV
GLdp
SP
LMmc
EM
ICo
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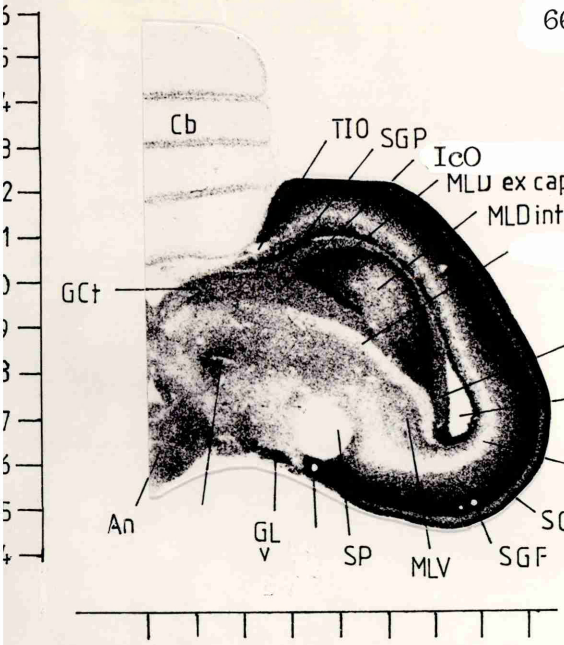
Cb
TIO
SGP
IcO
MLD ex cap
MLD int cor
GCt
GCt
V
SAC
SGC
SGF
MLV
SP
GL v
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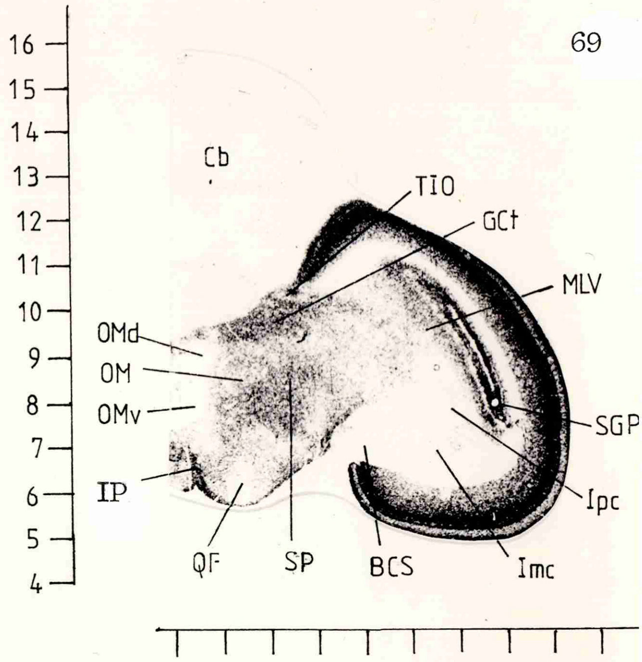
69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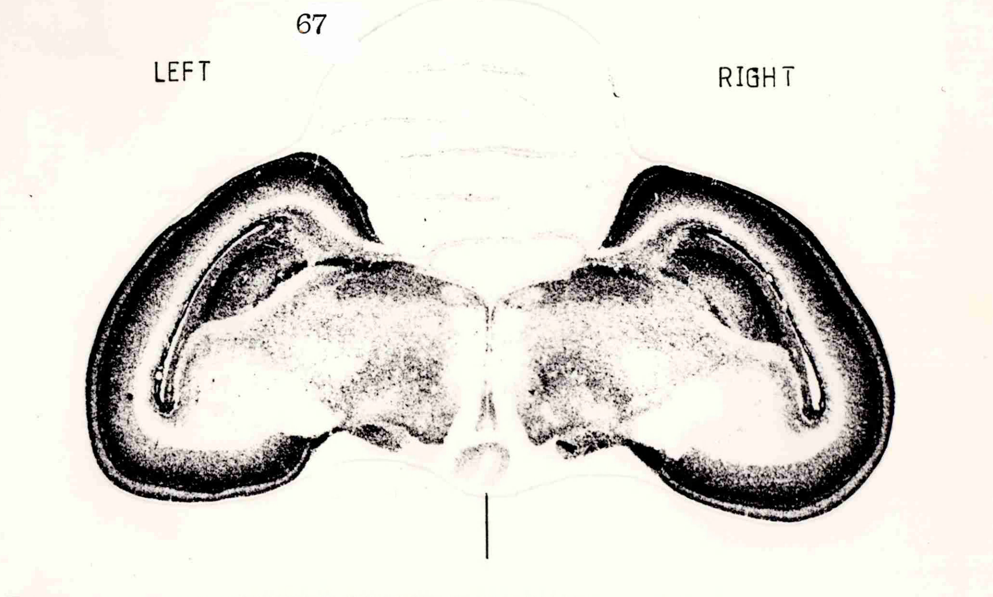
Cb
TIO
GCt
MLV
OMd
OM
OMv
SGP
Ipc
IP
QF
SP
BCS
Imc

67


LEFT
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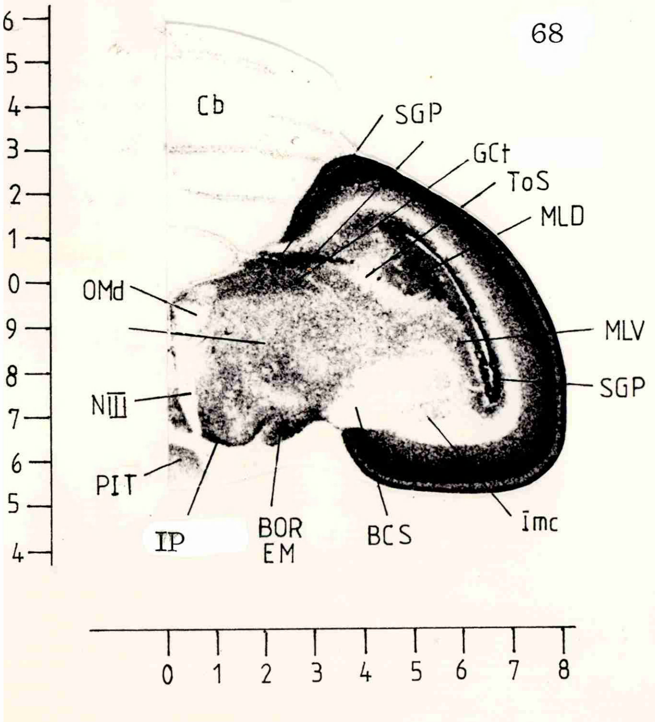
0

68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7
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4
Cb
SGP
GCt
ToS
MLD
OMd
MLV
SGP
NIII
PIT
IP
BOR EM
BCS
Imc
0
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3
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8

70

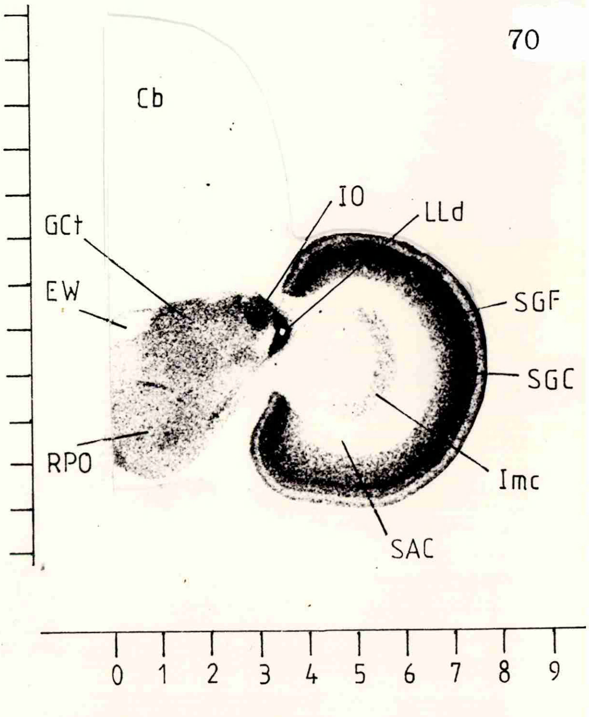
Cb
IO
LLd
GCt
EW
SGF
SGC
RPO
Imc
SAC
0
1
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Figure 71

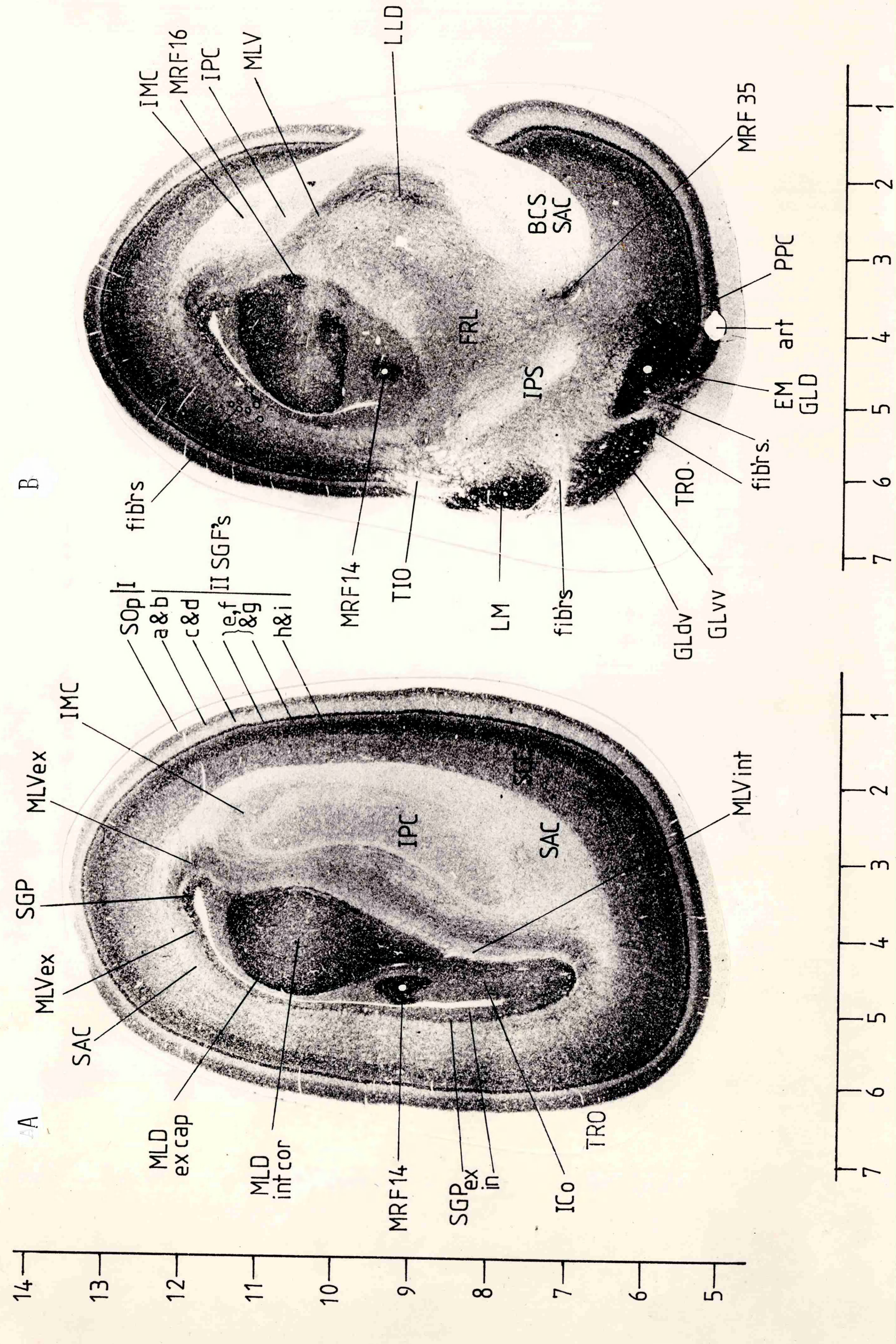
B
IMC
MRF16
IPC
MLV
LLD
MRF 35
BCS
SAC
PPC
art
FRL
IPS
EM
GLD
fib'rs.
TRO
fib'rs
MRF14
TIO
LM
fib'rs
GLdv
GLvv
1
2
3
4
5
6
7
SOp
a & b
c & d
}e, f &g
h & i
I
II SGF's
A
IMC
MLVex
SGP
MLVex
SAC
MLD ex cap
MLD int cor
MRF14
SGP ex in
ICo
TRO
IPC
SAC
SAC
MLVint
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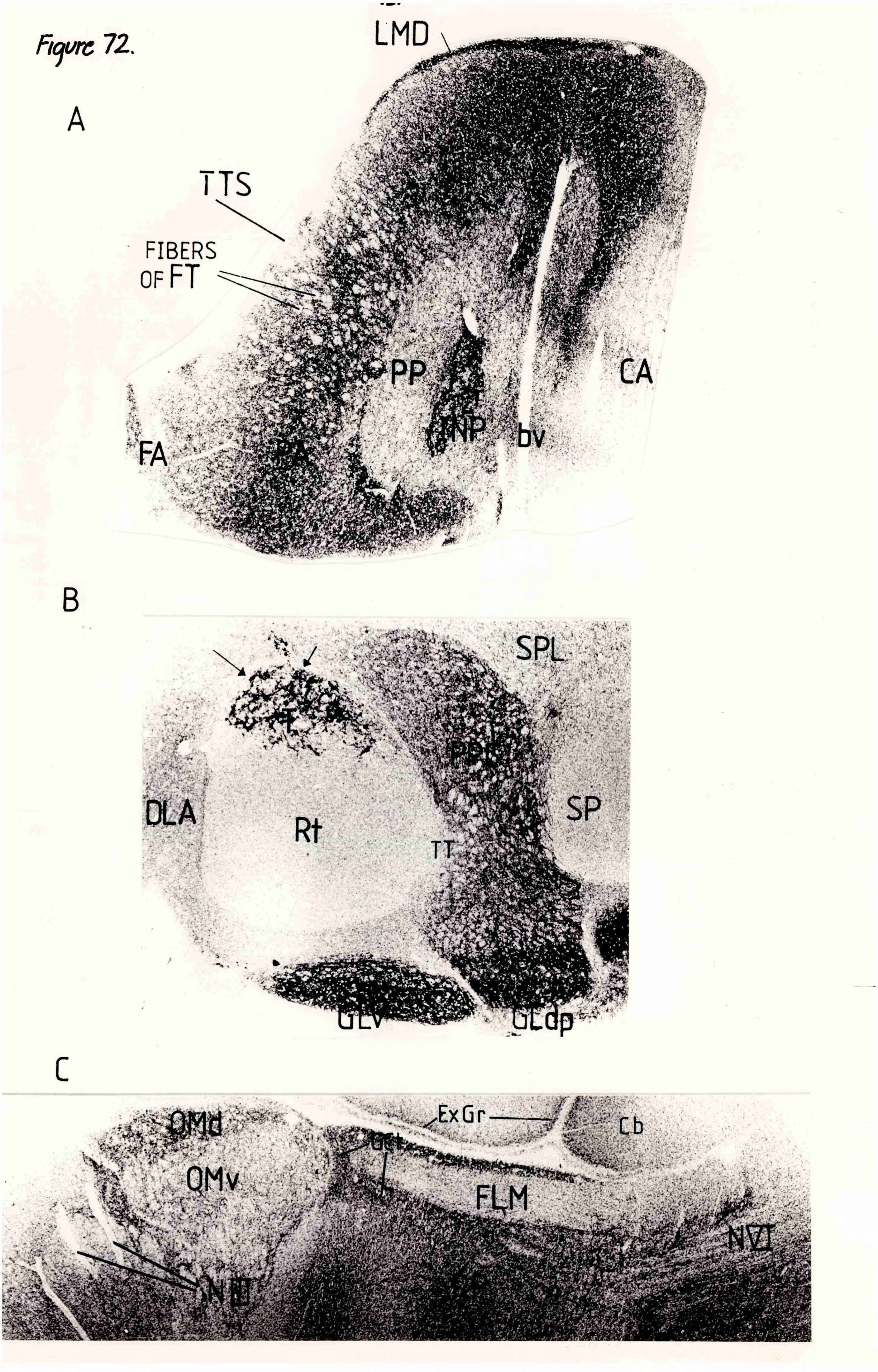
Figure 72 A to C. *Light field photomicrographs of regional details of the pattern of silver grains in parasagittal section across the paleostriatal complex (A), the antero-ventrolateral thalamus (B) and occulomotor nuclei (OM) and fasiculus longtitudinalis medialis (FLM) (C) of the 5 week post hatch chick brain, reflecting the pattern and cocentrations of MUSCARINIC CHOLINERGIC RECEPTOR. The arrows of figure B point to a dense dorsal MAChR field of the nucleus rotundus (Rt) which may correspond to the nucleus triangularis (T) but which quite clearly lies within the main body of the Rt. Scale bar = 1 mm.*

Figure 72.


A
LMD
TTS
FIBERS OF FT
PP
INP
CA
FA
PA
bv
B
SPL
T
PDc
DLA
Rt
TT
SP
GLV
GLdp
C
OMd
ExGr
Cb
GCt
OMv
FLM
NVI
NIII

Cerebellum, central nuclei and cortex.

The nucleus vestibularis (VS) is very low in MAChR (see figure 36: P 0-1, H 7-8). Areas immediately caudal to VS , through which traverse the fibres of the branchium conjunctivum (BS), are low to moderately dense in muscarinic receptor. The central cerebellar nuclei, cerebellaris lateralis (CbL) and medialis (CbM) are low in muscarinic receptor density. The central cerebellar white matter is completely devoid of muscarinic receptor in the 5 week post hatch chick brain (compare with MAChR distribution in the developing cerebellum, section 3.16)

Of the cerebellar cortical cell layers only the *external* aspects of the molecular cell layer (MCL) are populated throughout by moderate densities of MAChR (see figures 34-38 and 66). *Inner* aspects of the MCL are low in MAChR, although the *inner* aspects of MLC of folia I, X, and XI are clearly populated by higher densities of MAChR, ie. between low and moderate receptor densities. However, this folia difference in MAChR density may be an artifact of autoradiographic emulsion coating proceedure. The granula cell layer (GCL) is populated by very low densities of muscarinic receptor. However, there are very small and scattered moderate density patches of muscarinic receptor throughout the GCL, particularly along the purkinjie cell interface between the MCL and GCL.

3.15 Nicotinic receptor distribution in the post hatch chick brain.

The following is a comparatively brief description of the distribution and density of nicotinic cholinergic receptor (NAChR) in the two (2) week post hatch chick brain (N=4), as labelled by the partial irreversibly bound 'specific' nicotinic cholinergic antagonist ^{3}[H] α bungarotoxin (α BTX). The method used for the autoradiographic mapping of chick brain NAChR is the same as that used to map MAChR distribution. However, the partially irreversible binding character of ^{3}[H] α BTX for NAChR increases the probability of artifactual labelling, resulting from diffusion of ^{3}[H] α BTX away from original and 'specific' binding sites. For this reason, and others discussed elsewhere (see section 1.2) concerning the 'specificity' of the ligand α BTX for cholinergic receptor, the present findings for NAChR distribution should be seen as highly tentative, stressing the putative nature of nicotinic receptor as labelled by ^{3}[H] α BTX.

There are very few regions of the chick brain which are populated by "very high densities" of NAChR (ie. 150 pmoles/g protein), and these regions are mostly localised to midbrain structures. For this reason, and because the desciptive terms *high density, moderate density* and *low density* etc. are very limited, the following description of NAChR distribution in the telencephalon should be viewed independently of that for the midbrain. Table 6 gives a good idea of the marked difference in NAChR concentrations between the forebrain and midbrain of the post hatch chick. Where possible, I will try to indicate the approximate corresponding concentrations of nicotinic cholinergic receptor with the descriptive density term used.

3.15.1 Telencephalon.

The post hatch chick forebrain is in general populated by very low to low densities of NAChR (ie. 30 pmoles/g protein) (see figures 78-81: A 7-15, H 6-15 . However, there are very few regions of the telencephalon which are not populated by densities of αBTX labelled nicotinic cholinergic receptor in excess of non specific, ie. d tubocurarine displaced, αBTX binding densities. The distribution of nicotinic receptor is diffuse and, unlike MAChR distribution, comparatively undifferentiated by the underlying morphology of the forebrain.

Olfactory lobe and Tuberculum olfactorium.

The highest densities of NAChR in the chick forebrain are localised to the glomerular cell layer (GLomL) and external granular cell layer (OGrL) of the olfactory lobe (NAChR densities corresponding to > 80 p moles/g but < 130 pmoles/g protein) (see figures 74 and 75). The nicotinic receptor field of the OGrL is continuous with equally high NAChR populating the olfactory tuberculum (TO) (see figure 75). The mitral cell layer (Mit L) and inner granular cell layer (IGrL) are very low in α BTX nicotinic cholinergic receptor binding sites (ie. 10 pmoles/ g protein).

Caudal to the olfactory lobe, the moderate to high densities (for the telencephalon) of putative nicotinic receptor populating the TO are continuous with moderate NAChR densities populating ventral aspects of the lobus parolfactorious (LPO), paleostriatum augmentatum (PA) and nucleus accumbens (Ac) (see figures 76 ,77 and 81).

Paleostriatum.

The PA, LPO and nucleus interpeduncularis (INP) are populated by higher densities of NAChR than most other regions of the telencephalon, apart from that is the BO, TO and hyperstriatum dorsale (HD) (see figures 73-81: 8-12, 6-10). The moderate densities of putative NAChR populating dorsomedial aspects of the PA fall away abruptly at the juncture of the PA to the Ectostriatum (E) (see figure 81). However, between the PA and E, and in particular between the PA and neostriatum frontalis (NF), there are numerous fine 'streams' of silver grains, which correspond in direction with fibres entering and/or leaving the paleostriatum. Between the E and PA, these filamentous streams of NAChR correspond to an area massively pervaded by fibres of the tractus fronto-thalamicus (FT) and, between the PA and NF, to fibres of the tractus fronto-archistrialis (FA) (see figure 81).

Nicotinic receptor density in the PA is not uniformly moderately dense. For example, the juncture of the PA with the PP, over lateral aspects of the basal ganglia, is populated by very much higher NAChR densities than other aspects of the PA, a nicotinic receptor field which continues ventrally with the high NAChR densities populating the TO (see figures 77 and 81).

The LPO, in general, is populated by higher densities of NAChR than the PA. Again these higher densities of nicotinic receptor are differentiated as 'streams', orientated parallel to the basal ventral wall of the forebrain. Rostroventromedially, the LPO NAChR field continues beneath the forebrain ventricle to run, without any sign of a line of demarcation, into the equally high densities of NAChR populating the area accumbens (Ac) (see figure 81).

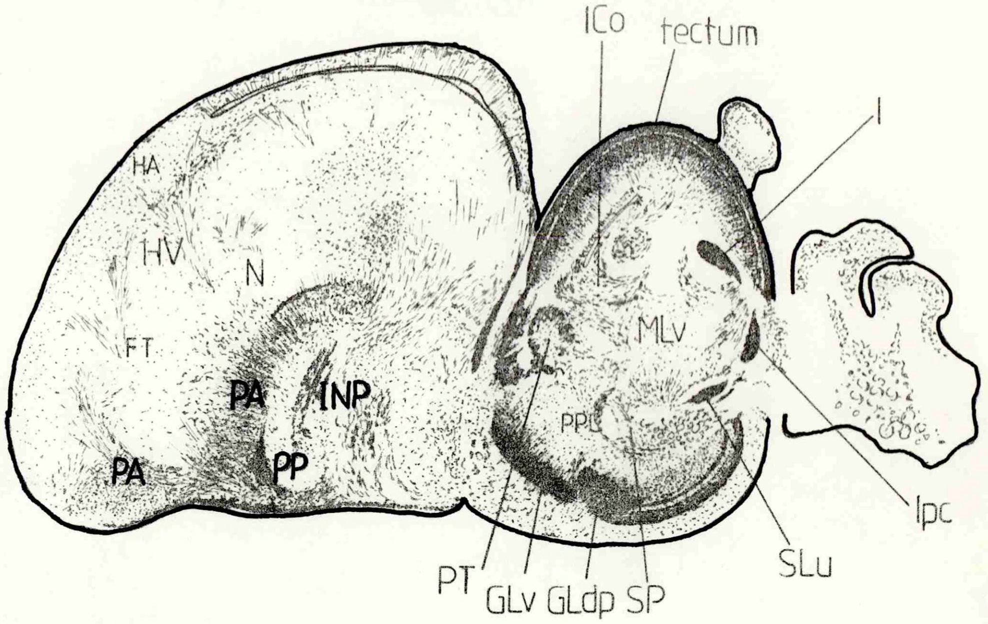
The paleostriatum primitivum (PP) is generally very low in α BTX labelled nicotinic receptor (see figures 73, 78, 79 and 81). However, similar

Figure 73. *Projected (camera lucida) drawing of the patterns and distribution of silver grains reflecting the distribution and regional concentration of α BTX labelled NICOTINIC CHOLINERGIC RECEPTOR in a parasagittal autoradiographed brain section of the 2 week post hatch chick brain. Wet emulsion coated. Compare with figure 78.*

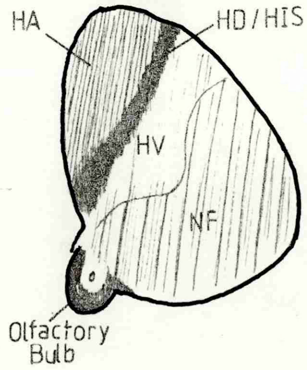
Figures 74 to 77. *A series of projected drawings, reproduced diagramatically, showing the distribution of α BTX labelled NICOTINIC CHOLINERGIC RECEPTOR in frontal autoradiographed brain sections of the telencephalon of the 2 week post hatch chick brain. These figures show only the regional distribution of α BTX labelled receptor , the apparent densities shown are neither proportional nor accurate representations of regional concentrations.*

Figure 73 to 77 NICOTINIC RECEPTOR DISTRIBUTION ; 2 WEEKS POST HATCH;
LIGAND 3H α Bungarotoxin

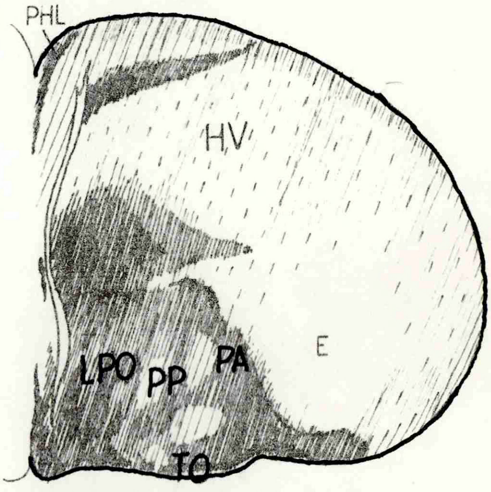
73


ICo
tectum
I
HA
HV
N
FT
PA
INP
PA
PP
MLv
PPL
Ipc
SLu
PT
GLv
GLdp
SP

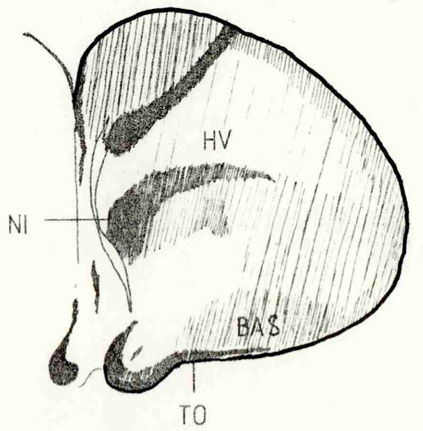
74


HA
HD/HIS
HV
NF
Olfactory Bulb

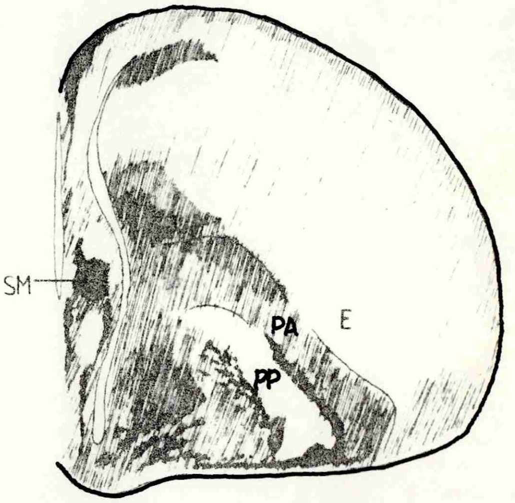
76


PHL
HV
LPO
PP
PA
E
TO

75



77



to muscarinic receptor distribution, rostroventral aspects of the PP are populated by slightly higher densities of NAChR than dorsal aspects. The INP is populated by moderate densities of NAChR, except along the INP interface to the PP, where NAChR distribution is once more differentiated as 'streams' of comparatively very high density nicotinic cholinergic receptor (see figure 77).

Hyperstriatum.

The hyperstriatum dorsale (HD) / hyperstriatum intercalatis superior (HIS) is populated by higher densities of NAChR than the adjoining hyperstriatum accessorium (HA) and hyperstriatum ventrale (HV) (see figures 74-77,and 81). Medial aspects of the HD/HIS, particularly where adjoining the forebrain ventricle, are more dense in NAChR than lateral aspects. In addition, the form of the medial HD/HIS NAChR field is 'bulbous' in outline, whereas, laterally, it is more laminated (see figure 75 and 76). The HA is populated by uniformly low densities of nicotinic receptor. The HV is almost devoid of specific α BTX nicotinic densities (compare with MAChR densities).

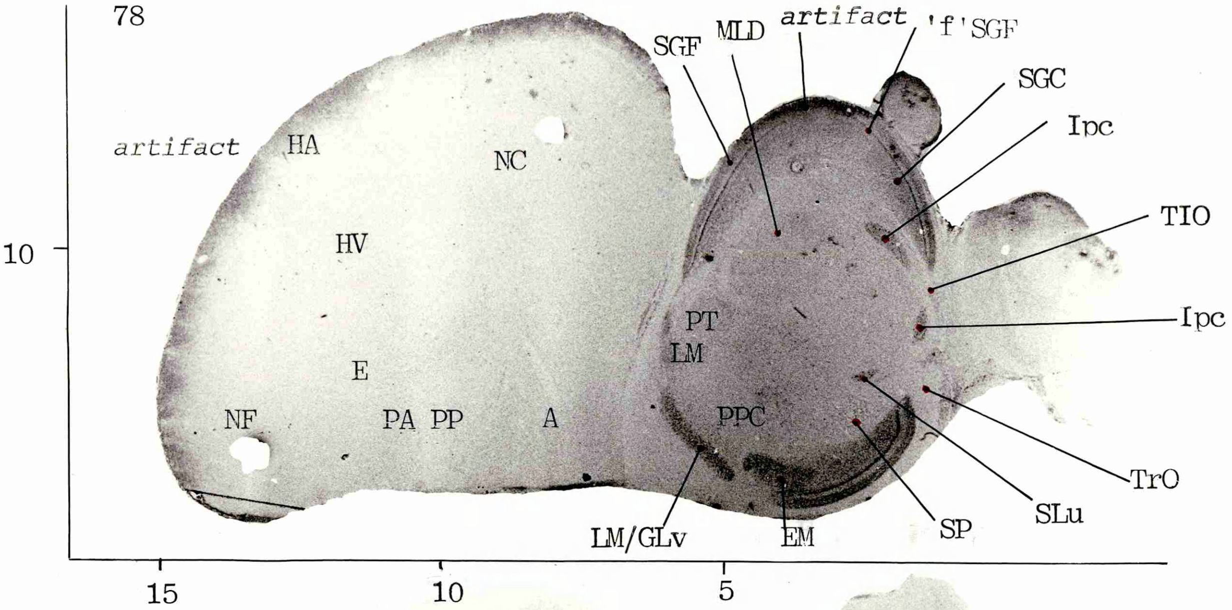
Neostriatum and Ectostriatum.

The neostriatum frontale (NF)and neostriatum intermedium (NI), apart from more medial and caudal aspects (see below), are populated by uniformly low densities of nicotinic cholinergic receptor (see figures 73- 77 and 81). Medial aspects of the NI are characterised by a relatively discrete higher density NAChR field which, ventrally, is continuous with NAChR densities populating dorsal aspects of the PA (see figures 76 and 77). More laterally, the higher NAChR densities of the medial NI

Figure 78 to 80. *A series of light field photomicrographs of parasagittal autoradiographed brain sections of the 2 week post hatch chick brain showing the distribution and regional concentration of silver grains reflecting the distribution and regional concentration of α BTX labelled NICOTINIC CHOLINERGIC RECEPTOR. Figure 80 is medial to figure 79 which is medial to figure 78. All three sections are from the same brain. The circumference of the telecephalon of all three sections is charaterised by a band of quite high density silver grains, which are probably an artifact of diffusion of 'non specifically ' bound ^{3}[H] α BTX ("halo effect "). These autoradiogram sections have been 'wet emulsion coated '.*

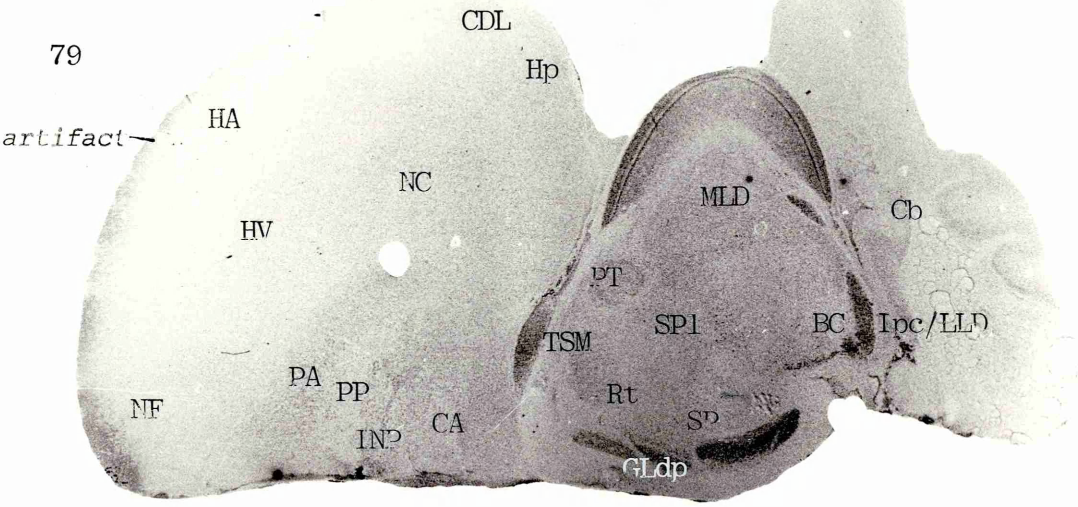
Figures 78 to 80. NICOTINIC RECEPTOR DISTRIBUTION : 2 WEEKS POST HATCH

78



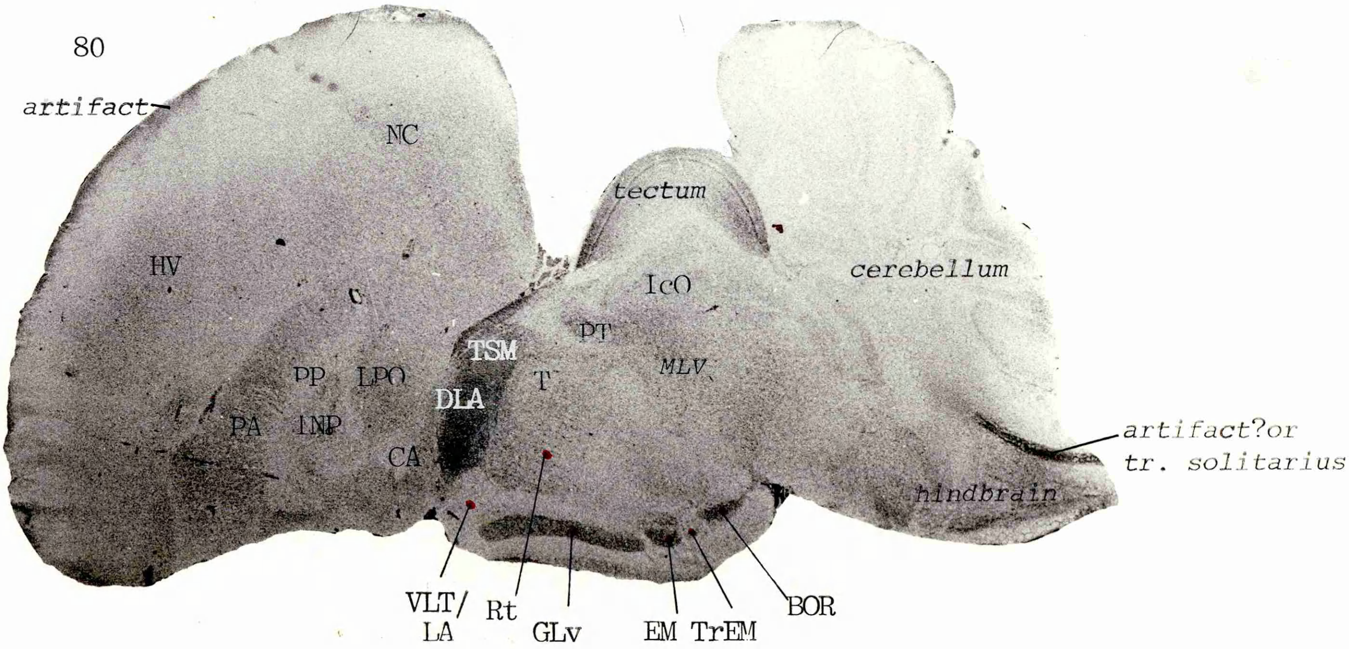
artifact
HA
NC
HV
E
NF
PA
PP
A
SGF
MLD
artifact
'f'SGF
SGC
Ipc
TIO
Ipc
PT
LM
PPC
TrO
SLu
SP
LM/GLv
EM
10
15
10
5

79



CDL
Hp
HA
artifact
NC
HV
MLD
Cb
PT
TSM
SP1
BC
Ipc/LLD
PA
PP
NF
INP
CA
Rt
SP
GLdp

80



artifact
NC
tectum
HV
IcO
cerebellum
PT
TSM
MLV
PP
LPO
DLA
T
PA
INP
CA
artifact?or
tr. solitarius
hindbrain
VLT/
LA
Rt
GLv
EM
TrEM
BOR

gradually decrease to eventually 'meld' into the lower NAChR densities of lateral aspects of the NI.

The ectostriatum (E) is completely devoid of specific αBTX labelled NAChR (see figures 79, 76 and 81). However, towards peripheral aspects of the E, a region known as the periectostriatal belt, there is a marked increase in NAChR density to levels equivalent to most other aspects of the neostriatum.

The area corticoidea or 'cortex' of the chick forebrain is populated by substantially higher densities of NAChR than underlying regions of the neostriatum and hyperstriatum (see figures 73 and 75-81). However, it is possible that the apparent higher densities of ^{3}[H] αBTX labelled NAChR in the 'cortex' is an artifact of receptor label (αBTX) diffusion (see figures 78-80).

The area parahippocampus (APH) is in general populated by very low densities of NAChR (see figures 77, 78, and 81- 82). However, the nucleus parahippocampus pars linearis (PHL) (Benowitz and Karten, 1976), situated along the most mediodorsal wall of the forebrain sulcus, is populated by moderate densities of NAChR. More caudally, the NAChR population of the PHL takes up a position along the dorsomedial margin of the APH (see figures 77-82).

3.15.2 Mesencephalon and Diencephalon.

Thalamus and primary optic nucleus (OPT).

The dorsal thalamus of the chick mesencephalon is dominated by a very high density NAChR field which largely corresponds to the nucleus dorsolateralis anterior thalami (DLA), but, in addition and without any break in continuity of nicotinic receptor density, to the dorsolateralis anterior thalami, pars lateralis (DLL), dorsolateralis anterior thalami, pars magnocellularis (DLAmc), lateral aspects of the nucleus dorsolateralis posterior thalami (DLP) and area pretectalis (AP) (see figures 78-80, 82 and 84). This group of dorsal thalamic nuclei, together with the nucleus lateralis anterior thalami (LA), has been functionally grouped and designated the primary optic nucleus (OPT) (Karten et al., 1968).

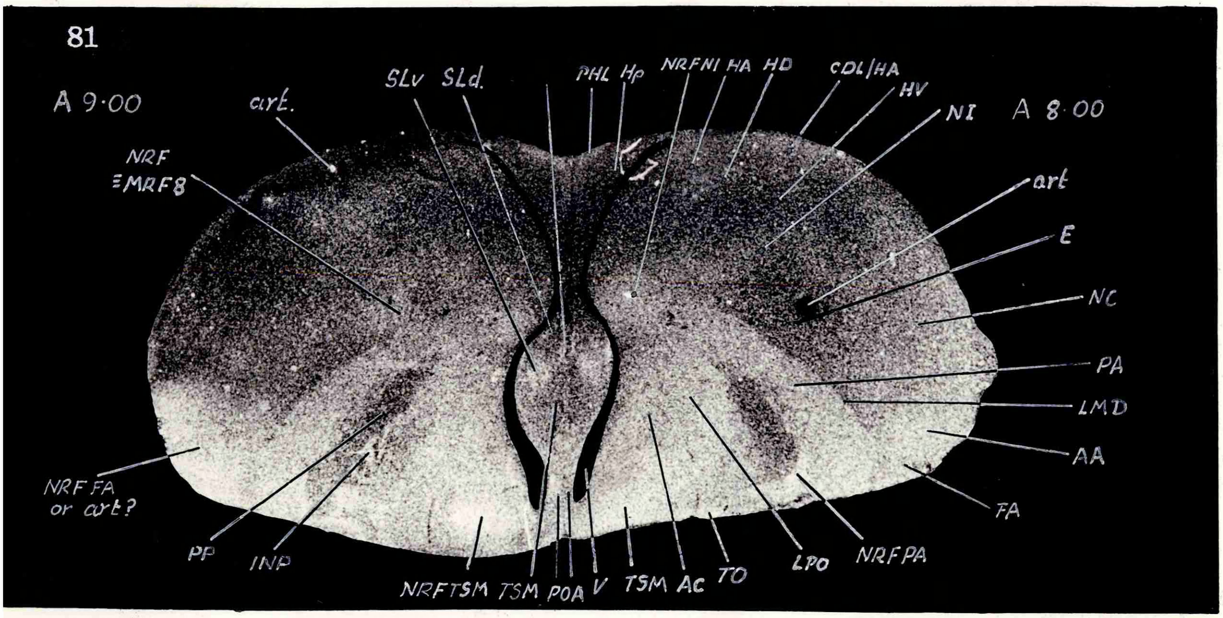
The NAChR field of the OPT is limited, ventrolaterally, by the nucleus ventrolateralis thalami (VLT), more medially by the main body of the fasiculus prosencephali lateralis (FPL), caudally by the nucleus rotundus (Rt) and dorsomedially by the nucleus dorsomedialis anterior thalami (DMA).

The limits of the OPT NAChR field are not, for most adjoining brain regions, sharply defined. Ventromedial aspects of the OPT high density NAChR field are heavily pervaded by fibres which, on first leaving or entering the main body of the FPL, are unlabelled for nicotinic cholinergic receptor (see figure 80). Dorsomedially, the very high densities of NAChR in the OPT extend towards and are continuous with nicotinic receptor densities populating medial aspects of the optic tectum (see figures 78 and 79). Dorsoanteriorly, the NAChR field of the OPT is heavily pervaded by unlabelled fibre tracts of the septomesencephalicus (TSM) (see figures 80: A 7-8, H 9-11, and figure 84 b). Dorsal to the TSM, the nucleus superficialis parvocellularis-tractus septomesencephalicus (SPC) is also populated by very high densities of nicotinic receptor, which, over

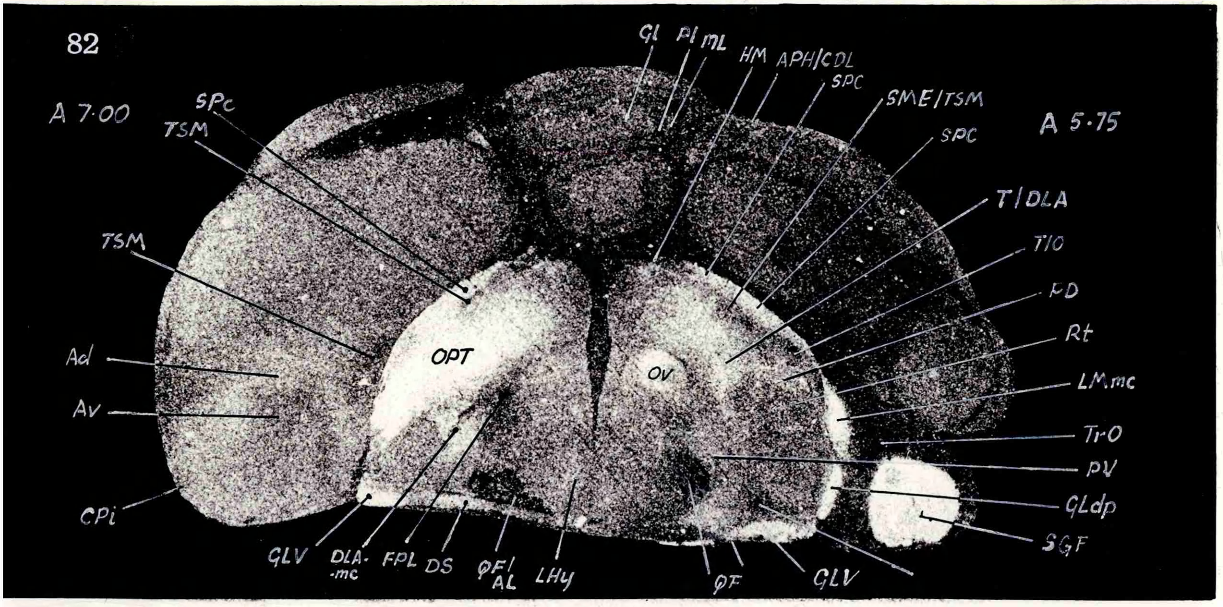
Figures 81 to 83. *A series of dark field photomicrographs of frontal autoradiographed brain sections of the 2 week post hatch chick brain, showing the distribution and regional concentrations of α BTX labelled NICOTINIC RECEPTOR across the forebrain , medial (81) and caudal (82), diencephalon (82), and optic lobe, mesencephalon, hypothalamus and cerebellum (83). The concentration of silver grains (seen in the figures as 'degrees of whiteness') are comparable between sections as shown, but to the cost of resolution of the regional detail of the optic tectum (83).*

Figures 81 to 83. NICOTINIC RECEPTOR DISTRIBUTION: 2 WEEKS POST HATCH.

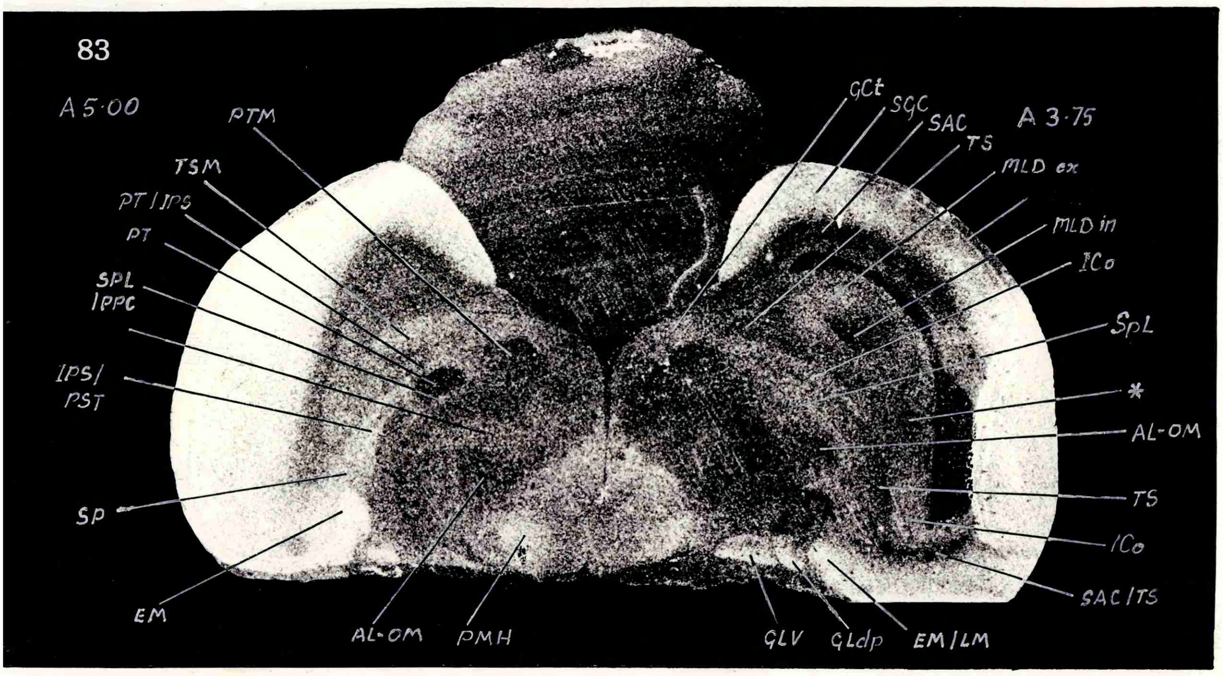
81


A 9·00
art.
NRF
≡MRF8
SLv
SLd.
PHL
Hp
NRFNI
HA
HD
CDL/HA
HV
NI
A 8·00
art
E
NC
PA
LMD
AA
FA
NRF FA
or art?
PP
INP
NRFTSM
TSM
POA
V
TSM
Ac
TO
LPO
NRFPA

82


A 7·00
SPc
TSM
Gl
Pl
ml
HM
APH/CDL
SPC
SME/TSM
SPC
A 5·75
TSM
T/DLA
T/O
PD
Rt
OPT
OV
Ad
Av
LM.mc
TrO
PV
GLdp
SGF
CPi
GLV
DLA-mc
FPL
DS
QF/AL
LHy
QF
GLV

83


A 5·00
PTM
GCt
SGC
SAC
A 3·75
TS
TSM
MLD ex
PT/IPS
MLD in
PT
ICo
SPL/PPC
SpL
IPS/PST
*
AL-OM
TS
SP
ICo
SAC/TS
EM
AL-OM
PMH
GLV
GLdp
EM/LM

more lateral aspects, are continuous with the very high nicotinic receptor densities of the OPT (see figure 82). The nucleus habenularis (Hb), situated at the most extreme dorsomedial margin of the anterior midbrain, is populated by high densities of nicotinic receptor (see figure 82).

The nucleus rotundus (Rt) is populated by low densities of αBTX labelled nicotinic cholinergic receptor (see figure 79, 80, 82 and 84b). However, dorsolateral aspects of this nucleus are populated by slightly higher NAChR densities than other aspects of the Rt (see figures 79 and 82), and throughout the Rt, there are very small patches of NAChR of higher receptor density, but which are not sufficiently concentrated as a group to constitute a 'receptor field'. Situated dorsoanteriorly to the Rt, there is a very high density NAChR field which corresponds to the tentatively identified nucleus triangularis (T). If this very dense NAChR field does correspond to the T, and not to some aspect of the DLA, then it is very closely associated and continuous with the very high density NAChR field of the OPT (see figure 82).

Of other thalamic nuclei, only the nucleus ovoidalis (Ov) is populated by high densities of NAChR (see figure 82), and, similar to MAChR densities in the Ov, it is the dorsal and medial aspects to this nucleus which are most dense in nicotinic receptor. The nucleus subrotundus (SRt), situated ventral to the Ov, is populated by generally very low densities of NAChR. Interestingly, there is a definite tail of high density NAChR extending from medial aspects of the Ov ventralward, which may correspond to tractus nuclei ovoidalis (TOv) (see figure 82).

The nucleus principalis precommisuralis (PPC)and nucleus spiriformis lateralis (SPL) are almost completely devoid of α BTX labelled

NAChR (see figures 77,79, and figure 83). This is a particularly significant finding, since the PPC has been shown earlier to be one of the most dense MAChR fields of the chick brain (see section 3.14.2. The nucleus spiriformis medialis (SPM) is completely devoid of nicotinic cholinergic receptor (see figure 80).

Geniculate, lemniscal and ectomamillary nuclei.

The complex of nuclei lying along the ventrolateral margin of the midbrain to ventromedial aspects of the optic lobe has already been extensively described in context with muscarinic receptor distribution. These nuclei, ie. the nucleus lentiformis mesencephali (LM), nucleus geniculatis lateralis, pars dorsalis (GLdp), nucleus geniculatis lateralis, pars ventralis (GLv) and nucleus ectomamillaris (EM), are populated by regionally discrete, very high densities of nicotinic cholinergic receptor (see figures 78-80, 82,83 and 84 b). The very high density NAChR field of the LM arises in the rostrolateral mesencephalon, but more caudally, comes to occupy a position between the GLdp and tectal laminae, a region corresponding to the nucleus ectomamillaris (EM). During the course of transistion of this dense NAChR field, from a position clearly identifiable as LM to one corresponding to the EM, there is no point of demarcation such as to suggest that the LM and EM are morphologically distinct nuclei. A similar continuity in the LM-EM NAChR field was observed for muscarinic receptor distribution. The LM/EM, GLdp and GLv NAChR fields are distinct and separated from one another by areas largely devoid of nicotinic receptor, which are probably inwardly projecting fibres arising from the main body of the optic tract (TrO) (see figures 79-80, 82 , 83 and 84b).

Within the main body of the TrO and particularly the optic chiasma, there are widely spaced, full and half circle patterns of moderately dense silver grains, which obviously suggest that nicotinic receptors are localised to regions between fibres of the TrO (see figure 73). Such silver grain patterns and densities are not apparent over consecutively cut brain sections,treated to show the distribution and density of non-specifically bound, ie. *d*-tubocurarine displaced, α BTX NAChR binding sites. However, these patterns of silver grains are very unusual and may result from the artifactual accumulation of unbound α BTX molecules between TrO fibre fasicles. Throughout all α BTX labelled autoradiograms of the chick brain, there is evidence for post emulsion coating diffusion of ^{3}[H] α BTX, seen most clearly as a 'halo' of silver grains around the circumference tissue sections (see figures 78-80, particularly the forebrain). It is probable that, during emulsion coating, lightly bound or unbound α BTX molecules become entrapped between the spaces of TrO fibres resulting from shrinkage during drying.

Similar patterns of silver grains of moderate density are localised to central aspects of the FPL, tractus tectothalamicus (TT), tractus septomesencephalicus (TSM) and central white matter of the cerebellum (see figure 80). The decussatio supraoptica (DS) is populated by uniform, moderate densities of nicotinic cholinergic receptor.

Preoptic, central nuclei and tracts of the optic lobe.

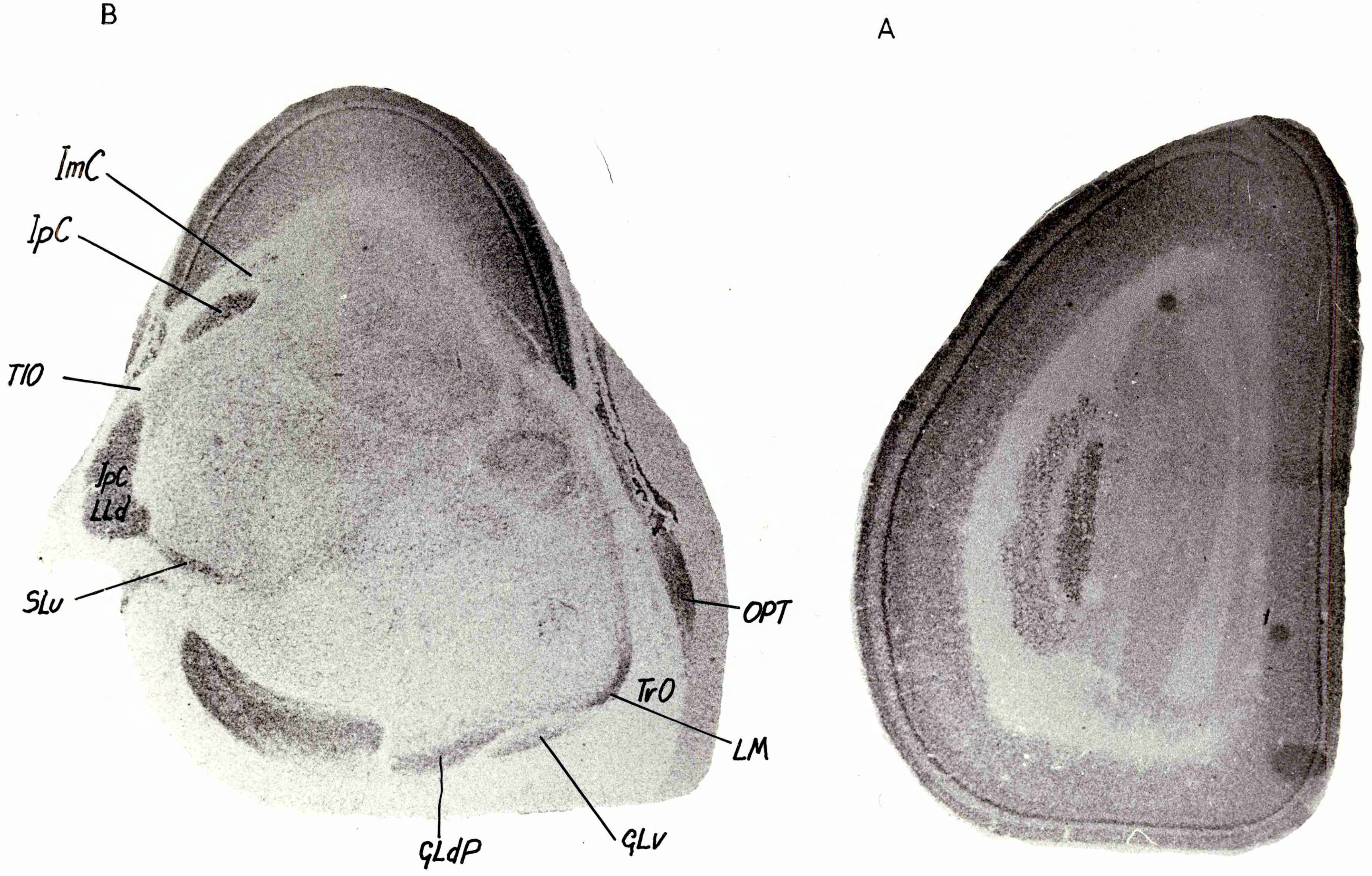
The circumference of the nucleus mesencephalicus lateralis, pars dorsalis (MLd) is populated by moderate densities of NAChR, while central aspects of this nucleus are completely devoid of nicotinic receptor (see figures 78-80 and 83). The circumventing moderate NAChR densities of the MLd are not sharply demarcated from adjoining regions of the optic lobe.

It is of particular interest to note that a small, circular and discrete area within the nucleus intercollicularis (ICo), immediately ventral to the MLd, is completely devoid of NAChR, a region shown to be populated by very high densities of muscarinic receptor (see figure 83 and compare with figures 65-66, region designated MRF 14).

The nucleus subpretectalis (SP) is populated by very low densities of NAChR (see figure 83). Situated dorsally to the SP, there is a very high density nicotinic receptor field localised to the nucleus interstitio-pretecto-subpretectalis (IPS) and tractus pretecto-subpretectalis (PST). The IPS/PST NAChR field is continuous, dorsally, with very high densities of nicotinic receptor populating the circumference of the nucleus pretectalis (PT) (see figure 84b). Central aspects of the PT, on the other hand, are completely devoid of α BTX binding sites. Dorsal to the PT and SPM is a further high density field of NAChR which corresponds to the nucleus pretectalis diffusus (PD) and TSM.

The nucleus lemnisci lateralis, pars dorsalis (LLd) is populated by very high densities of nicotinic receptor (see figures 78-80 and 84 b). The LLd NAChR field continues dorsolaterally to adjoin equally high densities of nicotinic receptor localised to the nucleus isthmi, pars parvocellularis (Ipc). NAChR densities in the Ipc continue, in a caudal direction, from a point adjoining the ventral margin of the LLd to an area immediately dorsal to the SP (see figures 78-80). The nucleus isthmi, pars magnocellularis (Imc) is also populated by high densities of nicotinic receptor, patterned as a reticulate network consistant

Figure 84 A and B. *Two light field photomicrographs of parasagittal sections across the optic lobe of the 48 hour post hatch (A) and 2 week post hatch (B) chick brain , showing the distribution of α BTX labelled NICOTINIC CHOLINERGIC RECEPTOR. Figure A (Lateral 5.75) is lateral to figure B (L. 3.75). Compare with figure 71 . Scale bar = 1mm.*


B
A
ImC
IpC
TIO
IpC
LLd
SLu
OPT
TrO
LM
GLdP
GLv

with the cellular morphology of this nucleus. Streaming from ventral aspects of the Ipc/LLd in a rostral direction, there is a 'wisp'-like high density NAChR field corresponding to the lemniscus lateralis (LL) and/or the nucleus semilunaris (SLu) (see figures 79 and 84 b).

Laminae of the optic tectum.

Very high densities of NAChR are localised to laminae of the stratum grisea et fibrosum (SGF) and stratum grisea centrale (SGC) (see figures 78-80, 83 and 84a) . Sublayer IIf of the SGF is the most dense NAChR populated lamina of the optic tectum (OT), similar to muscarinic receptor densities in the OT. Very high densities of NAChR are located to sublayer IIa and IIb of the SGF (see figure 84). However, between the very high NAChR fields of IIa and IIb is a region of lower receptor density. It is possible, although I think unlikely, that this region of lower receptor density corresponds to sublayer IIb, in which case the second of two high density NAChR fields of the peripheral SGF would correspond to sublayer IIc. However, it is my opinion that the thin band of lower receptor density merely marks a brief transition point between sublayer IIa and IIb and that the second of these NAChR fields does correspond to IIb and not to IIc, in which case sublayer IIc is populated by low to moderate densities of nicotinic cholinergic receptor, similar to sublayer IId. Sublayer IIg is low in nicotinic receptor and sublayers IIh and IIi of the SGF are populated by moderate to high densities of NAChR (see figure 84). The SGC is differentiated by NAChR density in two laminae; the uppermost, adjoining the SGF, is low to moderately dense in NAChR, while the more medial lamina, adjoining the stratum album centrale (SAC), is populated by moderate to high densities of nicotinic receptor. The SAC is largely devoid of α BTX labelled NAChR.

In general, nicotinic receptor distribution and density in the optic tectum is not as sharply localised to particular laminae as has already been described for MAChR distribution. Nicotinic receptors are also much less homogeneous, and the silver grain patterns, particularly under higher magnification, are much more 'patchy' than observed for muscarinic receptor distribution. The very high densities of NAChR localised to the sublayers of the SGF, similar to MAChR distribution, are continuous with the equally very high density receptor field of the lemniscal and ectomamillary nucleus complex.

Hypothalamus.

Lateral and caudal aspects of the chick hypothalamus are populated by moderate to high densities of nicotinic receptor (see figure 83). A particularly high density NAChR field is localised to a discreet area of the hypothalamus which corresponds to the nucleus lateralis hypothalami (LHy). This receptor field, however, is not diffuse and does not correspond to all aspects of the LHy as shown by the stereotaxic atlas of Karten and Hodos (1967) for the pigeon brain. Medial to the high NAChR densities of the LHy is an area populated by substantially lower densities of nicotinic receptor, corresponding to the nucleus lateralis hypothalami posterius (PLH) and nucleus medialis hypothalami posterius (PMH). Dorsal to the PLH and PMH, the stratum cellulare internum (SCI) is populated by moderate to high densities of NAChR; more caudally, the high density NAChR field of the SGI is continuous with a similarly high density NAChR field localised to the area hypothalami posterius (APH) (see figure 83). The stratum cellulare externum, lying lateral to the SCI, is very low in NAChR. Moderate densities of α BTX labelled NAChR are localised to the area ventralis (Tsai) (AVT) which adjoins and partly encloses the occulomotor nerve (NIII).

Cerebellum.

The granular cell layer (GCL) of the cerebellar cortex is populated by uniformly moderate densities of NAChR. The molecular cell layer is populated by low densities of receptor.

The fibrous central white of the cerebellum is very low in nicotinic receptor, as are the nucleus cerebellaris internus (Cbl) and commissura cerebellaris ventralis (CCV).

3.16 Ontogeny of muscarinic receptor distribution in the chick brain in ovo.

Because of the high percentage water content of the chick embryo brain, sectioning, in preparation for light microscope autoradiographic localisation of muscarinic receptor, proved to be very difficult. To resolve this problem the whole chick embryo head was sectioned which, in addition to maintaining tissue integrity, maintained the orientation of the brain as if *in vivo*.

The following is a provisional and general description of muscarinic receptor distribution in the *in ovo* chick brain from 10 days *in ovo* (10 DIO) to 1 day post hatch. Brain sections have been labelled by the irreversible antagonist ^{3}[H] PrBCM. Frontal and parasagittal sections were cut from at least three brains for each developmental day between 10 DIO and 1 day post hatch, composing, in total, several thousand receptor labelled autoradiographed brain sections.

Patterns of muscarinic receptor distribution in the chick brain in ovo.

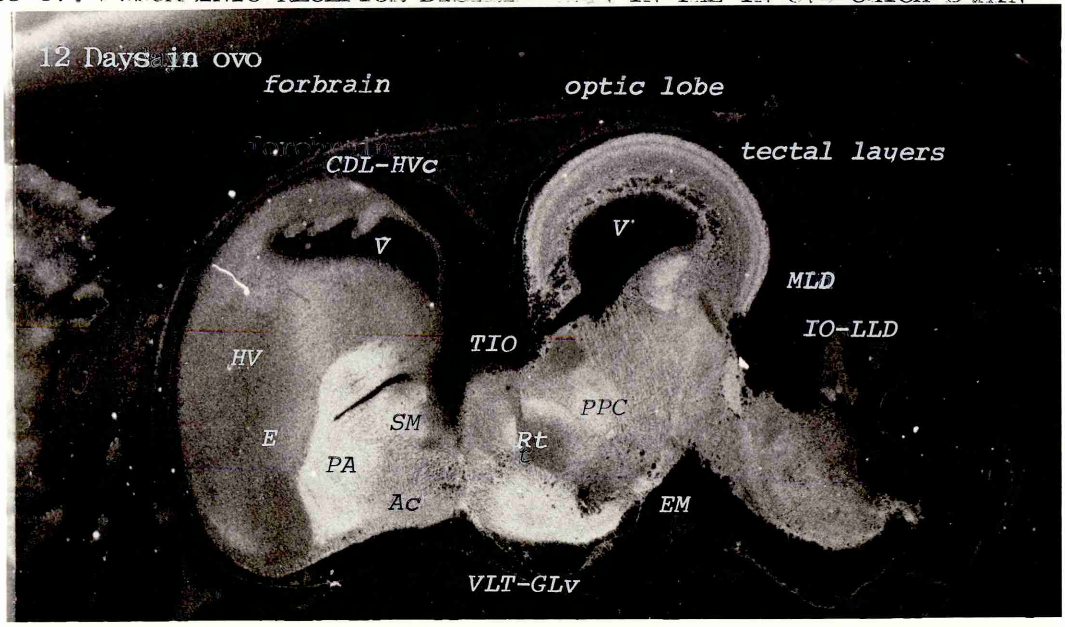
Between 10 DIO and 19 DIO all regions of the brain, whether broad fields of cells, laminated regions, relay nuclei and interstitial cell populations, expanses of white matter, fibre tracts, ventricular or subventricular proliferative zones are populated, at one time or another between these ages, by densities of specifically bound muscarinic antagonist which are considerably in excess of non specific binding densities (ie. those not displaced by nM concentrations of atropine). For many regions of the brain, the appearance of moderate to high densities of receptor is a transient phenomenon of in ovo development. Densities of receptor appear and then largely disappear within one or two developmental days. In addition, all regions of the chick brain, including those destined to be the main fibre bundles of the brain, for example the medial and lateral forebrain bundles, are populated by highly characteristic and unique *in ovo* patterns of muscarinic receptor densities (see figures 87, 89 and 96).

These patterns are concentrations of ^{3}[H] antagonist labelled receptor which, quite unlike receptor distribution in the post hatch chick brain, are not contained within and defined by morphological anatomical boundaries of particular brain regions, at least as discerned in the post hatch brain, but traverse wide expanses of the brain (eg. see figure 95). For midbrain regions in particular, these patterned receptor densities are indistinguishably intermingled between brain regions. There are in essence two types of regional receptor density pattern 1) homogeneous, where receptor density is distributed uniformly throughout any particular brain region, a pattern of receptor characteristic of post hatch muscarinic receptor distribution, and 2) heterogeneous which can be further divided into 'sheets' or 'patches' and 'parallel arrays'.'Sheets and patches'tend to be of higher muscarinic receptor density which may traverse wide areas of the brain, for example caudal forebrain (see figure 97) or be more regionally localised, while 'parallel arrays' tend to be of lower muscarinic receptor density and similar to 'sheets' can traverse large areas of the brain, but are concentrated in tightly packed parallel lines (see figure 95 and 96).

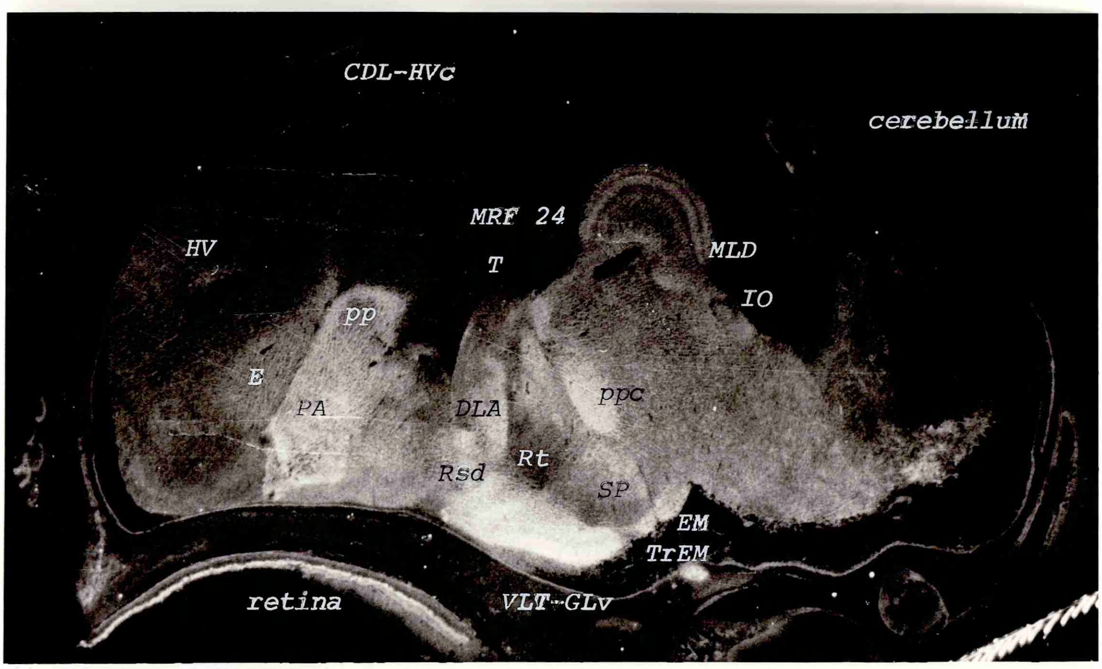
Although these heterogeneous patterns of muscarinic receptor density are characteristic of the embryo brain between 11 to 19 days *in ovo*, the appearance, Figures 85 to 87. *Dark field photomicrographs of parasagittal autoradiographed brain sections showing the distribution of silver grains ("whiteness") reflecting the pattern and comparative regional concentrations of ^{3}H PrBCM labelled MUSCARINIC CHOLINERGIC RECEPTOR in the 12 days in ovo (85), 14 days in ovo (86), and 16 days in ovo (87) chick EMBRYO brain. Although from different ages figure 87 is medial to 86 which is medial 85. The brain has not been dissected from the head for these autoradigraphed sections, consequently muscarinic antagonist labelled receptor are also evident in the Pia and Dura Mater, the retina (86) and ossifying bone of the cranium. Note the relative size of the olfactory lobe in comparison to the forebrain of the 16 days in ovo em$\overset{b}{\wedge}$ryo (87). These autoradiograms have been 'wet emulsion coated'. Scale bar = 1 mm.*

Figures 85 to 87. MUSCARINIC RECEPTOR DISTRIBUTION IN THE IN OVO CHICK BRAIN

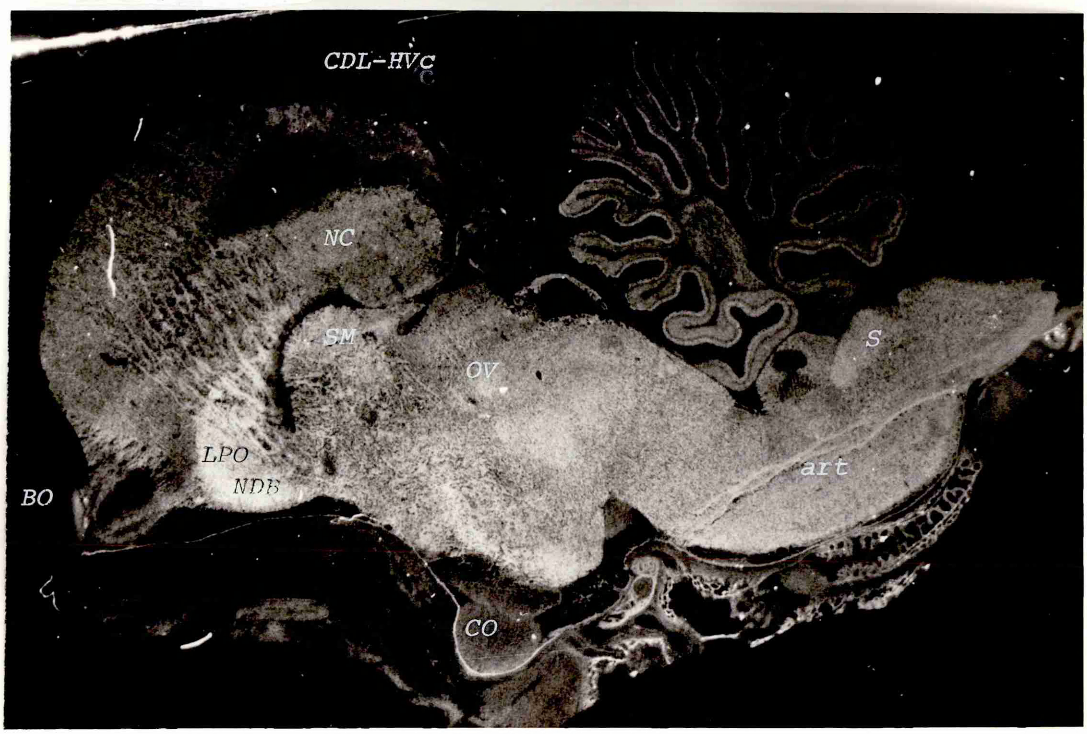
86


12 Days in ovo
forbrain
optic lobe
tectal layers
CDL-HVc
V
V
MLD
IO-LLD
HV
TIO
PPC
SM
E
Rt
PA
Ac
EM
VLT-GLv

87. 14 Days in ovo.


CDL-HVc
cerebellum
MRF 24
HV
MLD
T
IO
pp
E
ppc
PA
DLA
Rt
Rsd
SP
EM
TrEM
retina
VLT-GLv

88 16 Days in ovo.


CDL-HVc
NC
SM
OV
S
LPO
NDB
art
BO
CO

change in form, density or distribution of receptor exhibit a regionally distinct temporal and spatial order of development. It is quite impossible to describe the regional order of change in pattern and density of receptor, since considerable changes may occur on a highly regionally localised basis within one developmental day. However, between 17 and 19 DIO, for the vast majority of brain regions, there is a dramatic change from heterogeneous to homogeneous patterns of receptor distribution which is accompanied by post hatch distribution and densities of muscarinic receptor. There is quite a distinct caudal to rostral gradient of change from heterogeneous to homogeneous patterns of receptor distribution. For example, the dorsal anterior thalamus and habenular of the diencephalon are the last midbrain regions to show this transformation (see figures 97 and 98).

There appears to be some sort of interactive relationship between 'patches' or 'sheets' and the 'parallel arrays' of receptor density. For example, in the chick embryo forebrain the lower density parallel lines of receptor may at one point be 'disrupted' by a highly localised 'patch' of high density receptor (see figure 89 a and b). Between one developmental day and the next, the first 'patch' has either moved or disappeared and is displaced by another similar 'patch' of receptor interrupting the same field of parallel lines of lower density receptor, but at a different regional level.

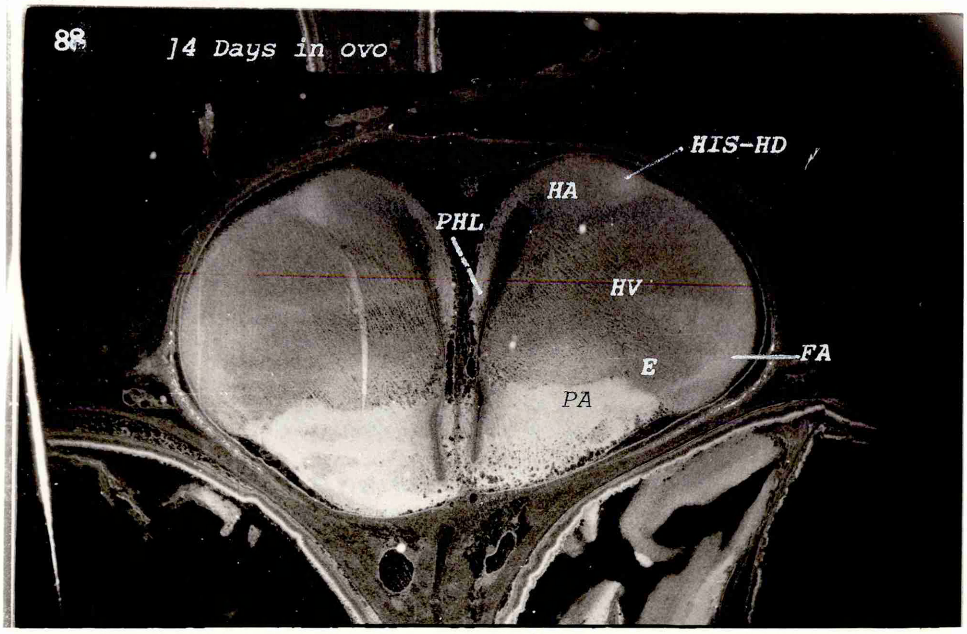
If figures 99 and 100 to 102 are compared, it can be seen that the distribution and form of muscarinic receptor distribution appears to correspond quite closely with the morphological differentiation of the brain, as stained by cryselecht violet and shown in figure 102 in light field and figure 99 in dark field. Figures 99 and 101 are consecutively cut sections to that shown in figure 100 which have not been labelled for receptor or subjected

Figure 88. *Dark field photomicrograph of a frontal autoradiographed section across the forebrain of the* *14 days in ovo* *chick brain, showing the distribution of* $^{3}[H]$ *PrBCM labelled MUSCARINIC CHOLINERGIC RECEPTOR. Note the moderate to high densities of MAChR to the hyperstriatum intercalatus superior (HIS) Scale bar = 1 mm.*

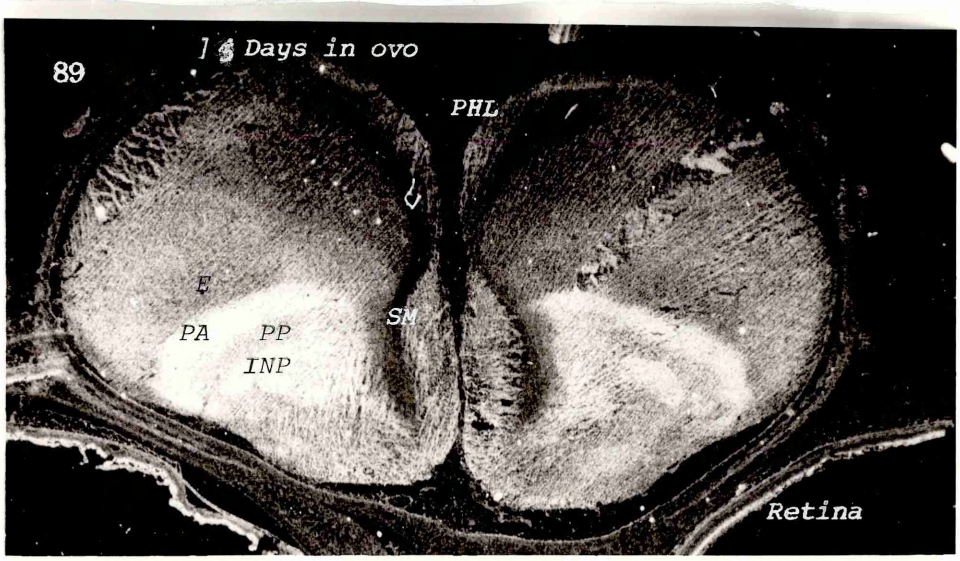
Figure 89 *A dark field photomicrograph of a frontal autoradiographed section across the forebrain of the* *15 days in ovo* *chick embryo brain, showing the patterns and concentration of silver grains reflecting* $^{3}[H]$ *PrBCM labelled MUSCARINIC CHOLINERGIC RECEPTOR distribution. This figure demonstrates quite clearly some of the developmental unique patterns of* $^{3}[H]$ *antagonist labelled receptor observed in the in ovo chick brain, but inparticular, shows the relationship (topographic) between "patch" like fields of higher density receptor and the near linear "parallel arrays" of lower receptor density. None of these patterns of silver grains are in any way artifacts of sectioning, receptor labelling or autoradiographic proceedures.*

Figure 88 and 89. MUSCARINIC RECEPTOR: IN OVO CHICK BRAIN

88


88
14 Days in ovo
HIS-HD
HA
PHL
HV
FA
E
PA

89


89
14 Days in ovo
PHL
E
PA
PP
INP
SM
Retina

to autoradiographic procedures. Figure 101, a dark field photomicrograph of a consecutively cut section to that shown in figure 100, shows the density and distribution of silver grains in a $^{3}[H]$ PrBCM labelled tissue section pre incubated with atropine (ie. non specific binding by $^{3}[H]$ PrBCM), subjected to the same autoradiographic procedure as the section shown in figure 101 Non specific binding by $^{3}[H]$ PrBCM in the *in ovo* chick brain was much less than that determined in the post hatch chick brain, as shown by silver grain densities. It is worth pointing out that in printing figure 105, the time of exposure of photographic paper to the negative image was reduced to show the outline of the brain section.

This demonstrates that the unique transient in ovo patterns of muscarinic receptor density observed in the chick embryo brain are not artifacts of tissue sectioning, receptor labelling or an artifact of autoradiography. Consecutively cut brain sections showed exactly the same patterns of silver grains and morphological differentiation, but if these had been artifacts due to tearing or some other procedural factor, they would not be faithfully reproduced. Tissue sections, whether subjected to autoradiographic procedure or not, showed patterns of silver grains or morphological differentiation which were similar in form. And finally, these specific silver grain patterns were not reproduced in sections treated to show the level of non specific binding by $^{3}[H]$ PrBCM. One other point: the brain section shown in figure 99 in dark field has not been immersed in mountant and or covered with a coverslip. The dark field image of the patterns of cellular structure are not seen in sections immersed in mountant.

Regional receptor density and distribution between 10 DIO and 2 day post hatch.

10 days in ovo. Low densities, but substantially higher than non specific ^{3}H PrBCM binding sites, of antagonist specifically labelled muscarinic receptor are distributed to all regions of the 10 DIO brain. In addition, high densities of muscarinic receptor are localised to motor column nuclei of the brainstem (probably hypoglossal and solitary nucleus), to two bands in the optic tectum corresponding to the SGF and SGC, and diffusely distributed to central and ventral aspects of the diencephalon, corresponding to the PPC, LA, VLT and GLv (see figure 85 of the 12 DIO brain, and figure 93 of the 10 DIO brain).

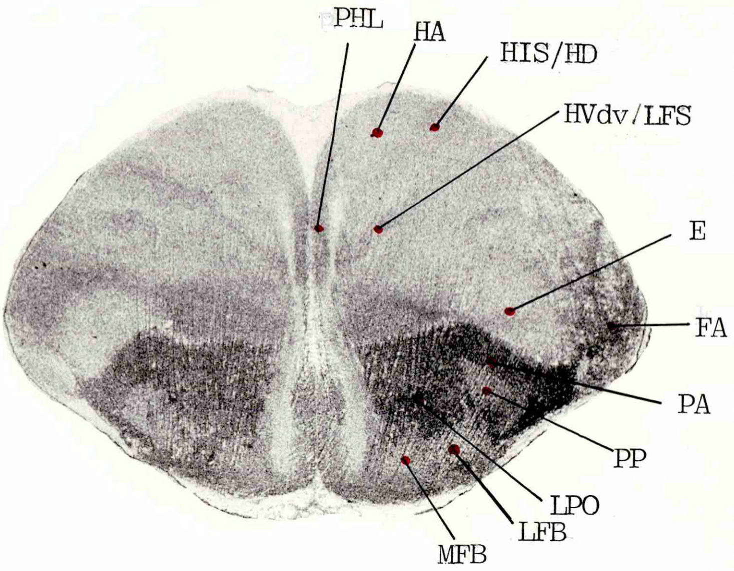
The cerebellum,consisting of little more than an oval swelling either side of the rhombencephalon at this age,is characterised by a small dense muscarinic receptor field localised to cell populations immediately beneath the external matrix cell layer.

In the forebrain, high densities of MAChR are present in most areas of the PA. The LPO is populated by lower densities of receptor, as is also the PP. Very high densities of muscarinic receptor appear in the region of the LMD between 10 and 12 DIO (see figure 85). Dorsal pallial regions of the forebrain are populated by generally low densities of receptor, although lateral aspects, particularly of the left hemisphere are populated by a higher density receptor field bending in towards the forebrain hemisphere midline.

The pattern of muscarinic receptor densities in the 10 DIO embryo brain are

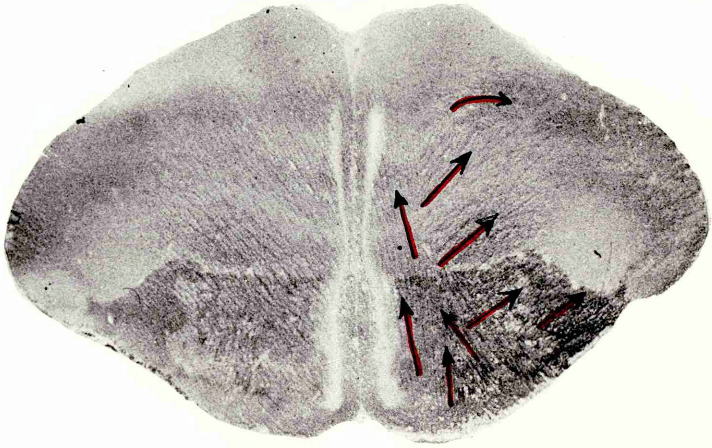
Figures 90 to 92. *A series of light field photomicrographs of autoradiographed frontal sections of the 16 days in ovo (90), 17 days in ovo (92) and 2 days post-hatch (92) chick embryo brain , showing the distribution and regional concentration of silver grains reflecting the distribution of ^{3}H PrBCM labelled MUSCARINIC CHOLINERGIC RECEPTOR.*

Figures 90 to 92. RECEPTOR DISTRIBUTION IN THE IN OVO CHICK BRAIN

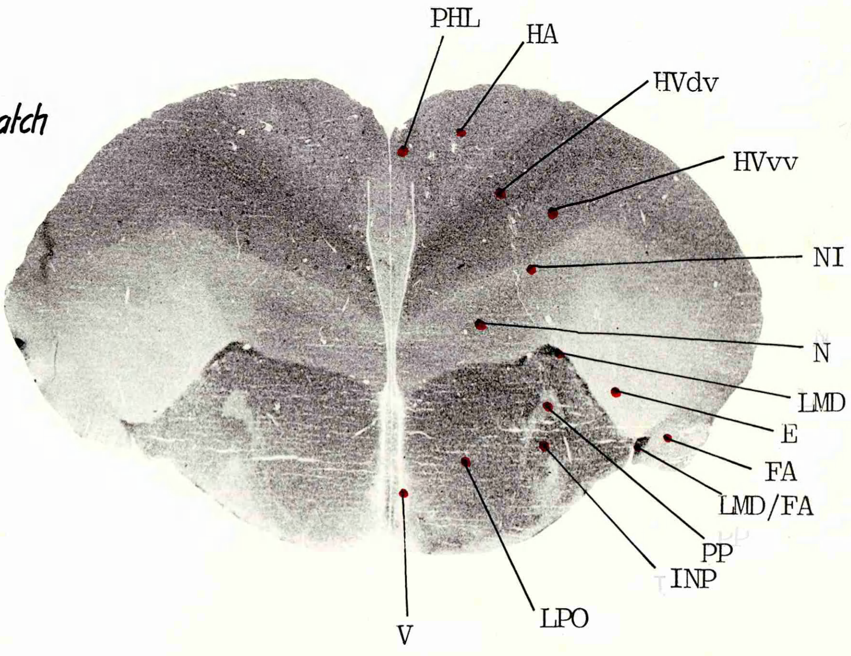


90. 16 Days in ovo

PHL
HA
HIS/HD
HVdv/LFS
E
FA
PA
PP
LPO
LFB
MFB



91. 17 Days in ovo



92. 2 Days *post hatch*

PHL
HA
HVdv
HVvv
NI
N
LMD
E
FA
LMD/FA
PP
INP
LPO
V

generally not characterised by the heterogeneous patterns of receptor observed over later stages of development, although there is evidence for the parallel arrays of lower receptor density described above.

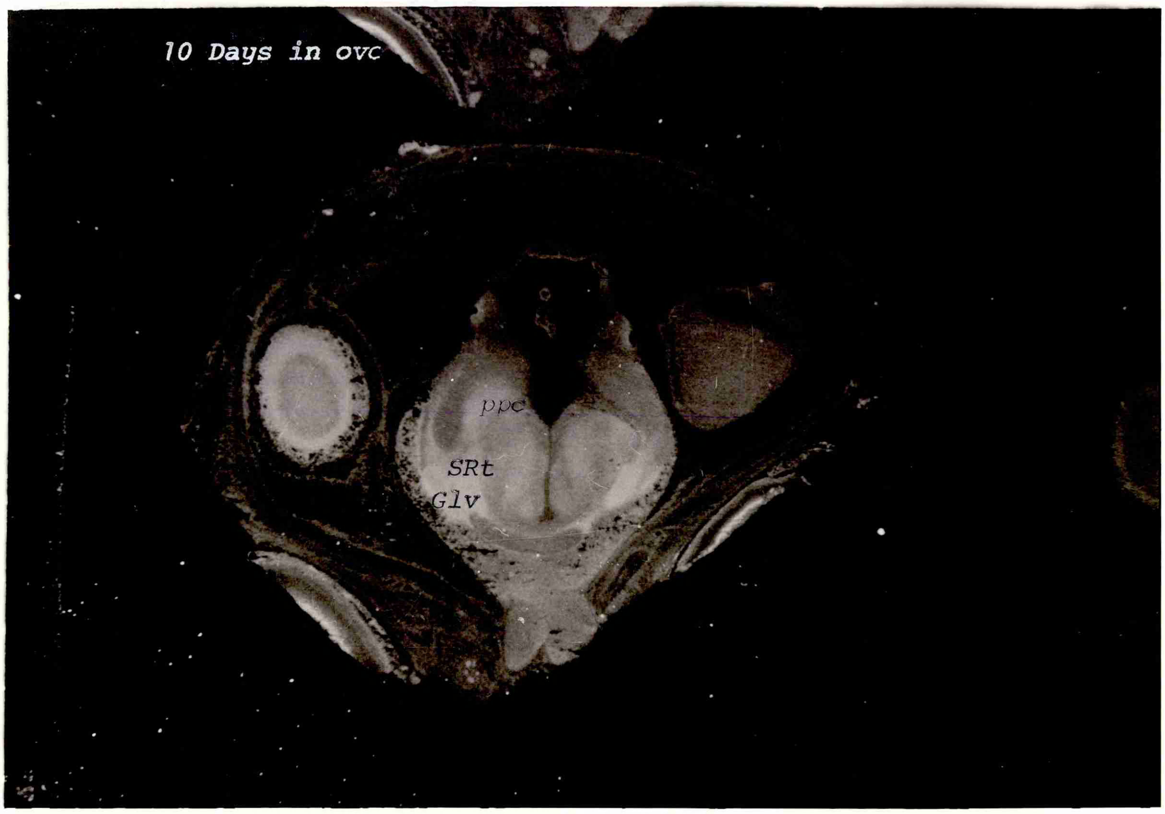
14 days in ovo. By 14 DIO mesencephalic nuclei have begun to differentiate towards adult patterns (see figure 86 and 94). The GLv and VLT are populated by high densities of muscarinic receptor, as is the PPC 'nucleus triangularis' (T) Nucleus Dorsolateralis anterior thalami (DLA), Sub Rotundus (SRt), Reticularis dorsalis (RSd) and the nucleus ovoidalis (Ov) is populated by high densities of receptor over central aspects and enclosed dorsoventrally by an undivided muscarinic receptor field (see figure 94). High densities of receptor are also localised to the MLd and IO (see figure 85 of the 12 DIO embryo brain). Moderate densities of receptor are present in hypothalamic areas and significantly in the optic nerve and chiasma (see figures 94 and 95).

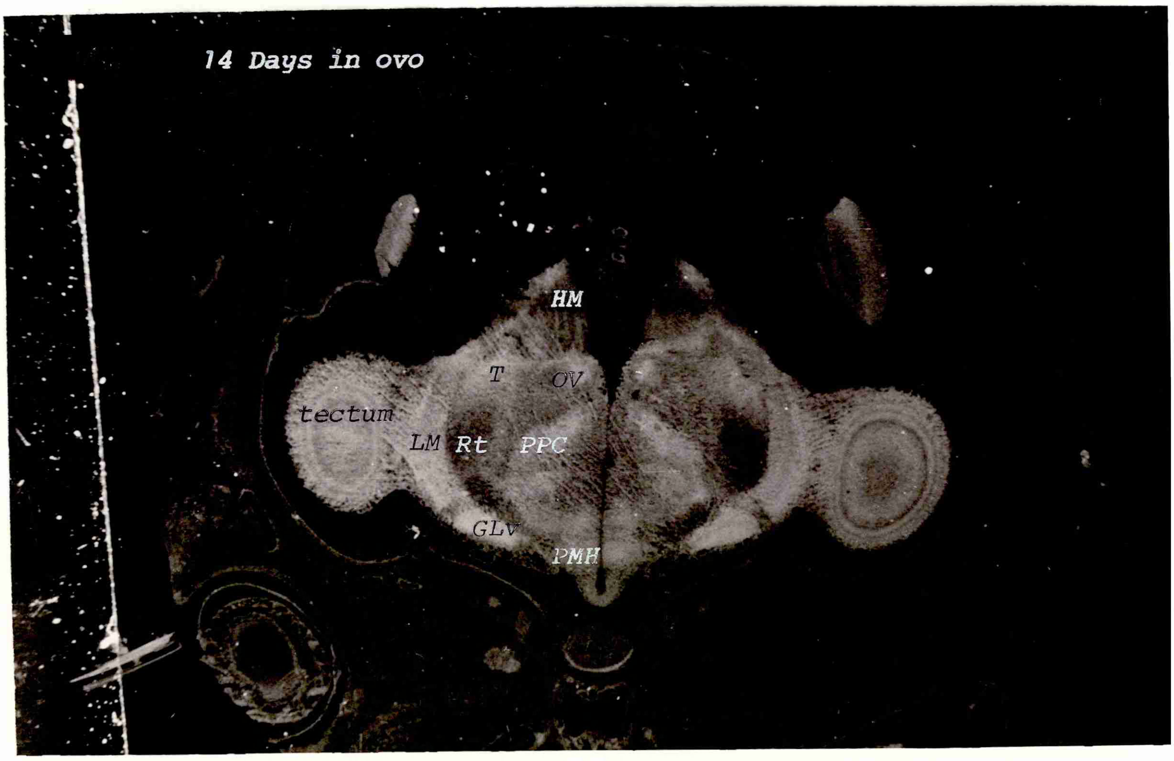
In the cerebellum, now expanded greatly in volume, the molecular cell layer (MCL) of medial aspects of folium II, IV, V, VI, VII and in particular IX is characterised by a thin and dense band of MAChR. This MCL band of receptor is discontinuous. Over more lateral aspects of the cerebellum only the MCL of dorsal aspects of the declive are populated by a high density band of receptor (see figure 103). The granular cell layer of all folia is moderately dense in MAChR. The central cerebellar nuclei CbL and CbM and processus lateralis cerebello-vestibularis are criss-crossed by complex patterns of moderate to high density receptor in 'sheet' and 'parallel array' patterns (see above and figure 104). Regions immediately dorsal to the ventricular surface of the cerebellar plate are populated by moderate to high densities of receptor as is also the choroid plexus.

Figures 93 to 98. *A series of dark field photomicrographs of autoradiographed frontal sections of the 10 days in ovo (94) , 15 days in ovo (95), 16 days in ovo (96) 17 days in ovo (97) , and 19 days in ovo (98) chick embryo brain; showing the distribution and regional concentration of silver grains reflecting the distribution of* ^{3}[H] *PrBCM labelled muscarinic receptor.*

Figures 93 to 98. *A series of dark field photomicrographs of autoradiographed frontal sections of the 10 days in ovo (94) , 15 days in ovo (95), 16 days in ovo (96) 17 days in ovo (97) , and 19 days in ovo (98) chick embryo brain; showing the distribution and regional concentration of silver grains reflecting the distribution of* $[^3H]$ *PrBCM labelled muscarinic receptor.*

Figures 93 to 98. MUSCARINIC RECEPTOR DISTRIBUTION IN YHE IN OVO CHICK BRAIN LIGAND 3[H] PrBCM.


10 Days in ovc
ppc
SRt
Glv


14 Days in ovo
HM
T
OV
tectum
LM
Rt
PPC
GLv
PMH

In the forebrain the high density receptor field of the PA and slightly lower densities of the PP are criss-crossed in linear patterns of receptor (see figure 86). The LMD is distinguished by very high receptor densities. The ectostriatum (E), completely devoid of receptor in the post hatch chick brain, is populated by moderate densities of MAChR (see figure 86). Immediately dorsal to the E is a slightly higher density field of receptor (MRF 8 in the post hatch chick forebrain) from which extends a low to moderate density field extending up towards a region which, post hatch, corresponds to HVc. Immediately opposite this region, across the forebrain ventricle, is a further small low density receptor field identified post hatch as MRF 1 of the CDL (see figure 86).

Curiously, another forebrain visual projection field, HIS, devoid of MAChR post hatch, is populated by comparatively high densities of muscarinic receptor at 14 DIO (see figure 88). From this figure it should also be noted that very high densities of receptor are localised to the retina (see also figure 89a) with moderate to high densities of ^{3}[H] antagonist labelled receptor localised to pia and dura mater and in addition cartilage and bone of the skull.

16 days in ovo. Muscarinic receptor fields of all regions of the brain between 15 and 17 DIO exhibit the most 'chaotic' and elaborately patterned fields of receptor than at any other time during development (see figures 87,90 ,92 , 95-97). At 16 DIO all regions of the brain ventral and caudal to the forebrain paleostriatum are populated by densities of muscarinic receptor equivalent to post hatch densities. Central and medial aspects of the optic lobe, in particular the stratum album centrale (SAC), which post hatch is devoid of MAChR, is populated by radially projecting high density

Figure 95. 15 DAYS IN OVO.

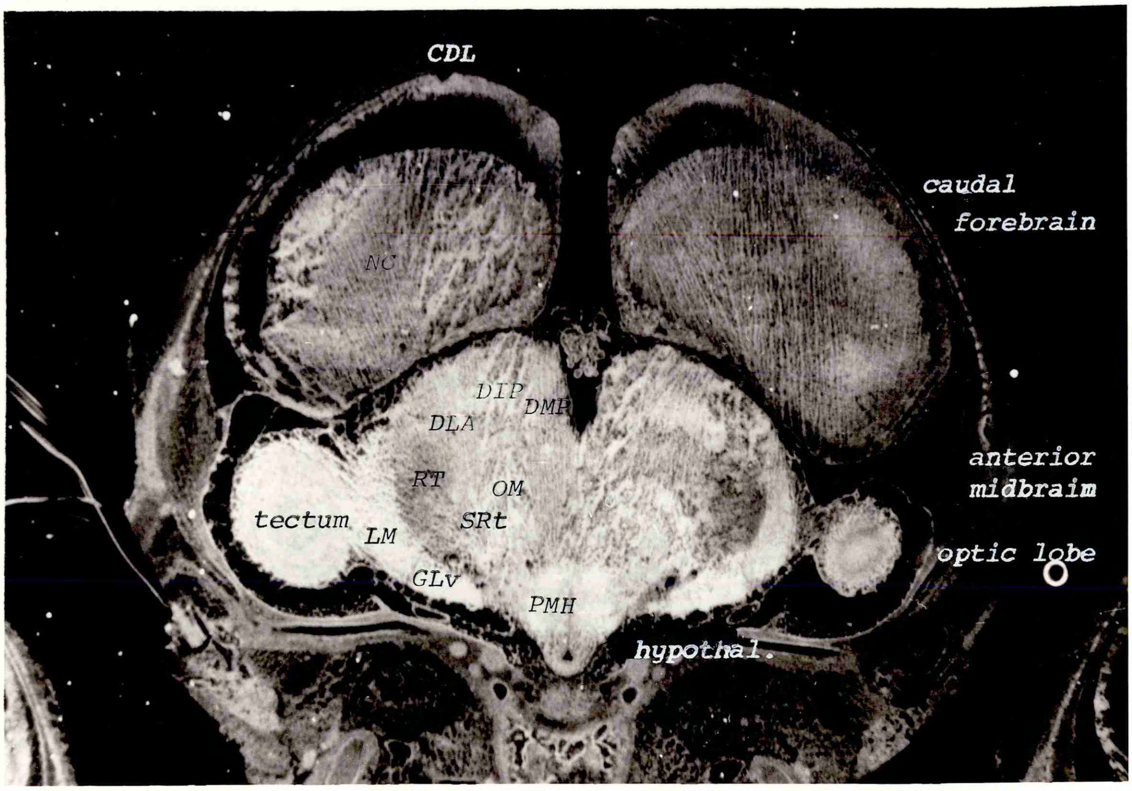
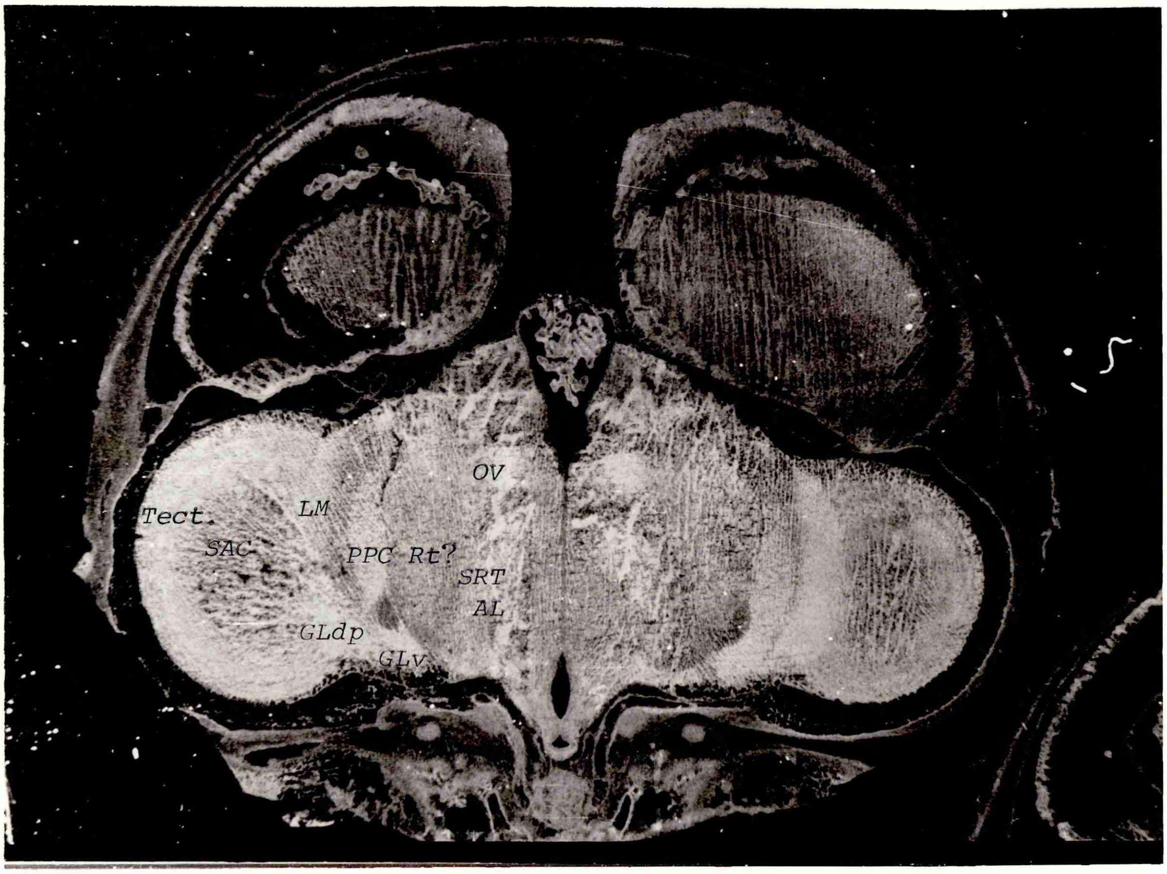

CDL
caudal forebrain
NC
DIP
DMP
DLA
RT
OM
anterior midbraim
tectum
LM
SRt
optic lobe
GLv
PMH
hypothal.

Figure 96. 16 DAYS IN OVO.


OV
LM
Tect.
SAC
PPC
Rt?
SRT
AL
GLdp
GLv

'sheets' of receptor extending towards tectal laminae from equally high densities of receptor localised to nuclei lentiformis mesencephali (LM) (see figure 96). Regions corresponding to the medial and lateral forebrain bundles, the ansa lenticularis (AC), the tractus frontoarchistrialis (FA) and even the tractus quintofrontalis (QF) are all similarly populated by high density and intricately patterned fields of receptor (see figure 87, 90, 95 and 96).

At 16 DIO the SP has lost its earlier moderate to high densities of MAChR. High muscarinic receptor densities appear for the first time in the interpeduncular nucleus (INP). MAChR densities in the E have largely disappeared by 17 DIO (see figure 91). The neostriatum is populated by moderate to high densities of receptor and lateral aspects of the right hemisphere are disrupted by a very high density patterned receptor field (see figure 91). The hyperstriatum ventrale and wulst remain populated by low densities of MAChR. However, thin bands of moderate to high density receptor within the HV may correspond to the lamina frontalis suprema (LFS). The HIS at 16 DIO is still populated by low to moderate receptor densities which are lost around day 18 *in ovo*.

The olfactory lobe is characterised by two moderate density bands of receptor, and the cortex prepiriformis by moderate to high density sheets of MAChR (see figure 87). Between 16 and 18 DIO moderate densities of muscarinic receptor are localised to the nucleus basalis (BAS) which is devoid of receptor in the post hatch brain.

In the cerebellum, the MCL of all folia is populated by very high densities of MAChR, the highest for any stage during development (see figure 87).

Figure 97. 17 DAYS IN OVO.

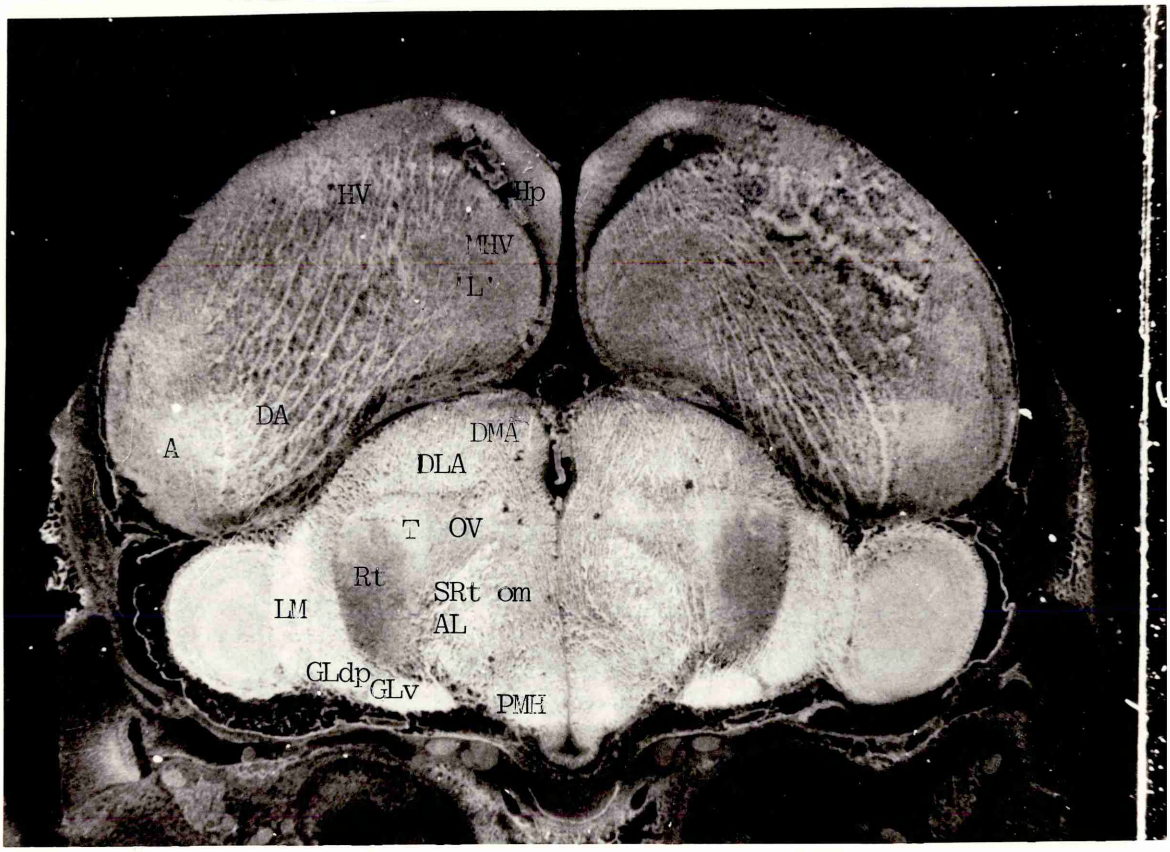
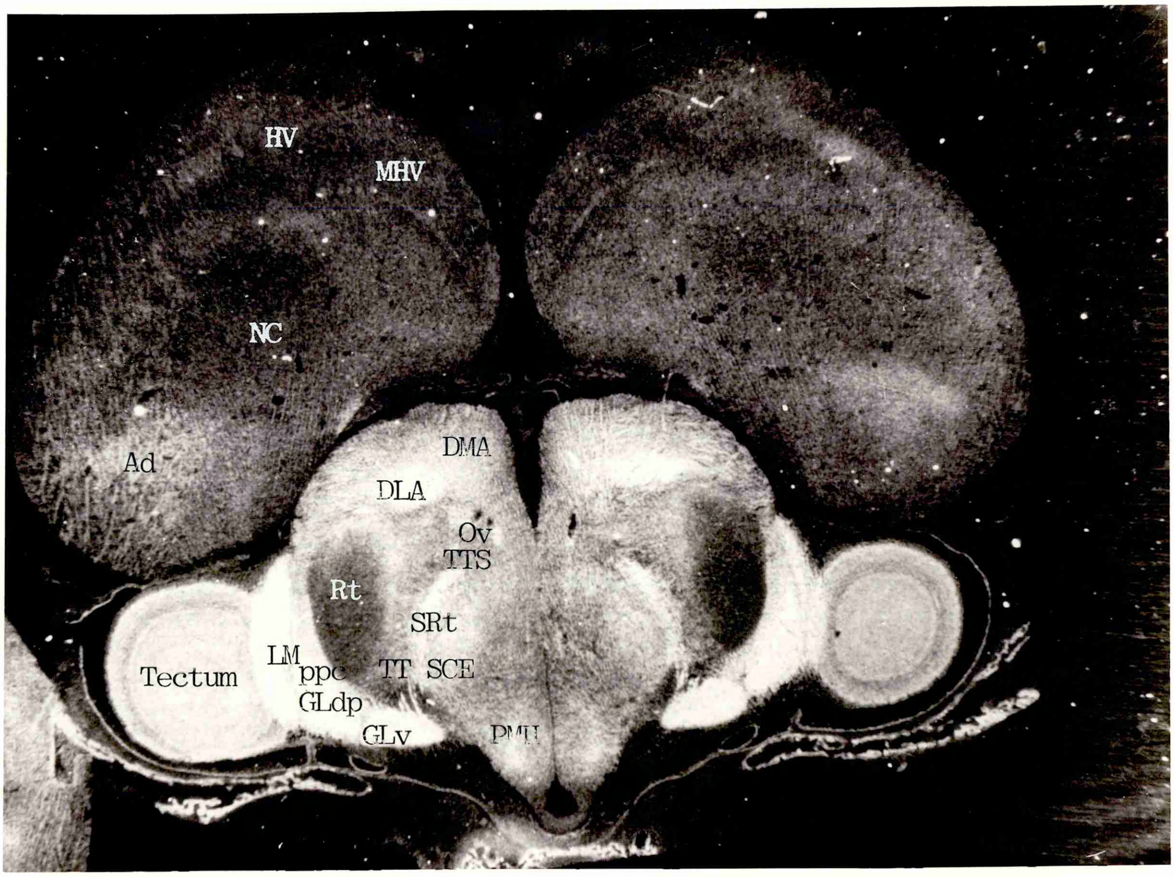

HV
Hp
MHV
'L'
DA
A
DMA
DLA
T
OV
Rt
SRt
om
LM
AL
GLdp
GLv
PMH

Figure 98. 19 DAYS IN OVO.


HV
MHV
NC
Ad
DMA
DLA
Ov
TTS
Rt
SRt
LM
Tectum
ppc
TT
SCE
GLdp
GLv
PMH

The granular cell layer is populated by moderate to high receptor densities. The purkinje cell layer is very low in MAChR. Central cerebellar nuclei are still characterised by high density lines and sheets of receptor(see figure 87).

18 - 21 days in ovo. From 15 DIO in the hindbrain, from 17 DIO in the ventral midbrain and from 18-19 DIO in the dorsal anterior midbrain and forebrain, a dramatic change in the pattern of muscarinic distribution takes place. On a regional basis within periods of one day the 'chaotic' heterogeneous high density patterns of receptor become homogeneous, regionally well defined populations of receptor (see figure 92 for the 21 DIO embryo, and compare figure 97 (18 DIO) with figure 98 (19 DIO)). By 19 DIO the post hatch distribution and comparative regional densities of antagonist labelled receptor are present to all brain regions, with the most notable exception of the hyperstriatum ventrale (HV).

Moderate densities of muscarinic receptor appear in most aspects of the HV between day 20 and 21 *in ovo* (day 21 is the day of hatch) (see figure 92), medial aspects of the HV are populated by moderate densities of receptor a little earlier, but not caudomedial aspects (see figure 98). High densities of muscarinic receptor do not appear in the HV until well past 48 hours post hatch.

In the cerebellum, by 18 DIO, the MCL has increased considerably in thickness and is populated throughout by moderate to high densities of receptor. The GCL is now populated by even increasingly lower densities of receptor. Between 16 and 18 DIO the criss-cross moderate to high densities of MAChR disappear from the central cerebellar nuclei and medulla.

Figures 99 to 102. *A series of consecutively cut frontal sections of the 15 days in ovo chick embryo brain, showing in dark field (99) and light field (102) the cellular morphology of the brain; and the distribution of SPECIFIC ^{3}[H] PrBCM labelled MUSCARINIC RECEPTOR (100) and NON SPECIFIC ^{3}[H] PrBCM labelled MUSCARINIC RECEPTOR (101) in dark field photomicrographs of autoradiographed sections. Figures 99 and 102 are stained with creysel-echt violet, neither section of which have been overlaid with a coverslip. The points to notice in this series of photographs include the 'equivalence' of morphological dfferentiation and silver grain patterning, that the optic tract (TeO) is intensely refractive in figure 99, whereas in figure 100,showing muscarinic receptor distribution, the TeO is not refractive (ie. black not white). The reverse is true, for example , in the hypothalamus between figures 99 and 100. Note also the lack of refraction of the pia and dura mater in figure 99, shown to be quite dense in silver grains (= MAChR) in figure 100.*

The section shown in figure 101 has been preincubated with 125 nM atropine sulphate before incubation with ^{3}[H] PrBCM (see methods). Note the complete absence of patterning of silver grains and in point of fact the almost complete absence of silver grains over the TISSUE SECTION. This photomicrograph has been under exposed (ie. photographically, not autoradiographic exposure) compared with that shown by figure 100. Note the high densities of silver grains around the ventral margin of the tissue section of figure 101, reflecting the diffusion of " non specifically " bound ^{3}[H] PrBCM apparently not removed from the tissue section during washing. Surprisingly, similar diffusion patterns are not seen around the circumference of the tissue section shown by fgure 100, although this section has been treated the same way as that in figure 101 but without preincubation with excess and saturating concentrations of atropine sulphate.

Figure 99.

15 Days in ovo

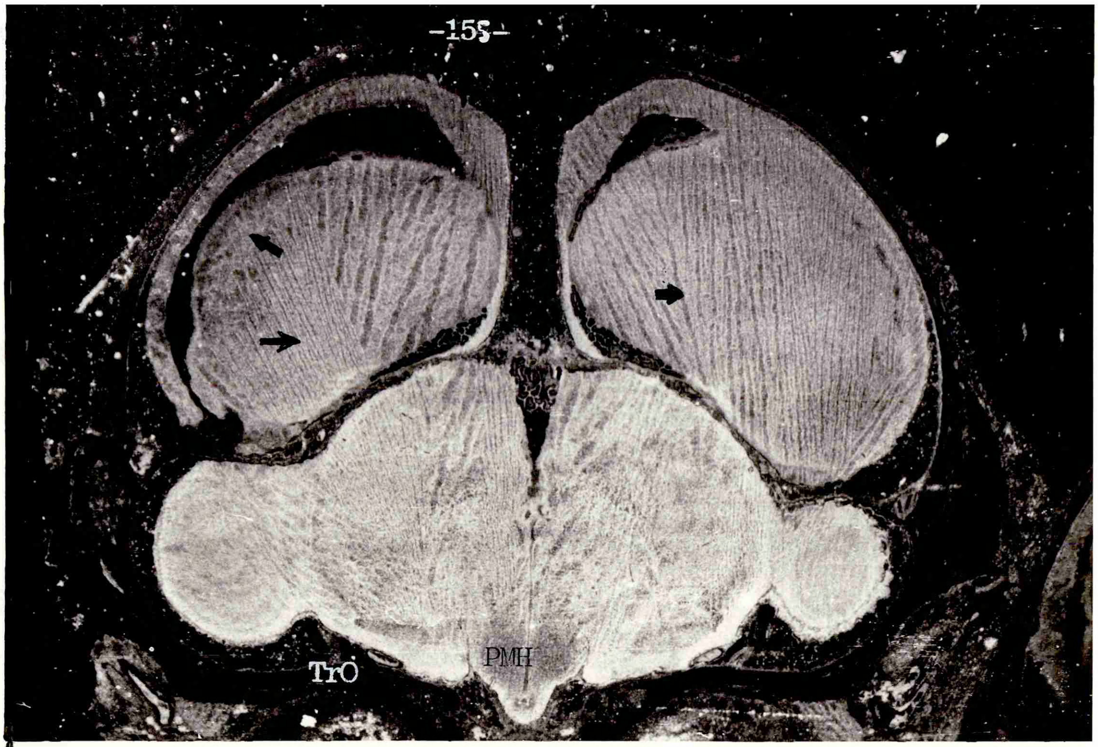


Figure 100

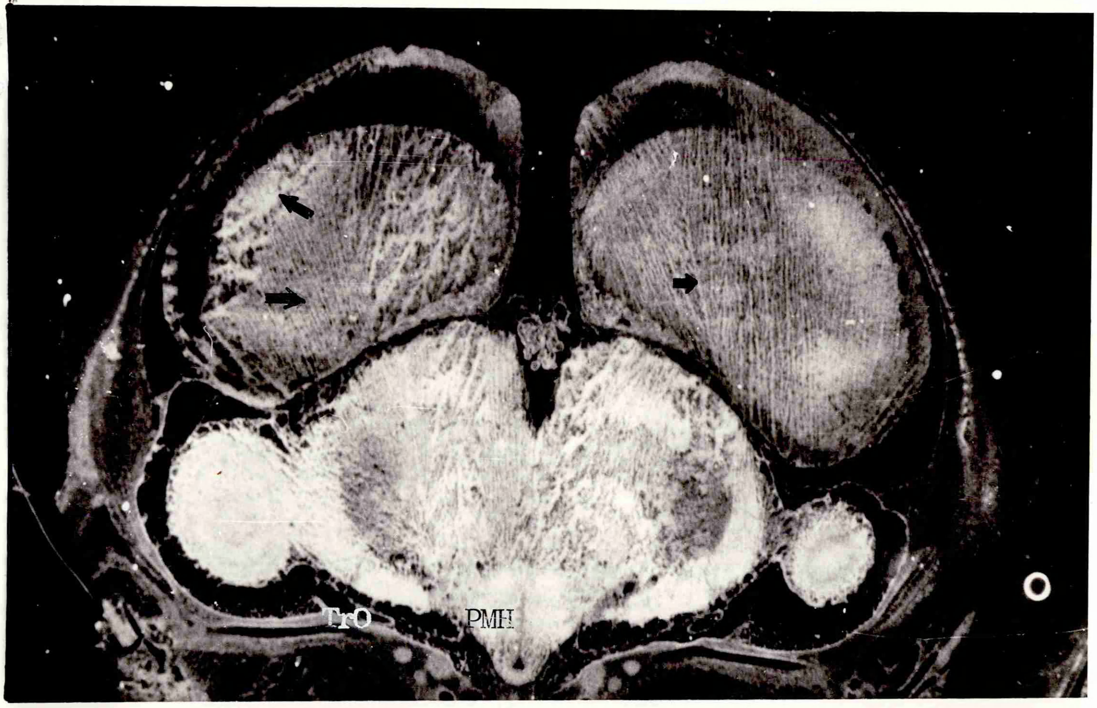
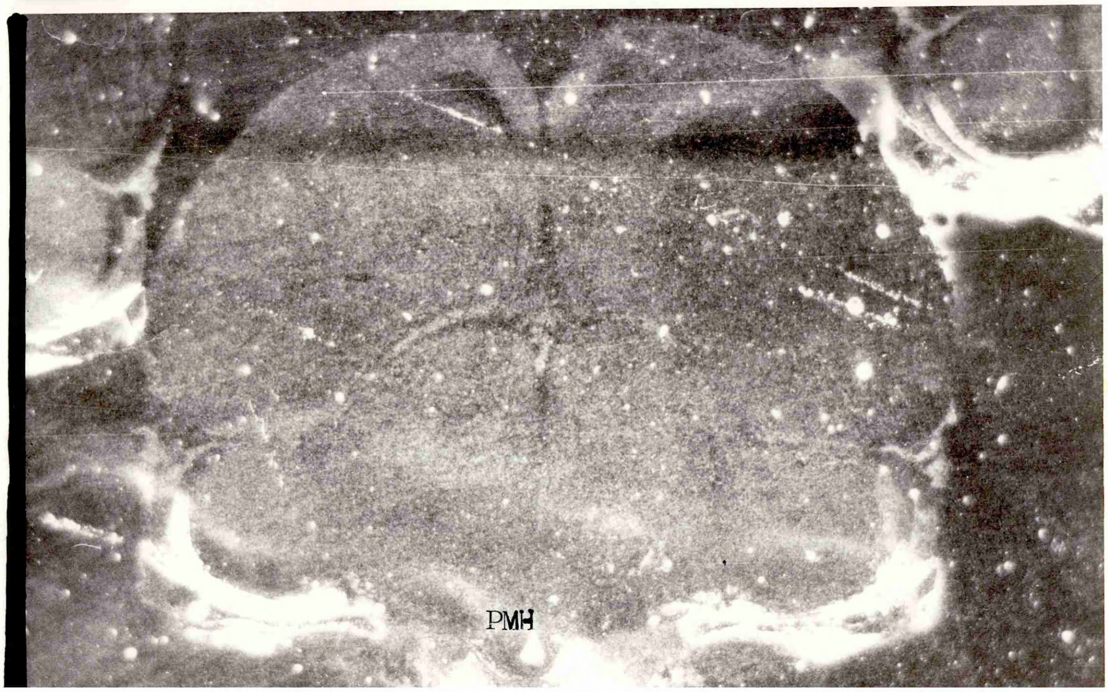


Figure 101.



At 21 DIO, higher densities of muscarinic receptor are localised to peripheral aspects of MCL rather than to deeper layers. Muscarinic receptor density in the GCL is now low with occasional moderate density patches of receptor.

At two weeks post hatch - peripheral aspects of MCL are moderately dense in receptor while deeper layers are populated by low densities of receptor, and by 5 weeks post hatch only the most peripheral aspects of the MCL are populated by low to moderate densities of $[^{3}H]$ antagonist labelled muscarinic receptor and deeper aspects of MCL by low densities of receptor.

Not at any time during chick cerebellar cortical development was there evidence for a 'migration' of $[^{3}H]$ antagonist labelled MAChR from one cortical layer to another (see figure 106 a and b).

Figure 102.
15 Days in ovo.

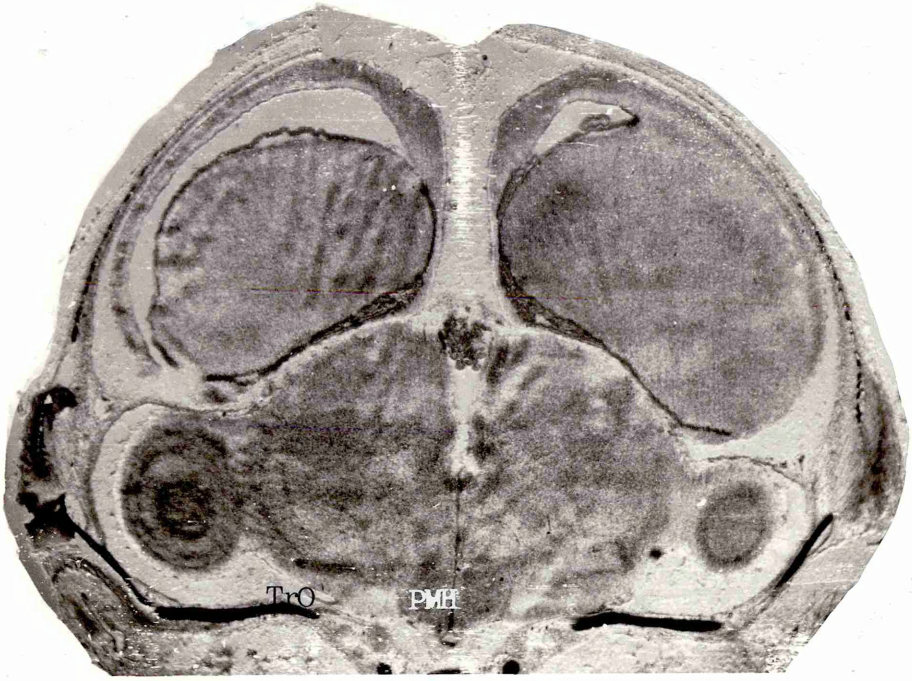


Figure 103.
14 Days in ovo.

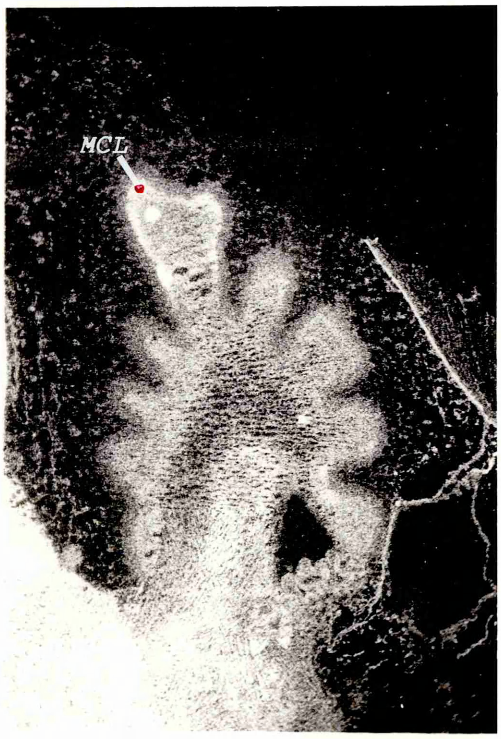
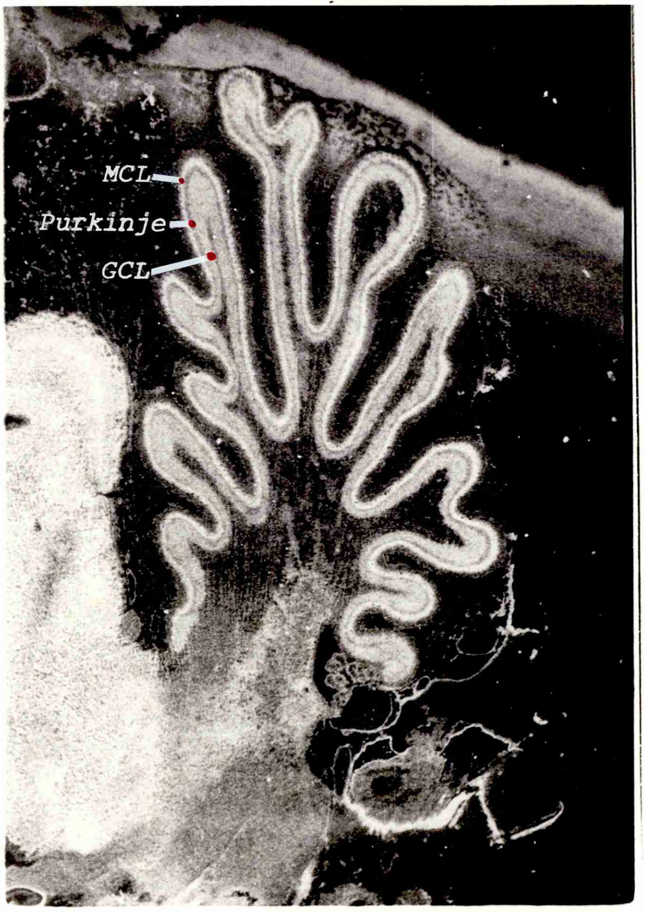


Figure 104.
16 Days in ovo.


MCL
Purkinje
GCL

4. Discussion.

4.1. An overview.

For the most part the following discussion will be directed towards comparing the results of this report for the distribution of ^{3}[H] antagonist labelled cholinergic receptor with other evidence for cholinergic distribution in the vertebrate brain and, furthermore, how the distribution and development of ^{3}[H] antagonist labelled receptor corresponds with current concepts of the 'identity' of cholinergic receptor. However, I feel that it is important not to lose touch with one of the more general aims of this study which is to contribute to our understanding of the role of cholinergic molecules in brain functions. Hopefully, the sections discussing antagonist labelled MAChR distribution in terms of vertebrate brain region homology and antagonist labelled muscarinic cholinergic development in ovo and early post hatch chick brain contribute to this aim.

Here, however, I wish to discuss an earlier objective of this study, the results of which are not included, but which substantially influenced the contexts and content of this report, namely to replicate the observation of a transient change in the number of ^{3}[H] antagonist labelled muscarinic receptor (MAChR) in the forebrain of young chicks trained to a behavioural learning discrimination task (see Rose et al., 1981). With a successful replication in mind, the second objective was to develop a procedure for *in vitro* labelling light microscope autoradiography by which to localise with greater precision than that afforded by homogenate studies, the precise regional locus of ^{3}[H] muscarinic antagonist binding changes following learning. In the event the replication of the observations of Rose et al., (1980) proved negative for reasons which remain unclear. However, the observations directed towards the second objective, to show the distribution of $[^3H]$ antagonist labelled MAChR, in conjunction with the observations on the developmental distribution of muscarinic receptor in the in ovo and early post hatch chick brain are relevant to the findings of Rose et al., (1980).

The hyperstriatum ventrale (Horn et al., 1979; Bradley and Horn, 1979) and basal ganglia (paleostriatum) (Goodman, 1970; Saltzen and Parker, 1975; Collias, 1979) have been strongly implicated as sites for processes concerned with learning in young chicks, while apparently the basal ganglia alone suffices for early socialisation in birds (see Collias, 1979; and see below). These regions of the chick forebrain are populated by very high densities of $[^3H]$ antagonist labelled muscarinic receptor in the 5 weeks post hatch chick brain (see results). However, during development (see section 4.6) $[^3H]$ antagonist labelled MAChR of the basal ganglia, tectal layers and thalamic relay nuclei, maintain a constant density from many days before hatch (from approx. 16 days in ovo onwards). Moreover, with an increase in regional volume over this period, there is undoubtedly an overall increase in regional concentration of $[^3H]$ antagonist binding sites (see section 4.2). In section 4.5 of this discussion I have suggested the possible homology between cell populations of the avian HV with certain cell populations of the mammalian cortex (Benowitz, 1980) on the basis of equivalent high densities of $[^3H]$ antagonist labelled MAChR, low AChE stain and CAT activity, very low α BTX labelled nicotinic cholinergic receptor and an equivalent late developmental expression of post hatch, or late post natal densities of antagonist labelled muscarinic receptor.

Learning in the chick, in particular filial imprinting, is thought to occur during a well defined early post hatch sensitive period (Bateson et al.,(1973) It is a phenomenon not confined to birds, for the maturation of cognitive functions in primates also occurs over a very protracted period of development (Goldman-Rakic, 1982). There is some debate as to whether the HV is required for early social behaviour in chicks or not; according to Collias (1979) early socialisation in birds depends only upon the integrity of the basal ganglia (paleostriatum). However Bateson et al. (1978) have shown that after bilateral destruction of the inner medial hyperstriatum ventrale, a region in tight apposition to the septum and the tractus septomesencephalicus, chicks failed to acquire an imprinted preference. Stettner and Schultz (1966) suggested that lesioning of the hyperstriatum did not affect aquisition of discrimination, but the ability to reverse a learned discrimination. The HV is thought to be a polysensory associative centre (see Benowitz, 1980), while the basal ganglia of birds, similar to mammals, is concerned with motor coordinated behaviours with respect to externally viewed objects (Saltzen and Parker, 1975). As already mentioned, the high densities of ^{3}H antagonist labelled MAChR in the HV are not observed until several days after hatch (at least 4) (see Jerusalinsky et al.,1981) which might suggest that functional coupling of cell populations in the HV during this period is less than complete (but see Bradley et al., 1982). On the other hand, MAChR densities in all other regions of the chick brain, including the basal ganglia, have attained a constant high density before hatch.

The recently hatched chick will form an attachment to any conspicuous object as a result of being exposed to it (Bateson, 1966) and will approach for hours on end without additional reward (see Horn et al., 1979), a behaviour which probably does not involve associative or cognitive processes, but one entirely consistent with circuits 'pretuned' to respond to a conspicuous external object with the correct motor response. This might involve tectal, thalamic and basal ganglia circuits, without perhaps the need for reference to 'higher' brain centres, eg. the HV. In this context it is of considerable interest to note that deficit learning in the Rhesus monkey depends only on subcortical functions in the infant (ie. basal ganglia), and it is only in the adult that this function becomes more essentially cortical (Goldman and Galkin,1978; Goldman-Rakic, 1982).

If mechanisms underlying learning and memory involve alterations in the pattern of neuronal connections, or their efficacy, the experiences (and associated neural activity) must somehow govern their appropriateness. Of two possibilities, 1) activity directed growth and 2) selective preservation (stabilisation) from a continuously forming population of transient synapses (see Changeux, 1977; Greenough, 1982), the latter seems more likely. Later in this discussion (see section 4.3 and 4.6) I have discussed the possibility that the ligand binding properties of MAChR change during development and that the change may be brought about by the electrical activity of developing brain circuits.

Rose et al. (1980) suggested that the increase in binding of ^{3}[H] antagonist to MAChR in the forebrain of chicks subjected to a learning discrimination task (see earlier) may represent either de novo synthesis of receptor, unmasking or activation of preexisting receptor. The transience of that change, beginning at 30 minutes post training and lasting 3 hours, is critical. The present findings show that the rate of appearance of ^{3}[H] MAChR binding sites in the HV of the chick is probably maximum between hatch and at least 48 hours post hatch, which suggests that functional synaptic transmission, at least muscarinic cholinergic transmission, between cell contacts of the HV is very recent and perhaps incomplete at 24 hours post hatch.

According to Csaba (1980)membrane receptors may be 'pliable' during maturation and consequently alter their structure to an extent that accounts for a changed responsiveness of cells to a given transmitter. A number of authors (see Giacobini, 1970; Le Douarin et al., 1975; Paterson, 1978) have emphasised the critical nature of environmental factors in determining whether neurons become cholinergic or adrenergic, in particular the influence of electrical activity. Recently, Bateson (1982) (after Kasamatsu and Pettigrew, 1979), in adressing the question of how a 'stable behaviour' can be changed, suggested that the release and modulatory action (for distinction see Dismukes, 1979) of noradrenalin accompanying behavioural stress, may reintroduce plasticity. The modulatory actions of noradrenalin and dopamine on cholinergic neuronal response is well established (see Vizi et al., 1977; Iversen, 1979).

With the above points in mind it is quite probable that any differences during the course of a paradigm between the levels of stress experienced by trained as opposed to untrained birds with an appropriate increase in the concentrations of stress related neuromodulators, might alter the ligand binding properties of ^{3}[H] antagonist labelled MAChR according to the difference in stress levels. In attempting to distinguish between chemical/morphological correspondents of learning, independent of stress (for example see aversant taste control in the study of Rose et al., 1981), it is important that attention/orientation/motor behaviours are as similar as possible between 'stress control' birds and birds subjected to the behavioural paradigm, because the difference in neuronal activity of circuits subserving these behaviours is likely to be critical in any 'activity' induced change in ^{3}[H] antagonist binding MAChR properties.

4.2 Antagonist muscarinic receptor capacity.

A fundamental criterion for proof of receptor ligand specificity is that ligand binding should saturate over increasing concentrations of free radio-labelled ligand. Apparently, for many reports of receptor ligand binding studies today, this criterion has been modified to read that a *component* of binding should show saturation (see Burt, 1978). That component is the *specific* binding component, or as in the present study, the component of $[^{3}H]$ *l* QNB and $[^{3}H]$ PrBCM binding to tissue slices of the chick brain which is displaced by, blocked by, or sensitive to excess and saturating concentrations of the established muscarinic receptor antagonist, atropine sulphate (Dale, 1914).

As anticipated, *specific* binding by $[^{3}H]$ PrBCM and $[^{3}H]$ *l* QNB to chick brain tissue slices increases rapidly over low concentration increments of free ligand, but over increasingly higher concentrations the rate of increase in the number of additional binding sites declines to reach a constant number between 4 and 6 nM free $[^{3}H]$ ligand concentration. The resulting classic equilibrium hyperbola is taken to indicate that $[^{3}H]$ *l* QNB and $[^{3}H]$ PrBCM are binding to a finite population of receptors with logand capacities of 695 pmoles/g and 585 pmoles/g protein respectively. The close equivalence between chick brain tissue slice receptor capacity for both $[^{3}H]$ antagonists is good evidence that both ligands are binding to a single finite population of receptor. The receptor capacities for $[^{3}H]$ muscarinic antagonists reported here are slightly higher than those reported for other tissue preparations of the chick brain: 265 pmoles/g protein (Francis et al., 1980), 300 pmoles/g protein (Longstaff and Rose, 1981) and 300-600 pmoles/g protein (Jerusalinsky et al., 1981). These latter studies used the mixed stereo-optical isomer ^{3}DL (±) QNB, while the present study used the L (-) isomer of QNB known to exhibit a higher selectivity and affinity for muscarinic receptor (eg. see Gilbert et al., 1979; Aronstam et al., 1979). It might be expected that total binding between these stereo-optical forms of QNB should not differ, only the apparent affinity. However, the concentration of non specific binding is likely to be considerably higher for ^{3}[H] DL (±) QNB than ^{3}[H] *l* QNB, and therefore, reported values for specific receptor antagonist capacity correspondingly lower in the former compared with the latter.

The ^{3}[H] muscarinic antagonist capacities for chick brain tissue slice receptor reported here corresponds well with values reported in homogenate preparations of goldfish brain, 685 pmoles/f protein (Francis et al., 1980), frog, 670 pmoles/g protein (Birdsall et al.,1980) and rat brain, max. 600 pmoles/g protein (Kobayashi et al., 1978)). The present values, however, do not correspond closely with ^{3}[H] muscarinic antagonist binding capacities reported for rat brain tissue slices, 2,130 pmoles/g protein (Rotter et al., 1979) and 150 pmoles/g protein (Gilbert et al., 1979). The differences between the present values and those given by Rotter et al. (1979) and Gilbert et al. (1979) are unlikely to be attributable to ionic media (see Birdsall et al., 1979; Aronstam et al., 1979; Gilbert et al., 1979), because all three studies used a similar complex Krebs-Henseleit ringer. The only difference between these studies, apart from species, is in the method of protein estimation. Rotter et al. (1979) assumed a protein concentration of 75% that of tissue slice dry weight, Gilbert et al. (1979) used wet weight as an estimate of protein, and in this report protein was calometrically assayed and determined from a protein versus tissue slice calibration curve (see results).

In contrast to specific binding, non specific binding by ^{3}[H] *l* QNB and ^{3}[H] PrBCM to chick brain tissue slices (ie. that component not displaced or blocked by atropine) is shown to increase slowly and as a linear function of free ligand concentration increments. In accordance with other reports (see Birdsall and Hulme, 1976; Hulme et al., 1978; Yamamura and Snyder, 1974), the present findings suggest that both ^{3}[H] *l* QNB and ^{3}[H] PrBCM are binding to a similar finite population of chick brain tissue slice receptor, the vast majority of which is atropine displaced or sensitive and, therefore, probably muscarinic cholinergic receptor.

A second criterion of receptor ligand specificity is that ligand binding should be shown to be inhibited by pharmacologically active concentrations of muscarinic drugs, whereas there is no such inhibition by drugs whose pharmacological properties suggest a different primary site of action, at least at those concentrations at which they exert their predominant pharmacological effects (see Birdsall et al., 1976). A comprehensive treatment of this criterion is beyond the scope and aims of this report. Nevertheless, data included show that the potency of the established muscarinic antagonists N-methyl scopolamine (NMS) and atropine, determined from the concentration at which 50% of ^{3}[H] antagonist binding is inhibited (I_{50}), are considerably greater than that of the established peripheral nicotinic cholinergic receptor antagonist d tubocurarine, namely 300 and 0.6nM for NMS and atropine respectively and 3mM for d tubocurarine. This is evidence in support of the view that ^{3}[H] *l* QNB does not bind, at least to any great extent, to the same binding sites as d tubocurarine.

Neither atropine nor NMS inhibition of ^{3}[H] *l* QNB binding to muscarinic receptor of chick brain tissue slices appear to correspond to a simple Langmuir isotherm (see Hulme et al., 1981). The Hill coeficient (N_H) for Atropine, 1.27, suggests that either binding is to a heterogeneous population of antagonist binding sites or that binding is cooperative. The Hill coeficient for NMS inhibition deviates even further from unity, N_H 0.5, but in contrast to atropine inhibition, suggests negative cooperativity amongst NMS binding sites.

4.3 The pharmacological identity of muscarinic receptor.

In direct contrast to the above observations indicating heterogeneity or cooperativity amongst muscarinic antagonist binding sites, recent evidence has stressed that the binding of $[^3H]$ antagonists in general and the binding of atropine in particular is to a homogeneous population of receptor which bind antagonists with one affinity (Berrie et al., 1973; Birdsall and Hulme, 1976; Gulper et al., 1977; Birdsall et al., 1978). On the other hand muscarinic agonists apparently bind to at least two sites, termed 'high' and 'low' with a third, but minor, 'super high' affinity site (Birdsall et al., 1978; Hammer et al.,1980).The homogeneity of antagonist binding largely contradicts the observations of Paton and Rang (1966) in showing that atropine binding to cholinergic receptor of ileal smooth muscle was not a clearly saturable process and could not be described in terms of a single binding site but of three, two of which showed limited binding capacities for atropine, while the third component represented simple membrane partition.

Scatchard analysis of the equilibrium curve for $[^3H]$ *l* QNB and $[^3H]$ PrBCM binding to chick brain tissue slice receptor revealed, like the Hill plot of atropine and NMS inhibition of $[^3H]$ *l* QNB binding (see above), a substantial non linear component to $[^3H]$ antagonist binding which could be accomodated by two sites of limited antagonist capacity and, of course, a third minor site, not included in the Scatchard analysis, corresponding to the 'non specific' (non atropine displaced) binding by these $[^3H]$ antagonists.

Antagonist binding heterogeneity has also been shown for a number of structural analogues of N-2-chloroethyl derivatives of benzilycholine, such as benzilycholine mustard (BCM) (Fewtrell and Rang,1973; Burgen et al.,1974) and benhydryl mustard (BHM) (Gill and Rang, 1966; Moran and Triggle, 1970; Gupta, 1973). BCM binding to cholinergic receptor of ileal smooth muscle, similar to BHM and atropine, was shown to be a non saturable process, but described in terms of two binding sites, one the acetylcholine (muscarinic) receptor proper, the other an allosteric or regulatory site (Gupta et al., 1976), after Monod et al. (1965). Of perhaps even greater significance to the present observations is the suggestion made by Gupta et al. (1976) that alkylation of the ACh receptor site only occurs over higher concentrations of BCM ($>$ 5nM), below which binding is confined to the regulatory site (the concentrations of $[^3H]$ *l* QNB and $[^3H]$ PrBCM used in this study to show the distribution of muscarinic cholinergic receptor in the chick brain are 1.6 and 3.0 nM respectively)).

According to Burgen et al. (1974) and Rotter et al. (1979), propylbenzilycholine mustard (PrBCM), also a close structural analogue of benzilycholine, in contrast to BCM, BHM and atropine, binds to a single finite population of receptor in rat brain homogenates and tissue slices. However, this is not strictly true, since the above reports, similar to observations in this study, show that $[^3H]$ PrBCM binding is distinguishable into two components, one atropine sensitive and one insensitive. Rotter et al. (1979) reported that the atropine insensitive component of $[^3H]$ PrBCM binding was in excess of 30% that of specific binding; significantly, over low $[^3H]$ PrBCM concentrations (>5nM) atropine inhibition deviated substantially from linearity (see above ref. Gupta et al., 1976) which was explained by Rotter et al. (1979) in terms of discrepancies in initial uptake and binding by ^{3}[H] PrBCM to rat brain tissue slices. The high level of non specific binding reported by Rotter et al. (1979) was not observed in the present study for either ^{3}[H] PrBCM or ^{3}[H] *l* QNB binding to chick brain tissue slice receptor.

The full significance of ^{3}[H] antagonist binding heterogeneity to CNS MAChR is difficult to ascertain at the present time (see Hammer et al., 1980). This phenomenon may prove to have considerable bearing on how we view receptors in general. For example heterogeneity of antagonist binding undermines the critical pharmacological criterion of a strict, mutual complimentarity of antagonist-agonist binding sites and hence the established view that the sites of action of different chemical transmitter-modulators on the membrane receptor surface are entirely discrete (see Triggle and Triggle, 1976 and ref. therein; and Introduction). Moreover, other observations suggest that heterogeneity of antagonist binding may be reflecting different cellular sites of location of muscarinic receptor. For example, according to Szerb (1977) the affinity of the muscarinic antagonist ^{3}[H] (±) QNB for 'presynaptic' receptor is 10-20 times less than for postsynaptic receptor. This observation appears to be related to the findings of Walmsley et al. (1980 and 1981) in showing that ^{3}[H] antagonist labelled MAChR are transported along the axon of the sciatic and vagus nerve of the rat to presynaptic sites, in additon to the observation that MAChR, associated with central white matter tracts of the mammalian brain, eg. corpus callosum, are of the agonist high affinity type. According to Snyder (1975), heterogeneity of muscarinic antagonist binding is a consequence of antagonist binding with high affinity to the antagonist form of receptor, but lower affinity to the agonist form, with the reverse true for agonists. Heterogeneity of muscarinic ligand binding is discussed further in section 4.6 with respect to apparent developmentally transient [^{3}H] antagonist binding sites associated with white matter tracts.

4.4 Receptor versus other cholinergic marker distribution in the avian brain.

In conjunction with pharmacological criteria for receptor ligand specificity (see sections 4.2 and 4.3), an additional and pertinent criterion is that the location and distribution of antagonist labelled receptor should correspond with other evidence for neurotransmitter-receptor distribution.

It has been suggested (Aprison and Takahashi, 1965) that cholinergic systems in the avian brain are in excess of other transmitters. The extremely wide and concentrated populations of muscarinic receptor (MAChR) shown here for the chick brain might be seen to support this view. However, as has already been discussed (see section 4.2), the overall concentration of MAChR corresponds closely with values reported for the brains of other vertebrates, including the chick (Jerusalinsky et al., 1981), for which the consensus of opinion is that cholinergic systems are less prolific than, for example, catecholaminergic (Jurio and Vogt, 1967; Shute, 1975; but see also McGeer and McGeer, 1979).

The concentration of acetylcholine (ACh) and its synthetic and degradative enzymes CAT and AChE have been shown to be highest in the mesencephalon of the pigeon brain (Aprison et al., 1964; Aprison and Takahashi, 1965). This observation does not correspond with the results of the present study in showing that MAChR are present in approximately equivalent concentrations in the forebrain: 339 ± 75, midbrain: 398 ± 137, and hindbrain: 205 ± 96 pmoles/g protein. But the report of a greater concentration of AChE and CAT to the pigeon midbrain (Aprison and Takahashi, 1965) does correspond with considerably higher concentrations of α BTX labelled nicotinic cholinergic receptor in the chick midbrain, 160 ± 25, compared to the forebrain, 15 ± 2 pmoles/g protein (this report). This is a particularly curious correspondence, since, as will be discussed more extensively below, one of the denser MAChR fields of the chick brain, the hyperstriatum ventrale, has been shown by other studies to be devoid of AChE (Kusunoki, 1969; Karten and Dubbeldam, 1976) (see figures 3 a-c) and by the present finding to be similarly devoid of nicotinic α BTX labelled receptor, a result also observed by Bradley and Horn (1981) in the chick brain.

On the basis of these observations, therefore, there appears to be a close correspondence between AChE and nicotinic receptor concentration,but not necessarily muscarinic receptor. On the other hand, comparison of MAChR distribution shown here for the chick brain with one of the very few detailed histochemical studies of AChE staining in brain tissue sections of other avian species, *Uroloncha domestica* and *Anas platyryncha v domestica* (Kusunoki, 1969) reveals a remarkably close correspondence between the regional distribution and concentration of these two markers, particularly considering the extensive criticism of the use of AChE as a specific cholinergic/cholinoceptive marker (see introduction,) and Silver, 1974).

It cannot be overemphasized that it is not only the precise regional localisation of AChE to the brain regions of these different avian species which corresponds exactly with MAChR localisation in the chick, but the near identical correspondence of intensity of AChE stain and MAChR density, as for example to all regions of the archistriatum, paleostriatum, in particular, the lamina medullaris dorsalis and nucleus interpeduncularis, and, curiously, MRF 9 and the dense, discrete MAChR field of the area corticoidea dorsolateralis (see figures 3 a - c and section 4.5). As mentioned earlier, the HV has been shown to be devoid of AChE (Kusunoki, 1969; Karten and Dubbeldam, 1976). However, in Kusunoki's study caudal aspects of the medial hyperstriatum ventrale (see Bradley and Horn, 1981) are shown to stain moderately for cholinergic enzyme. Apart from the HV, there are only two other regions between these avian species which do not show corresponding densities of MAChR and AChE stain, the nucleus isthmi, shown in Kusunoki's study to stain intensely for AChE, but in the chick to be devoid of MAChR (this report), and the nucleus rotundus, shown to stain moderately for AChE, but again in the chick to be largely devoid of MAChR. However, the nucleus isthmi and lemniscal complex of the chick are dense in ^{3}[H] α BTX labelled nicotinic cholinergic receptor (this report), and even the rotund nucleus is populated by low densities of nicotinic receptor.

While AChE distribution and intensity of stain corresponds very closely with cholinergic receptor distribution at a regional level, it is apparent that, over laminated regions of the avian brain, the optic tectum and cerebellar cortex, the correspondence is far less convincing. The distribution of CAT and AChE activity/stain in the pigeon optic tectum (Henke and Fonnum, 1976; Kusunoki, 1969; Shaerer and Sinden, 1949), compared with MAChR distribution (this report) and ^{3}[H] α BTX nicotinic cholinergic receptor distribution (Poltz-Tejera et al., 1975; and this report), is shown in figure 105 . Neither CAT nor AChE correspond with muscarinic or nicotinic distribution. Surprisingly, MAChR and nicotinic receptor show quite a close correspondence, as also do AChE and CAT (Henke and Fonnum, 1976). The present findings show high densities of nicotinic cholinergic receptor localised to tectal sublayers (II a and 6, or 2 and 3) of the stratum grisea et fibrosum (SGF) which differ from the findings of Poltz-Tejera et al. (1975) in showing high α BTX binding to sublayers II b and c (3 and 4 in their report). However, the results of this study and that of Poltz-Tejera et al. (1975) agree in showing that sublayer II f is the most densely populated nicotinic receptor lamina of the chick and pigeon tectum, in addition to sublayer II f being the most dense MAChR lamina of the chick tectum (this report).

The cerebellar cell layers of many vertebrates stain intensely for AChE ((see Silver, 1974), and it was largely the observation of Curtis and Crawford (1965) and Crawford et al. (1966), showing the unresponsiveness of cerebellar cells to the iontophoretic application of ACh , together with the observation of low CAT levels (see Hebb and Silver, 1967), that led to the widely held view that AChE is an imperfect marker for cholinergic systems. Friede and Fleming (1964) revealed that almost all AChE staining of the pigeon, canary and parakeet cerebellar cortex is localised to the MCL, but, in particular, to regions adjoining the Purkinje cell layer. The granular cell layer (GCL) of the chick (Phillis, 1965)) also stains for AChE, but the intensity of stain was still only shown to be half that observed in the MCL. The results of the present study in showing moderate densities of MAChR localised to most peripheral aspects of the MCL, clearly does not correspond well with the above reports for the distribution and intensity of AChE stain, although moderate AChE stain in the GCL (Phillis, 1965) might be seen to correspond with the low densities of nicotinic receptor localised to the GCL (this report).

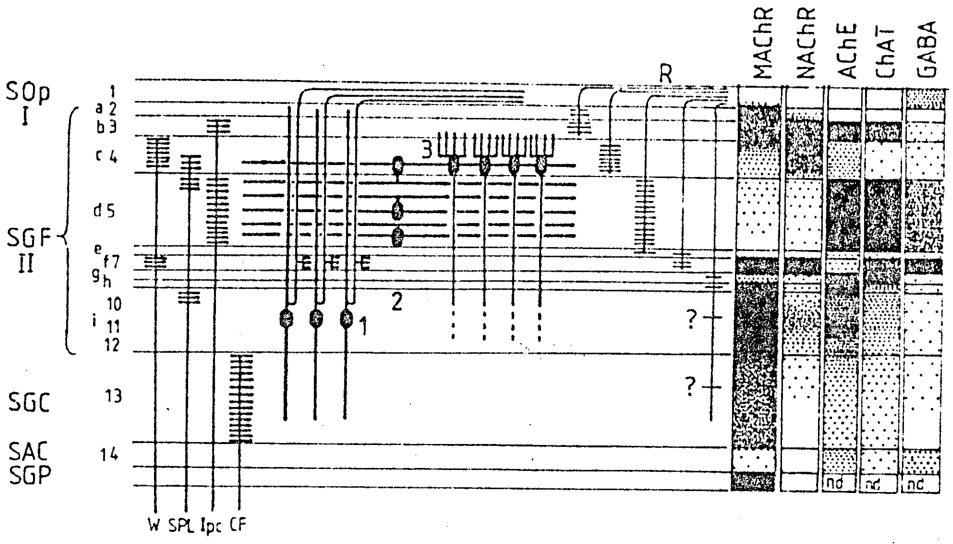


Figure 105. *Diagramatic representaion of ^{3}[H] PrBCM and ^{3}[H] 1 QNB labelled muscarinic receptor (MAChR) (this report), α BTX labelled nicotinic receptor (NAChR) (Poltz-Tejera et.al., 1975), acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activity (Henke and Fonnum , 1976) and gamma amino butyric acid (GABA) (Hunt and Kunzle, 1979) distribution in layers of the optic tectum of the chick and pigeon brain. To the left of this figure is a diagramatic representation of three proposed inhibitory networks of the tectal cortex (1,2 and 3) (after Hunt and Kunzle, 1979), in addition to retinal afferents (R).Other afferents shown: W : visual wulst; SpL : nucleus spiriformis lateralis; Ipc ; nucleus isthmi; CF : commisural fibres. Note that sublayer IIf is the most dense MAChR, NAChR and GABA populated lamina of the tectum, a lamina in receipt of axonal arborisations from IIi (Γ) inhibitory system.*

SOp : stratum opticum; SGF : stratum grisea et fibrosum; SGC : stratum grisea centralis; SAC : stratum album centrale; SGP : stratum grisea et periventricularis.

While MAChR distribution and density in the chick cerebellar cortex, apparently does not correspond with AChE distribution in the adult avian cerebellum (adult here is taken to be represented by the 5 weeks post hatch chick brain), distribution of MAChR during in ovo development is substantially different from adult patterns in the chick (this study) and corresponds much more closely with the patterns of AChE staining in the cerebellum of adult birds (see above). In addition, the density of MAChR is higher in the in ovo chick cerebellum than in the post hatch chick brain, an observation which corresponds with that of Elkes and Todrick (1955) and Hamwhich and Aprison (1955) for higher concentrations of cholinesterase in the developing cerebellum than those observed in the adult. However, perhaps of even greater significance is the observation from this study that the 14 day in ovo chick cerebellum is characterised by high densities of MAChR to the MCL of folium IX (uvula), folium VI and folium VII (tuber vermis) (see figures 87,103), a pattern of MAChR density and distribution which corresponds precisely with that of AChE distribution shown in the adult Uroloncha brain (Kusunoki, 1969, see figure 3 a - c).

Exactly why the pattern and density of MAChR in the chick cerebellum should correspond more closely with AChE staining in the adult avian cerebellum, only during a critical stage in development, is not clear. But it has been suggested, in order to explain low CAT levels in the adult, that, during development, cerebellar cholinergic neurons, speculated to be 'non functional', lose their enzyme complement (Hebb and Ratkovic, 1964, see section 4.6). On the other hand, as will be discussed later, it is as likely that the ligand recognition properties of cholinergic receptor alter during development, a change which may be coincidental to that shown for substrate preference and inhibitors of acetylcholinesterase in the developing chick brain (Kasa and Csillik, 1968; Krnjevic and Siegel, 1965). The axonal specific 11 S form of AChE has been shown to develop around day 16 in ovo (Courand et al., 1979; Villafruela et al., 198), 8 days after the 4 and 6 S molecular species. Curiously, the high affinity agonist form of muscarinic receptor, reported to be in nerve axons, also appears some seven days after the appearence of the low affinity forms of receptor (Walmsley et al., 1980; 1981).

No attempt will be made in this report to relate the distribution of ^{3}[H] antagonist labelled cholinergic receptor with possible cholinergic pathways. It might be possible to associate regional density of ^{3}[H] antagonist receptor with the size of a particular afferent input or efferent output. However, the observations of this report give no indication of whether ^{3}[H] antagonist labelled MAChR are localised to pre afferent contacts or post afferent sites. In addition, the assumption that ^{3}[H] antagonists are labelling synaptic receptor is by no means proven (see introduction, section 1.2). For example, a substantial number of ^{3}[H] muscarinic antagonist receptor may be 'acceptors', or binding to non neuronal cells, in particular glia (Repke and Maderspeck,1982)

In the following section evidence is given which suggests that cholinergic pathways of the mammalian brain (see Lewis and Shute, 1978; McGeer and McGeer, 1979) are to a large extent present in the chick.

4.5 Muscarinic receptor distribution between brains of vertebrate species.

Recent decades have seen a greater emphasis placed on common patterns of neurotransmitter type and brain distribution as additional, but strong supportive evidence for vertebrate brain region homology (Koelle, 1954; Dahlströme and Fuxe, 1965; Jurio and Vogt, 1967; Parent and Oliver, 1970; Karten and Dubbeldam, 1973; Wächtler, 1979; Ellison, 1979). By homology I mean equivalent characters having a common phylogenetic origin (see Hodos, 1967). According to Sakharov (1974) the presence of several neurotransmitter substances in the vertebrate nervous system is a relic of primitive neuronal organisation and that those neurons which share a common and distinct set of specific chemical characteristics are of common ancestry. In other words, similarity between neurons with respect to their transmission chemistry is a good indication that these neurons are homologous. This may not, however, be true for receptors which, it has been suggested, evolved independently of transmitters and, during development, may be 'imprinted' according to the transmitter released from the innervating presynaptic terminal (see Csaba, 1980). The pattern and density of receptor may be considerably modified as a consequence of environmental sensory influence. Nevertheless, receptor development is probably an integral part of phylogenesis and common patterns of receptor distribution are probably as valid a criterion as comparing morphology and topography, criteria most commonly employed in denoting homology.

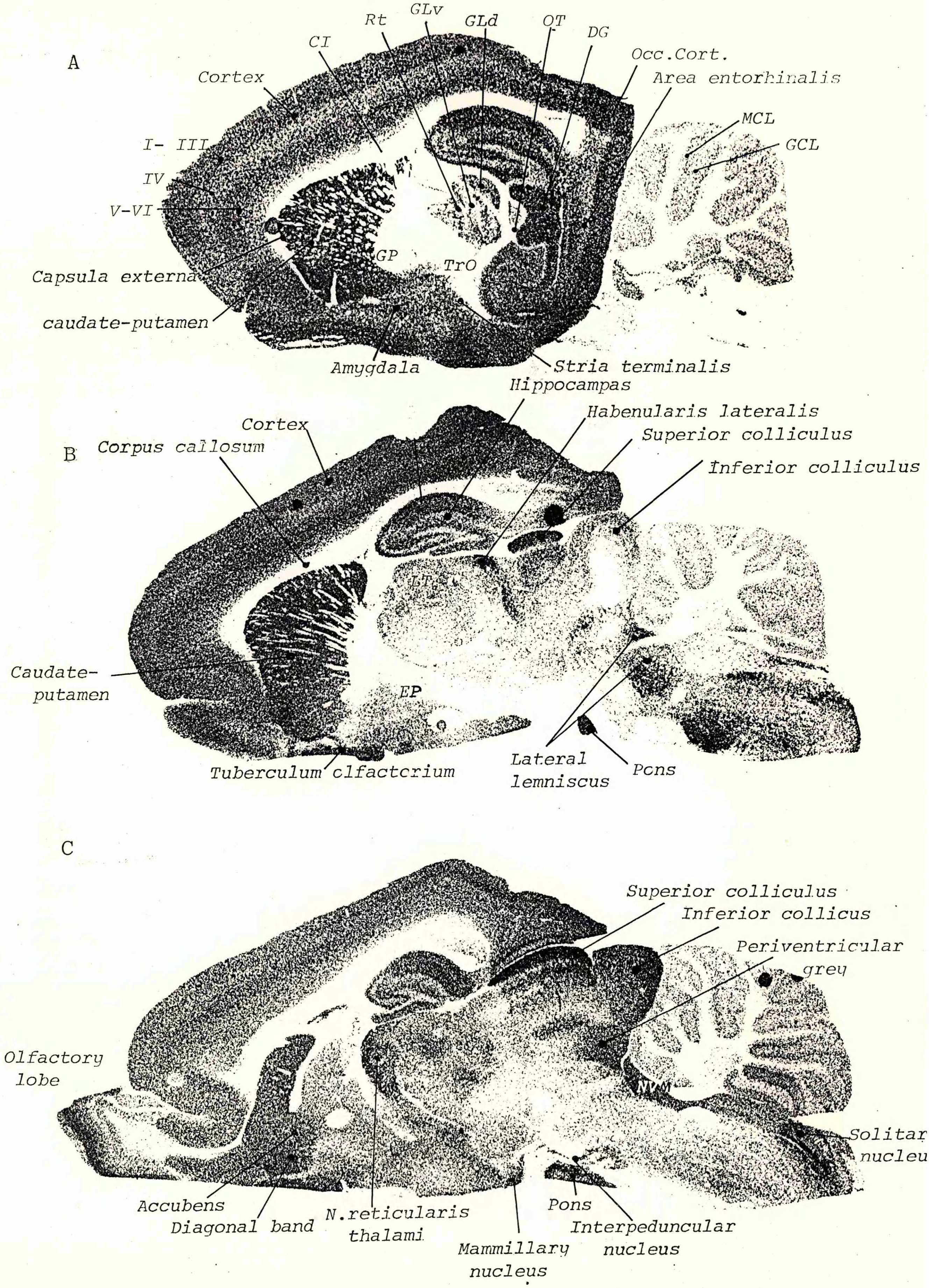
On the other hand, it should be stressed that the concentration of neurotransmitter, enzyme or receptor has been shown to change substantially as the result of experience (Rose and Stewart, 1978; Rose et al., 1981) or seasonal variation (Dryer and Peper, 1974) or postmortem changes (Stavinhoe and Weintraube, 1974). In addition, in comparing highly divergent species of vertebrate brain, it is possible, as shown for catecholamines (see Burnstock, 1979 ; and section 4.1), that there may be a change in the predominence of one neurotransmitter-receptor type over another. However, the case for using ^{3}H antagonist labelled muscarinic receptor distribution in particular for comparative study is perhaps strengthened by the observation of Birdsall et al. (1980) that the ligand binding properties of MAChR have probably changed very little during the course of vertebrate evolution (but see section 4.3 and 4.6). With these points in mind, the following discussion will be restricted to comparatively well established homologues of vertebrate brain with respect to distribution and density of ^{3}H antagonist labelled cholinergic receptor.

Kappers (1936) suggested that the basal ganglia of mammals are relatively unmodified representatives of one of the most ancient features of the vertebrate brain. Kallen (1953; 1962); in comparing reptilian, avian and mammalian forebrain development, proposed that the paleostriatum augmentatum (PA) and paleostriatum primitivum (PP) of birds are homologues of the mammalian caudate nucleus and putamen (CP) and globus pallidus (GP) respectively. The results of the present study have shown that,while the PA is populated by very high densities of muscarinic receptor and differentiated by numerous unlabelled fibrous striations, the PP is largely devoid of receptor. This observation corresponds with evidence for MAChR distribution and density in the mammalian basal ganglia where the CP has been shown to be very dense in MAChR but not the GP (Kuhar and Yamamura, 1976; Kobayashi et al., 1978; Rotter et al., 1979; and this report see figures 105,106). The above evidence adds further support to the CP-PA, GP-PP homology and in addition complements

Figure 106. *A series of light field photomicrographs of the pattern and regional density of silver grains in LKB tritium film exposed and apposed to parasagittal tissue sections of the 50 day post natal rat brain labelled by ^{3}H 1 QNB for MUSCARINIC CHOLINERGIC RECEPTOR. (Coulter, unpublished observations). The section shown in C is medial to B which is medial to A. (For a comparison with the chick see text of discussion).*

Abbreviations;

Figure 106. MUSCARINIC RECEPTOR DISTRIBUTION IN THE 50 DAYS POST NATAL RAT: LIGAND ^{3}[H] 1 QUINUCLIDINYL BENZILATE.


A
Rt
GLv
GLd
OT
DG
CI
Occ.Cort.
Area entorhinalis
Cortex
MCL
GCL
I- III
IV
V-VI
GP
TrO
Capsula externa
caudate-putamen
Amygdala
Stria terminalis
Hippocampas
Habenularis lateralis
Superior colliculus
Cortex
B
Corpus callosum
Inferior colliculus
LT
Caudate-
putamen
EP
Tuberculum clfactcrium
Lateral
lemniscus
Pcns
C
Superior colliculus
Inferior collicus
Periventricular
grey
Olfactory
lobe
Solitar
nucleu
Accubens
Diagonal band
N.reticularis
thalami
Pons
Interpeduncular
nucleus
Mammillary
nucleus

other histochemical evidence for their equivalence. For example, high concentrations of catacholamines are shown to be localised to the mammalian striatum (Fuxe, 1964; Jakabowitz and Palkovits, 1974) and the avian PA (Jurio and Vogt, 1967) in addition to the equivalence of intense AChE staining and localisation to the PA (Jurio and Vogt, 1967; Karten and Dubbeldam, 1973) and mammalian CP (Lewis and Shute, 1978). High concentrations of monoamines and intense AChE staining have also been shown in the basal ganglia (ventro-lateral area) of *Caimen crocodilis* (Brauth and Kitt, 1980).

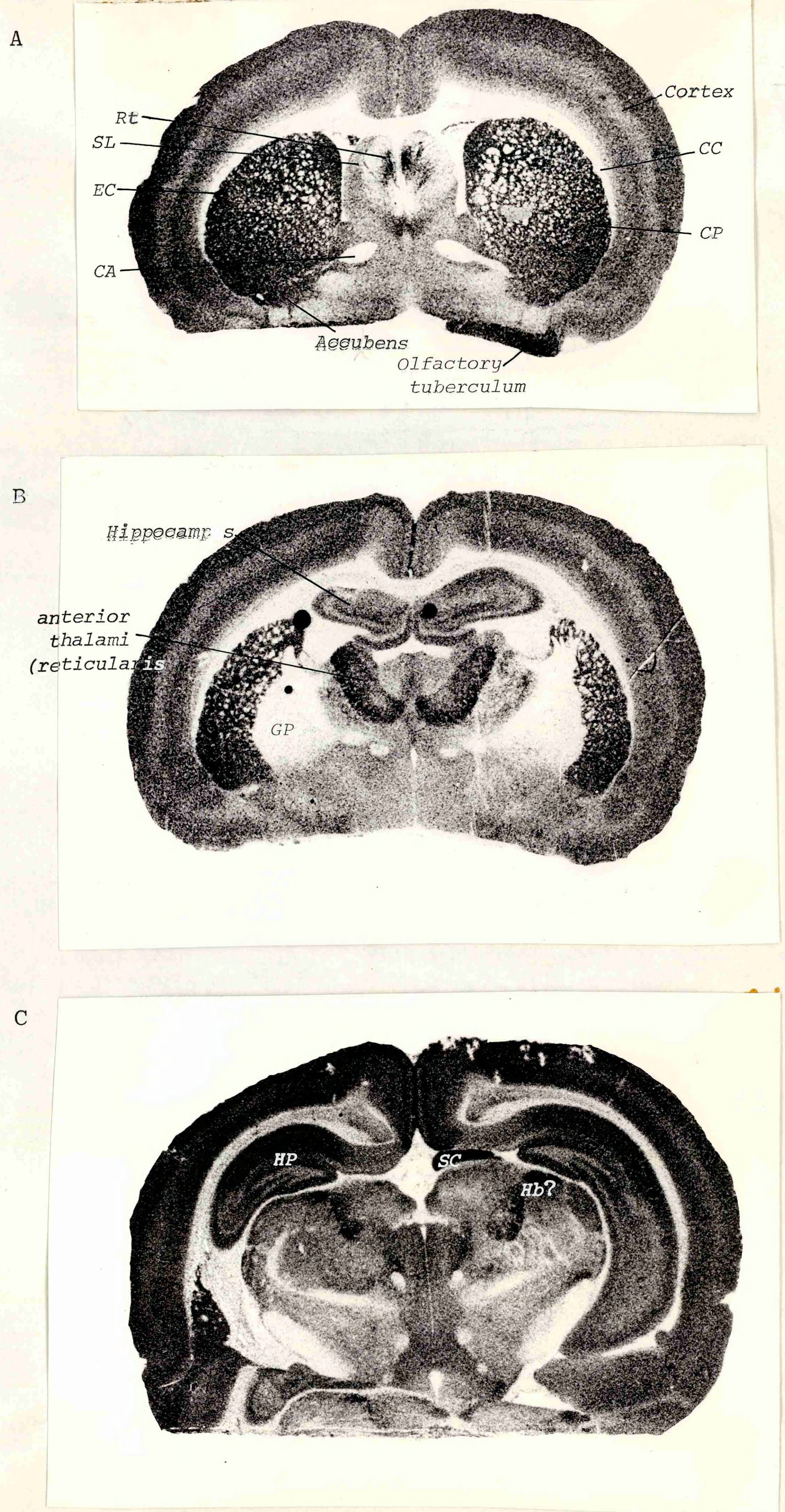
Parent and Oliver (1970) specifically proposed that the PP of birds corresponds to the external segment of the mammalian globus pallidus, and Karten and Hodos (1967) suggested that the nucleus interpeduncularis (INP) of birds corresponds to the internal segment of the globus pallidus. While, in the chick brain,the INP is extremely dense in muscarinic receptor, MAChR density over all regions of the rat globus pallidus has been reported to be very low (Rotter et al., 1979, see figure 2). The entopeduncular nucleus of the rat has been shown to stain intensely for AChE (see Webster, 1973). The inner segment of the globus pallidus is sometimes referred to as the entopeduncular nucleus. Lewis and Shute (1976) have shown intense AChE staining to the inner segment of the GP, staining pervaded in similar manner to MAChR densities in the chick INP (this report) by unstained/unlabelled large diameter fibre bundles.

The dense MAChR field of the chick PA has been emphasised as equivalent to the mammalian CP. The PA however is a small MAChR field compared with the extensive and equally dense MAChR field localised to the Lobus parolfactorius (LPO) of the chick, a region lying medial and rostral to the PA, PP and INP. Although muscarinic receptor density at the PA-LPO juncture does not distinguish a sharp dividing line between these regions, Karten and Dubbeldam (1973) stress the importance of distinguishing the LPO from the PA on the basis that the two regions possess a different cytology and hodologic relationship. Of particular significance is the difference in efferent projections between the LPO and PA, the former contributing almost exclusivly to the medial forebrain bundle, while the latter project massively upon the PP and INP, points of origin of the ansa lenticularis (AL) (Karten and Dubbeldam, 1973). On the basis of this and other evidence, Karten and Dubbeldam have proposed that the LPO corresponds only to the head of the caudate of mammals in contrast to the view of Johnston (1926) and Kappers et al. (1936) who proposed that the PA contained the equivalent of the head of the caudate putamen and bed nucleus of the stria terminalis. The bed nucleus of the stria terminalis is suggested to lie medial to the LPO (Zeier and Karten, 1971). Rotter et al. (1979) report moderate densities of MAChR to the bed nucleus, and *my own results have shown* the bed nucleus to be almost *devoid of MAChR* in the *rat*.

Nauta (1979) has suggested that the nucleus accumbens septi of mammals may only be artificially separated from the caudate putamen complex by the frontal horn of the lateral ventricle.The accumbens of *Caimen* (Brauth and Kitt, 1980) and of *Chelonea* (Kusunoki, 1971) have been shown to stain intensely for AChE. In the rat brain the accubens septi has been shown to be very dense in muscarinic receptor (Rotter et al., 1979). The present study, like that for AChE distribution in the turtle brain (Kusunoki, 1971), shows that, rostrally, the dense MAChR field of the LPO continues beneath the forebrain ventricle to occupy a position equivalent to anterior limits of the accumbens septi.It is possible that the LPO of the chick does not corres-

Figure 106. A series of light field photomicrographs of the pattern and regional density of silver grains in LKB tritium film exposed and apposed to parasagittal tissue sections of the 50 day post natal rat brain labelled by [^{3}H] l QNB for MUSCARINIC CHOLINERGIC RECEPTOR. (Coulter, unpublished observations). The section shown in C is medial to B which is medial to A. (For a comparison with the chick see text of discussion).

Figure 107 . MUSCARINIC RECEPTOR DISTRIBUTION IN THE 50 DAYS POST NATAL RAT BRAIN: LIGAND 3 [H] QUINUCLIDINYL BENZILATE


A
Rt
SL
EC
CA
Cortex
CC
CP
Accubens
Olfactory tuberculum
B
Hippocamp s
anterior thalami (reticularis
GP
C
HP
SC
Hb?

pond to either segment of the caudate putamen but to the accumbens.However, the form and density of MAChR in the LPO is equally supportive of the suggestion of Karten and Dubbeldam (1973) that the LPO corresponds only to the head of the mammalian caudate.

A further observation from the results of this study is that the Lamina medullaris dorsalis (LMD), adjoining and limiting the PA dorsally from the overlying ecto- and neostiatum, is populated by substantially higher densities of MAChR than the PA itself. Similarly, the external capsule of the rat is also more dense in muscarinic receptor than underlying aspects of the PC (see figure 106 a,b). Both the LMD of birds (Kusunoki, 1969; Karten and Dubbeldam, 1973) and the external capsule of the rat (Paxinos et al., 1980; and see figure 2) have been shown to stain more intensely for AChE than adjoining regions of the PA or CP respectively.

To my knowledge no study has advanced the suggestion that the avian LMD and mammalian external capsule might be homologous. It is interesting to note here that the ascending cholinergic reticular system is thought to project to cortical regions via the external capsule and cingulum of mammals (Shute and Lewis, 1967). If the LMD and external capsule are homologous, the observation that the only two regions of the chick and rat forebrain above the LMD and external capsule which are devoid of muscarinic receptor are the ectostriatal core of the chick and corpus callosum of the rat . which might raise some interesting questions concerning the phylogeny of the ectostriatum of birds shown to stain heavily for myelinated fibres.

Neuroanatomical (Raisman et al., 1965; and Raisman, 1966) and electrophysiological evidence (Anderson et al., 1961) have demonstrated a septo-hippocampal pathway in mammals originating in the medial septal nucleus and nucleus of the diagonal band (Raisman, 1966) which is cholinergic (Lewis and Shute, 1978). It is of considerable interest therefore to note that, similar to muscarinic receptor distribution in the rat (Rotter et al., 1979), the diagonal band nucleus (Broca) (NDB) and medial septal nucleus of the chick are populated by high densities of MAChR . The NDB of the pigeon has been shown to be the site of afferents and efferents to and from the parahippocampalis linearis (PHL) in particular (Benowitz and Karten, 1976) and adjacent areas of the parahippocampus (Kraniak and Siegel, 1978). Both Benowitz and Karten (1976) and Kraniak and Siegel (1978) came to the same conclusion that the parahippocampal area of birds is more likely a homologue of the mammalian subicular cortex which in the rat is populated by comparatively low densities of MAChR (see this report, figure 105) compared to other regions of the mammalian hippocampus. The mammalian hippocampus is one of the few regions of the forebrain to be populated by both muscarinic and nicotinic receptor (Kuhar and Yamamura, 1975, Arimatsu et al., 1981) and it is therfore perhaps significant that the only region dorsal and medial to the chick forebrain ventricle to possess both muscarinic and nicotinic receptor is the PHL (this report).

On the other hand, the hippocampus of mammals is very dense in MAChR (Rotter et al., 1979; and this report), while the parahippocampus and hippocampus of the chick are generally populated by low densities of muscarinic receptor (this report). This is a result which might be seen to undermine any direct equivalence between the hippocampus of the chick and hippocampus of mammals, but which is entirely consistant with the proposed PHL-subiculum homology (Benowitz and Karten, 1976; Kraniak and Siegel, 1976). This correspondence raises interesting possibilities for comparison of the very high density

MAChR field shown to be localised to the area entorhinalis (MRF 1) of the chick forebrain with the mammalian hippocampus, particularly in view of the close association of this MAChR field with both septal and hyperstriatal regions of the chick forebrain.

Despite morphological dissimilarities between the broad cell masses of the avian telencephalon and the laminar organisation of the mammalian cortex, comparative embryological, anatomical and histochemical studies all suggest that most of bird telencephalon is comparable with elements of the mammalian cortex (see Benowitz, 1980). Nauta and Karten (1970) have suggested that the embryological zone designated D 1 by Kuhlenbeck (1938), from which arises the hyperstriatum ventrale (HV), neostriatum (N), ectostriatum (E) and area corticoidea, may, during evolution, also have given rise to certain layers of the mammalian cortex (see figure 7, section 1.6).

The cerebral cortical layers of mammals have been shown to be populated by high densities of MAChR (Hiley and Burgen, 1971; Kuhar and Yamamura, 1976; Rotter et al., 1979) and the majority of cortical cells responsive to acetylcholine are muscarinic cholinergic (Krnjevic and Phillis, 1963). Many studies in comparing cholinergic distribution in vertebrate brains have remarked upon an apparent shift in relative concentrations of cholinergic systems from the hindbrain of lower vertebrates to the forebrain of mammals (eg. Wächtler, 1980), corresponding with a similar shift in location and elaboration of integrative and associative systems of the vertebrate brain, culminating in the primate cortex (see Hebb and Ratkovic, 1964). Apart from the basal ganglia (see above) and archistriatum of the avian brain suggested to be homologous with the mammalian amygdala (Haefelfinger, 1968; Zeier and Karten, 1971), the only other regions in the chick forebrain which are both derived from Kuhlenbeck's D 1 (see earlier) and populated by equally high densities of MAChR as the mammalian cortex, are the hyperstiatum ventrale (HV), and corticoidea dorsolateralis (CDL). I have already indicated that the dense MAChR field of the CDL (MRF 1) may be equivalent to some component of the mammalian hippocampal complex other than the subiculum. The neostriatum of the chick is populated by low to moderate densities of MAChR, and the ectostriatum is almost devoid of muscarinic receptor. The ectostriatum, on the basis of an apparent equivalent visual afferent thalamic input, has been suggested to be homologous with cells in layer IV of the mammalian cortex (see Benowitz, 1980). In addition, the auditory projection field L of the avian neostriatum shown to be very low in muscarinic receptor (this report) has been suggested to be equivalent to the thalamorecipient cells in layer IV of the auditory mammalian cortex (Karten, 1969). Layer IV of the rat cortex is populated by substantially lower densities of MAChR than other cortical layers (Rotter et al., 1979; and this report). This leaves the more densely populated MAChR layers of the mammalian cortex and the very high density MAChR field of the hyperstriatum of the chick as possibly homologous. (see below).

There are several other points which indicate that the cell populations of the chick hyperstriatum may be homologous with those of the mammalian cortex. Wächtler (1980) in adressing the "cholinergic shift" described above concluded from a comparison of AChE distribution in the vertebrate brain that the main increase in AChE neurons in mammals is not to the cortex but the basal ganglia. This is a conclusion which is obviously not consistent with the reports of very high densities of MAChR shown to the mammalian cortical layers (see above). However, as discussed earlier, there appears to be a consistent discepancy between AChE staining in cortical layers and MAChR distribution and density which is not seen in the basal ganglia of mammals (see figure 2 , taken from Lewis and Shute, 1978; see also Parent and Oliver, 1969; Cotman and Nadler, 1978; Paxinos et al., 1980). This discrepancy is matched by a lack of AChE staining in the HV of birds (see figures 3b - c), taken from Kusunoki, 1969; Karten and Dubbeldam, 1973) shown by the present study to be dense in muscarinic receptor. This observation is difficult to explain, but it may suggest that the properties of cortical cholinergic systems are different from those of the basal ganglia (see section 4.3), a difference reflected it seems between species of vertebrate brains. McCamin and Aprison (1964) in showing that cholinergic systems in the rabbit telencephalon develop later than the rest of the brain commented that this phenomenon may be seen to indicate a parallelism between phylogeny and ontogeny.In the chick brain it is only the MAChR densities of the hyperstriatum which are expressed late in chick brain development not those of the basal ganglia (see section 4.6).

There are many other examples of proposed homologous regions between the chick and rat brain which show equivalent densities and distribution of cholinergic receptor, each as pertinent to questions of ontogeny, phylogeny and function as the examples already discussed. These include the optic tectum (chick) and superior colliculus (rat), the mesencephalic nucleus (chick) and inferior colliculus (rat), the nucleus rotundus (chick) and the LP pulvinar (rat), the principle optic nucleus (OPT) and perhaps the nucleus geniculatis lateralis ventralis (GLV) (chick) and corpus geniculatis lateralis ventralis (LGN) (rat), and for both species, the lateral lemniscus, the interpeduncular nucleus, the habenular, the pons, the principle trigeminal nucleus, and the solitary nucleus.

In contrast to the above correspondences, the density of ^{3}[H] antagonist labelled muscarinic receptor between the chick and rat olfactory bulb is large, the latter populated by the highest densities of MAChR in this vertebrate brain ((Rotter et al., 1979), while in the chick the olfactory lobe is very low in receptor. In contrast, the olfactory lobe of the chick (this report) and mouse (Arimatsu et al., 1980) are both populated by high (in the chick comparatively) densities of α BTX labelled nicotinic cholinergic receptor. The difference in MAChR density between the rat and chick olfactory lobe may be seen to undermine the use of MAChR distribution as an indication of homology. However, it is the density of receptor which differs, not the distribution, which may more correctly be interpreted as a reflection of the difference in functional emphasis upon olfaction between these vertebrate species. This, if correct, suggests that the elaboration of neuronal systems in the rat olfactory lobe, functioning to integrate olfactory input, corresponds more closely with the number of muscarinic than nicotinic receptors.

To conclude this section, muscarinic receptor distribution probably does reflect upon vertebrate brain phylogeny and this is most clearly seen where the functional emphasis of these brain regions has in essence remained unchanged. But where there has been a substantial change in functional emphasis, then there may be a marked difference in receptor density, but not necessarily distribution of receptor.

4.6 Muscarinic antagonist receptor development in the in ovo and early post hatch chick brain.

The observations and comments of this study in reporting the development of ^{3}[H] antagonist labelled muscarinic cholinergic receptor in the chick brain are both preliminary and tentative for the following reasons:

1) recognition of the changes in patterns and density of ^{3}[H] antagonist labelled muscarinic receptor for each region of the chick brain between 10 days in ovo (10 DIO) and 5 weeks post hatch is far from complete;

2) while the distribution and *comparative regional densities* of ^{3}[H] antagonist labelled receptor are clearly described by the figure photomicrographs of this report, the regional *concentrations* of ^{3}[H] antagonist binding sites during chick brain ontogenesis have yet to be determined;

3) while there is a wealth of evidence for all aspects of avian brain development, the given form of that evidence for brain maturational processes is not easily related to the detail of evidence from this report concerning the extremely localised patterns and changes of antagonist labelled MAChR distribution and density observed during chick brain ontogenesis.

The following discussion therefore is directed towards certain generalised observations on ^{3}[H] muscarinic antagonist labelled receptor development which with ongoing analysis may require modification,if hitherto unrecognised highly localised events in chick MAChR development subsequently prove to run contrary to the observations discussed here. Only a few regional examples of MAChR ontogeny are included in order to illustrate the following summary observations:

1) many regions shown to be populated by comparative high densities of ^{3}[H] antagonist labelled muscarinic receptor in the post hatch chick brain are also populated by comparative high densities of MAChR in the 10 DIO chick embryo brain, a developmental time point which is well in advance of the main period of synaptogenesis;

2) at different stages during development and almost without exception, all regions of the chick embryo brain are populated by densities of specifically

bound $[^3H]$ PrBCM which are markedly higher than non specific binding densities (ie. non atropine displaced), and for a number of regions these antagonist MAChR densities are developmentally transient;

3) all populations of antagonist MAChR, whether developmentally transient or not, exhibit unique developmental patterns of muscarinic receptor distribution which appear in certain instances to correspond spatially and temporally with growing afferent or efferent cell processes, while in other instances these antagonist labelled receptor densities appear to correspond with the changing patterns of regionally localised cell bodies;

4) while there in no evidence from this study in support of an obvious caudal-rostral gradient of antagonist labelled MAChR during development, the appearance of $[^3H]$ antagonist binding sites in the hyperstriatum ventrale, derived from Kuhlenbeck's (1978) embryological zone D1, is much later than in other regions of the forebrain.

Opinion is divided as to whether there is a close correlation between the appearance and increase in the concentration of cholinergic molecules (eg.ACh, CAT etc) in the developing chick brain with the onset or major period of synaptogenesis.There is evidence both for (Nachmansohn,1939;Rogers et al.,1960;Birdick and Strittmatter, 1965; Marchand et al., 1977; Enna et al., 1976; Haywood, 1978) and against (Filogamo, 1960; Bonichon, 1960; Marchisio, 1967; Turbow and Burthalter, 1968; Burt et al., 1968; Enna et al., 1976; Leah et al., 1980) such a correlation. Opinion is also divided as to whether cholinergic molecules develop at the same time or increase at the same rate. For example, Enna et al. (1976) report a near identical parallel increase in the number of $[^3H]$ (±) QNB labelled muscarinic receptors in the chick embryo brain with the time and rate of increase in both AChE and CAT. On the other hand, Burt et al. (1968) reported that maximal AChE activity recorded at 8 days in ovo (8 DIO) preceeds maximal CAT activity by 11 days.

The present autoradiographic study for antagonist labelled MAChR development is not easy to relate to the above and other reports for concentrations of receptor and other functional cholinergic molecules. The major difficulty is that any small change in silver grain density represents a much larger increase in the total number of receptors, since the brain regions in question are greatly increasing in volume between 10 DIO and 5 weeks post hatch. Nevertheless, the present results show regionally comparative high concentrations of antagonist labelled muscarinic receptor to the paleostriatum augmentatum, anterior and ventral thalamus, and nucleus principalis precommissuralis in the 10 DIO chick embryo brain, a developmental time point well in advance of the major period of synaptogenesis in the chick brain (see Corner et al., 1967), but not necessarily the onset of neural function (see Burt, 1968; Hamburger, 1970). The present findings are in accord with those of Sugiyama et al. (1977) and Enna et al. (1976) in showing substantial concentrations of $[^3H]$ (±) QNB labelled MAChR as early as 5 DIO in the chick retina and brain respectively. It is interesting to note here that Enna et al. (1976) reported no "quantitatively detectable" concentration of $[^3H]$ (±) QNB binding in the 10 DIO embryo brain, when binding was expressed per brain and not as a function of protein content (see figure 4). As Enna et al. (1976) and Marchisio and Giacobini (1969) have pointed out, homogenate studies of transmitter-receptor development cannot easily distinguish regionally localised developmental changes in cholinergic molecules, the major advantage of the present autoradiographic study.

The present observations suggest that MAChR development largely precedes anatomical synaptogenesis which is in agreement with the findings of Rotter et al. (1979) for the rat brain (but see below).

The relative constant density (but not necessarily regional concentration) of ^{3}[H] antagonist labelled MAChR in the basal ganglia, thalamus and tectum during the latter stages of in ovo chick brain development contrasts sharply with the marked loss of ^{3}[H] antagonist labelled MAChR in the chick cerebellar cortex on the one hand, and the very late appearance of high densities of MAChR in the forebrain hyperstriatum ventrale (HV) on the other. These two regional examples of ^{3}[H] antagonist labelled MAChR development are of particular interest, because the marked loss and dramatic late increase in antagonist binding sites to the cerebellar cortex and HV respectively contrasts with reports for AChE activity and stain in the adult brain. The adult cerebellar cortex of all vertebrate species has been shown to stain intensely for AChE (Burgen and Chapman, 1951, Cavanagh and Holland, 1961; Friede and Fleming, 1964; Shute and Lewis, 1965; Kasa and Silver, 1968; Altman, 1970; Kusunoki, 1969), while the HV of birds has been consistently shown to be low in AChE activity and intensity of stain (Kusunoki, 1969 ; Karten and Dubbeldam, 1976; Haywood, 1978) . Earlier in this discussion it was suggested that the chick HV may be homologous with certain cell populations in the mammalian cortex (see section 4.5) and,as observed here in the HV,MAChR in most cerebellar cortical cell layers appear late in development (Rotter et al., 1979), are dense in the adult, and, again similar to the HV, cortical cell layers have consistently been shown to be comparatively low in AChE activity and stain (Lewis et al., 1967; Parent and Oliver, 1969; Cotman and Nadler, 1978; Lewis and Shute, 1978; Johnston et al., 1981).

This remarkable correspondence in the concentration of cholinergic molecules continues with CAT which, like AChE activity but in contrast to MAChR density, has been shown to be low in the mammalian cortex (Lewis et al., 1967; Goldberg and McCaman, 1969; Palkovits et al., 1974; Yamamura et al., 1974; Kobayashi et al., 1975; McGeer and McGeer, 1976) and similarly low in the anterior roof of the chick forebrain (Haywood et al., 1978), a division made up almost entirely by the hyperstriatum. How the late development of MAChR in mammalian cortical cell layers and the HV of birds is related to the clear discrepancey between the high concentrations of MAChR and low concentration and activity of cholingergic enzymes is unclear (but see section 4.5 and below).

The apparent marked loss in the number of $[^3H]$ antagonist labelled muscarinic receptors in the granular cell layer of the chick cerebellar cortex during development (this report) corresponds with the reported loss of $[^3H]$ antagonist labelled muscarinic receptors in the granular cell layer of the rat during post natal development (Rotter et al., 1979). Rotter et al. also reported that with the loss of MAChR in the granular cell layer there was a concomitant increase in the number of $[^3H]$ muscarinic antagonist binding sites in the molecular cell layer (see figures 108 a,b). This finding is in contrast to those of the present study, where the molecular cell layer of the chick is shown to be populated by high densities of MAChR from very early stages of cerebellar cortical ontogeny (ie. from 10 DIO onwards). In addition, by 5 weeks post hatch, antagonist labelled MAChR in the chick are localised only to most external aspects of the molecular cell layer. Rotter et al. (1979) reported that in the adult rat MAChR are localised (in low densities) to all aspects of the molecular cell layer and in particular to the vestibulocerebellum. Rotter et al. could find no cholinergic afferent input whether extrinsic, eg. axons of the primary vestibular afferents (mossy fibres) (Kun et al.,

108 A MUSCARINIC RECEPTOR DISTRIBUTION, RAT & CHICK CEREBELLUM

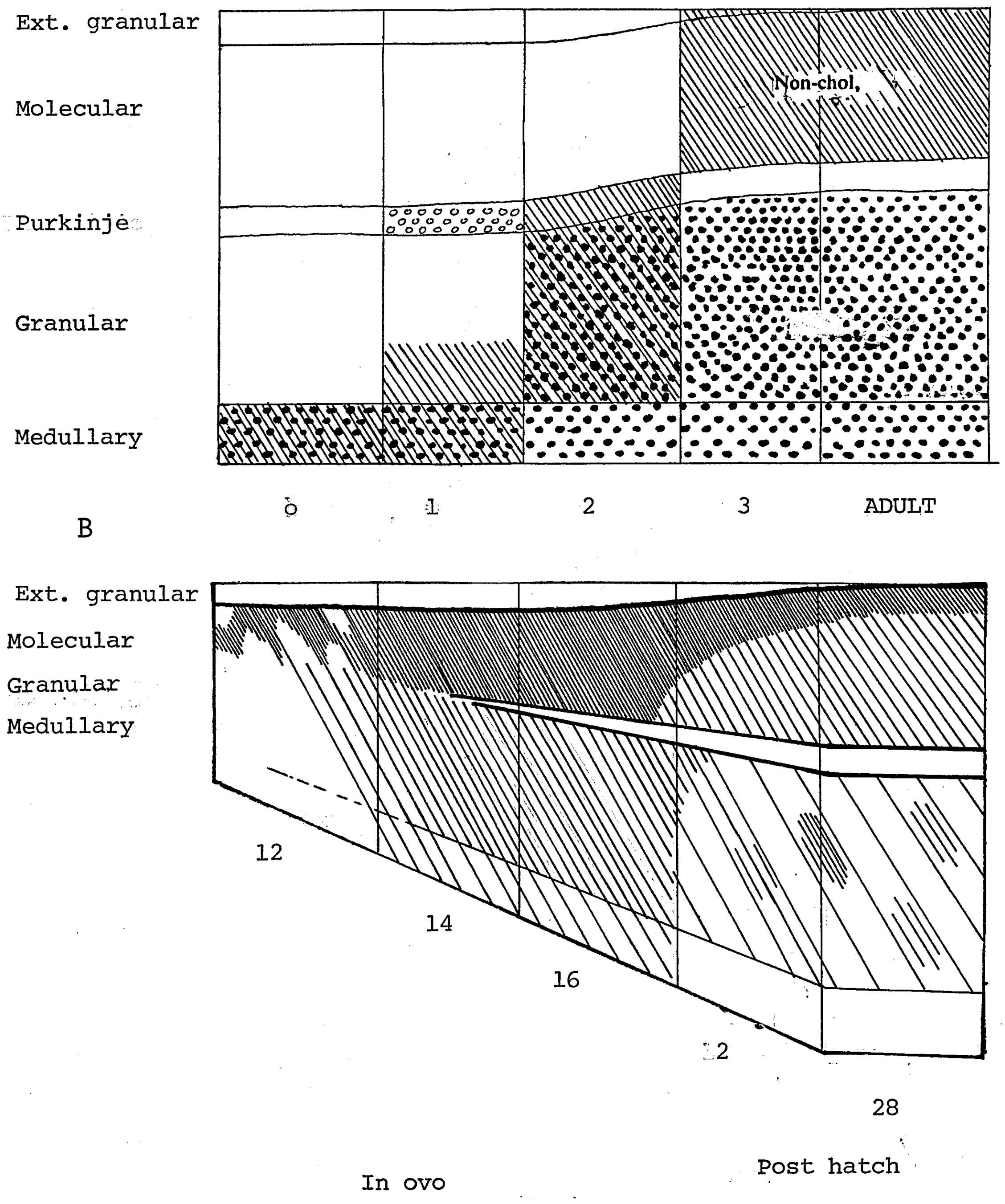

Ext. granular
Molecular
Purkinje
Granular
Medullary
Non-chol.
0
1
2
3
ADULT
B
Ext. granular
Molecular
Granular
Medullary
12
14
16
22
28
In ovo
Post hatch

FIGURE 108 A) The development of ^{3}[H] PrBCM labelled muscarinic receptor ▨, acetylcholinesterase containing mossy fibres ⁘, acetylcholinesterase Purkinje cells ∘∘∘, and synapses, in the various layers of the vestibulo cerebellum of the rat during the first 3 postnatal weeks after birth. (taken from Rotter et.al. 1979)

B) The development and distribution of ^{3}[H] PrBCM labelled muscarinic receptor ▨, in the various layers of the in ovo and post hatch chick cerebellum.

1978) or intrinsic, eg. Golgi cells (Shute and Lewis, 1965) which corresponds to the localisation and developmentally transient patterns of rat cerebellar cortical MAChR, and he concluded that MAChR localised to the molecular cell layer may be associated with Purkinje cell dendrites as another expression of histogenetic memory (see Silver, 1967). The evidence for this suggestion is that Purkinje cells display a pharmacologically muscarinic sensitivity to iontophoresis of ACh (Crawford et al., 1966) and that AChE reappears in Purkinje cells when the cerebellum is undercut (Kasa et al., 1968).

The cell types, organisation and majority of afferent and efferent pathways of the rat cerebellum are similarly represented in the chick (Fujita, 1969; Mugnaini, 1969) and yet it is clear that there are considerable developmental differences in the pattern of cortical MAChR distribution, which are not so marked in the adult. The present findings undermine the histogenetic memory hypothesis for the presence of cholinergic molecules to apparently non cholinergic/cholinoceptive cerebellar cortical cells (see Rotter et al., 1979, after Silver, 1967). Since, if such a hypothesis were correct, a similar pattern of MAChR distribution and density as that observed in the rat should be reproduced, if only transiently, at some point during the development of MAChR in the chick cerebellar cortical cell layers. I suggest that the differences in MAChR development in the cortex of these two vertebrate species is related to the 'altricial' as opposed to the precocial development of the rat and chick respectively, and reflects markedly different environmental influences upon cerebellar cortical cell maturation and patterns of connectivity (transient ?) between these two vertebrate species (see below).

Kuhar et al. (1980) (after Hebb and Rakovic, (1964) suggest that the more rapid maturation of MAChR in caudal as opposed to rostral regions of the rat brain during development corresponds with evidence suggesting that cell division ceases and synaptogenesis begins in the hindbrain well in advance of cortical layers(see Altman, 1969). The observations of the present study do not indicate any obvious caudal rostral progression of MAChR antagonist binding sites, apart from the very late development of ^{3}[H] antagonist labelled receptor already referred to in the hyperstriatum ventrale (HV). The HV is also distinguished by being the only region of the chick brain which is dense in muscarinic receptor in the post hatch brain (at 5 weeks post hatch), but whose population of ^{3}[H] antagonist labelled MAChR is not, unlike all other dense MAChR fields of the in ovo brain, disrupted by developmentally transient patterns, shown to occur between 12 and 18 DIO, since MAChR in the HV is largely expressed after this time point. In fact these transient patterns, recognised by changes in the distribution of silver grain densities (ie. ^{3}[H] antagonist binding sites) occur in all regions of the chick brain, whether dense in MAChR or not, including the HV.

The 'significance' of these patterns will be discussed further below, but for the moment they appear to indicate the passage of afferent or efferent cell processes which course through the HV to or from dorsal regions of the forebrain roof, prior to the appearance of the vast majority of post hatch ^{3}[H] muscarinic antagonist binding sites. As pointed out earlier (see section 4.1), the HV is thought to be a polysensory, non lemniscal integrative and association centre, interposed between sensory and motor regions of the chick forebrain(see Benowitz, 1980). The HV recieves neither direct sensory input from the brainstem nor projects out of the telencephalon (Zeier and Karten, 1971; Karten, 1969) and, like mammalian cortical layers, is composed mostly of Golgi type II interneurons (Kappers et al., 1936 but see Bradley and Horn, 1982).

It might be anticipated therefore that the organisation and functional coupling of cells and their processes in the HV would await the passage of afferents and possibly efferents to and from other regions of the chick forebrain, but in particular the dorsal hyperstriatum (visual Wulst) and 'cortex' in order to prevent disruption. However, it is more probable that the necessary inductive influences serving to specify the pattern of HV neuronal connections with accompanying appearance of the majority of $^{3}[H]$ muscarinic antagonist labelled receptor are not present until the arrival of the correct intracortical afferents to the HV. The microcircuit interneuron of the mammalian cortex is one of the last cell types to differentiate during brain ontogenesis (Rakic, 1978; Cowan, 1979).

The transient patterns of $^{3}[H]$ muscarinic antagonist binding sites, described here during chick brain development, were apparently not observed by Rotter et al. (1979) in showing autoradiographically the developmental distribution of $^{3}[H]$ PrBCM binding sites in the post natal rat brain. A plausible explanation for this discrepancy of observations between these two vertebrate species is not immediately evident, but then neither is the explanation for these extraordinary developmental patterns in the chick in the first place. It is possible that the failure of Rotter et al. (1979) to observe similar events during post natal development in the rat is related to proceedural difference between these studies with respect to antagonist labelled tissue slice exposure times to the overlying nuclear emulsion. The exposure times of Rotter et al. were perhaps of insufficient duration for these patterns, some of which in the chick are of comparatively low density, to be discerned. Of course, it is just possible, similar to the differences in MAChR development in the cerebellar cortex between the chick and rat (see above), that the present differences again reflect the altricial as opposed to precocial development of the rat and chick respectively. I believe this to be highly unlikely, since these patterns appear to be reflecting some very fundamental property of brain neurogenesis (see below).

At this stage in analysis it is not clear whether the transient patterns of $^{3}[H]$ muscarinic antagonist binding sites are reflecting the same phenomena as those underlying the transient loss of $^{3}[H]$ antagonist binding sites from certain brain regions, eg. neostriatum, fibre tracts, white matter of the stratum album centrale, central white of the cerebellum and as described above the cerebellar cortex. The majority of dense MAChR fields appear to maintain their density of antagonist binding sites throughout development, although similarly 'disrupted' by these transient developmental patterns. One explanation for these developmental patterns of $^{3}[H]$ antagonist binding sites is that between 12 and 18 DIO the distribution of MAChR is passively, ie. without any 'purposeful' active functional role, reflecting the movements of cell populations or their extending cell processes as the cell populations of the brain move into a predetermined order. Furthermore, the change from heterogeneously patterned to homogeneously patterned regionally localised fields of $^{3}[H]$ antagonist labelled receptor, occuring between 17 and 19 DIO for the majority of midbrain and forebrain regions, may reflect the moment of completion of gross cellular order. The evidence for such a view is inconclusive, for, as far as I am aware, no morphological or anatomical study has described or commented on a migration, grouping or organisation of cells or their processes in the avian brain which in any way resembles the patterns of $^{3}[H]$ antagonist labelled MAChR observed here for the developing chick brain. On the other hand, this study has given evidence to show that certain of the 'patch-like' fields of receptor do appear to correspond closely in form with the pattern of grouped cell bodies, as stained for Nissl substance, but not so obviously with the near linear, often multiple parallel arrays of comparativley low density antagonist labelled MAChR fields.

A hypothetical 'passive' role for the early appearance of post hatch densities and distribution of ^{3}[H] antagonist labelled MAChR (apart from anterior-dorsal forebrain) might be seen to be consistent with the view expressed among others by Fambrough and Rash (1971) that the commitment of cells to differentiate activates the 'set of genes' that encode for all special protein characteristics of the differentiated state. While the present evidence does not discount such a hypothesis, it should be noted that the temporal order of MAChR appearance and distribution,in the chick forebrain in particular,does not correspond well with regional neuronal 'birthdates' or isochrome maps of regional cellular differentiation in the developing chick forebrain (see Jones and Levi-Montalcini, 1958; Tsai et al., 1980; 1981 a,b). On the other hand, lateral aspects of the chick forebrain are populated by transiently high densities of antagonist labelled MAChR between 12 and 15 DIO which, at the same time, exhibit the almost global chick brain developmental phenomenon of apparent 'disruption' of regional cellular homogeneity, an observation consistent with evidence suggesting that lateral aspects of chick forebrain develop and differentiate in advance of more medial regions. (see Tsai et al., 1981 b).

Recently a number of studies have reported the localisation of ^{3}[H] muscarinic antagonist binding sites to splenic (Laduron, 1980), sciatic and vagus nerves (Walmsley et al., 1981), antagonist labelled receptor which apparently are transported distally from the neuronal cell body to *presynaptic* terminals. This observation might be seen to account for the linear patterns of ^{3}[H] antagonist MAChR observed in the developing chick brain between 15 and 18 DIO (this report), in particular the observation of moderate to high densities of ^{3}[H] *antagonist* labelled receptor to fibre tracts of the chick brain, eg. medial and lateral forebrain bundles, tractus occipito-mesencephalicus, ansa lenticularis, cerebellar white matter and even the optic tract. Particularly, since Walmsley et al. (1981) suggest that MAChR localised to the sciatic and vagus nerve axons are of the high affinity agonist type (see section 4.3), similar to MAChR reported to be associated with white matter tracts of mammals, eg. the corpus collosum of the adult rat (Walmsley et al., 1980)).

The significance of the high affinity agonist sites associated with axons and fibre tracts is that,during development,the appearance of high affinity agonist binding sites occurs six to seven days after the appearance of low agonist affinity forms of muscarinic receptor during post natal development in the rat brain (Walmsley et al, 1981),at around the same developmental time point when certain regions in the rat brain lose ^{3}[H] antagonist binding sites (see Rotter et al., 1979). If the view is held that receptors have atleast two ligand recognition sites (see section 1.2 and 4.3), the high affinity agonist binding site recognised with low affinity by antagonists and a high affinity antagonist binding site recognised with low affinity by agonists (see Snyder, 1975), it is possible that prior to synaptogenesis, and perhaps more correctly the onset of functional chemical transmission, ^{3}[H] antagonists are binding to both 'agonist' and 'antagonist' binding sites with one affinity. Following functional coupling however, during which an 'inductive' influence, perhaps the transmitter, a 'second messenger', or electrical activity (see Cuatrecasas, 1974; Henderson, 1976; Patterson et al., 1978; Csaba, 1980),alters the ligand recognition properties of receptor, ^{3}[H] antagonist binding is subsequently restricted to the antagonist binding site.alone, at least with high affinity. This might be seen to explain why during development and prior to 'functional transmission' ^{3}[H] muscarinic antagonists are binding to sites in the developing chick brain, in particular fibre tracts which, in the post hatch chick brain, are devoid of antagonist labelled receptor.

An 'inductive' change in MAChR ligand binding properties during brain ontogenesis is one of a number of speculative explanations put forward by Walmsley et al. (1981) to account for the lag in appearance of high affinity agonist forms of MAChR in the developing rat brain. As plausible as this explanation appears, there are problems with regard to this interpretation. For example, it is the appearance of high affinity agonist binding sites, not low affinity agonist sites, which apparently correspond with the major period of synaptogenesis in the rat brain (Walmsley et al., 1981) and yet it is thought that it is the low agonist affinity form of MAChR which is indicative of functional cholinergic transmission (see Birdsall et al., 1978). For example, Rodbell (1980) has proposed that in the absence of nucleotides, bimolecular complexes of'muscarinic receptor' and a guanine nucleotide (G-protein) associate to form oligomers which have *high affinity for agonists*. In the presence of both agonist and nucleotide (c GMP), the oligomeric complex is postulated to dissociate, yielding the free receptor G-protein which has *low affinity for agonists* (see Hulme et al., 1981). In this case ^{3}[H] muscarinic antagonists are binding to the 'functionally coupling' or more 'recently functionally coupled' (desensitised ?) receptor and in particular to the guanine nucleotide regulatory protein, irrespective of whether the view is taken that muscarinic antagonists bind with one affinity to all muscarinic agonist sites (see Birdsall et al., 1978; Hulme et al., 1981), or with high affinity only to the agonist form of receptor (Snyder, 1975; see also Hanley and Iversen, 1977; Gupta et al., 1976; Aronstam et al., 1981; Burguiser et al., 1982). In other words, it is possible that, during development and in the post hatch chick brain, $[^3H]$ muscarinic antagonists are identifying receptors which are functioning in the process of chemical transmission, but binding with high affinity to a guanine nucleotide regulatory protein.

This might be seen to explain why $[^3H]$ muscarinic antagonist binding maintains a constant density in regions known to be cholinergic, eg. basal ganglia, thalamic relay nuclei etc., but is lost from non cholinergic regions, eg. cerebellar cortex and fibre bundles in the developing chick brain. However, it does not explain why $[^3H]$ antagonists recognise these receptors during early development when they could not be functioning in transmission, unless, of course, these transient $[^3H]$ antagonist labelled receptors are localised to the surface of growing cell processes in receipt of transient contacts during development. Silver (1971) hypothesised that an immature neuron might develop a sensitivity to ACh, if it was located in or migrated through an area in which ACh was found (see also Csaba, 1980).

The 'functioning receptor'- high antagonist affinity correspondence might also be seen to explain why the high affinity agonist forms of receptor transported in the vagus and sciatic nerve (Walmsley et al., 1981), which according to Rodbell (1980) are functionally inactive, possess a low affinity for antagonists. Presuming that the low density antagonist labelled receptors, observed in this study to be localised to neuronal processes, are internal and not on the surface (see above), why is it that antagonists label these receptors only during a critical period in in ovo development, since pre muscarinic receptor proteins are being synthesized in neuronal soma and transported along nerve processes in the post hatch brain as well. The answer may be a question of access, antagonists binding (albeit with low affinity) to internalised receptor protein only before oligodendrogliagenesis and myelination, after which time access to the receptor for antagonists is restricted. It is quite possible that agonist binding is achieved by reuptake mechanisms at dendritic and axonal nerve endings to diffuse down nerve processes to bind with receptor being transported up the nerve.

Earlier (see section 4.5), the lack of correlation between AChE staining in the HV and high density of muscarinic receptor was discussed. In contrast to the observation of Kusunoki (1970) and Karten and Dubbeldam (1976), a recent study by McCabe et al. (1982) has shown the HV of the 24 hour post hatch chick brain to stain quite intensely for AChE. This may be seen to confirm the observations of Jerusalinsky et al. (1981) in showing that AChE activity and MAChR antagonist labelled concentrations correlate well during chick brain *in ovo* development (see Introduction). However, the study by Jerusalinsky used a very crude microsome enriched preparation and antagonist binding access would not have been restricted by factors such as myelination. On the other hand, tissue slice staining techniques would be.

Conclusions.

This study has shown very clearly where in the young post hatch chick brain muscarinic and nicotinic cholinergic receptors are concentrated. The problems encountered in achieving this primary objective were relatively straightforward and necessitated perfecting technical skills and ensuring clarity in the presentation of data. In contrast, questions directed towards asking why cholinergic receptors are concentrated to particular regions of the brain proved to be extremely difficult to frame, let alone attempt to answer.

In showing that muscarinic antagonist labelled receptors are distributed throughout the vertebrate brain, the observations of this study may be seen to be consistent with psychopharmacological evidence suggesting that cholinergic responsive neurons are part of brain systems subserving a wide variety of behaviours and homeostatic mechanisms. There is no evidence by which to suggest that muscarinic cholinoceptive cells are in receipt of inputs functioning to transmit signals of one particular sensory modality. Muscarinic receptor are localised to regions shown to be particularly rich in golgi type II interneurons. This apparent correspondence and the failure to associate the regional concentrations and distribution of muscarinic receptor with major efferent pathways of those regions, suggest that a considerable percentage of muscarinic receptors is localised to regionally intrinsic neuronal populations. If any functional correspondence were to be made with regard to muscarinic receptor distribution in the vertebrate brain, it might be to suggest that MAChR is most concentrated to regions serving to integrate and associate sensory input and, in addition, to regions in receipt of descending extrapyramidal motor afferents.

In contrast, antagonist labelled nicotinic cholinergic receptors are concentrated to regions of the brain primarily concerned with the relay of one particular sensory modality, vision. On the other hand,comparative high densities of nicotinic cholinergic receptor in the forebrain are localised to cell layers of the olfactory bulb of the chick, like other vertebrate species. Nicotinic cholinergic receptors may be localised to cholinoceptive efferents, but probably not, as for muscarinic receptors to regionally localised cholinoceptive circuits serving to integrate certain classes of sensory input. The continuing debate as to whether muscarinic and nicotinic receptors should or should not be regarded as quite distinct molecular entities has been largely avoided in the Introduction and Discussion of this report. From the distribution of antagonist labelled muscarinic and nicotinic receptors, it is only possible to conclude that, while nicotinic antagonist binding sites are never concentrated in regions populated by low densities of muscarinic antagonist labelled receptors, high densities of muscarinic antagonist binding sites are present in many regions of the brain populated by very low densities of α-bungarotoxin labelled binding sites.

Two analytical approaches have been emphasised in this study in attempting to discover the causal influences specifying muscarinic distribution in the post hatch/post natal vertebrate brain, one a comparative approach between species of vertebrate, the other a study of the distribution of muscarinic rec receptor during the latter stages of in ovo chick brain ontogenesis. The view is taken that influences operative during phylogeny, ontogeny and experience are closely and perhaps inseparably related in serving to specify the regional distribution of muscarinic cholinergic receptor in the brain. With regard to species comparison, a conclusion is that homologous neurons in, for example, the rat and chick brain,as indicated by common patterns of afferent- and efferentation, most often also correspond with respect to whether they are muscarinic cholinoceptive.Where there are differences, eg. olfactory lobe, it is receptor density which differs, which is probably a reflection of differences in the concentrations of a particular cell type and its function is that region or, alternatively, a difference in environmental experience serving to 'induce' or 'stabilise' a particlular ligand identified class of receptor to these regional afferent receptive cells.

A similar conclusion may be drawn from the development of muscarinic receptor distribution. This is particularly well illustrated by the patterns and density of muscarinic receptor in the cerebellar and cerebral cortex and dorsal pallial forebrain of the rat and chick during brain ontogenesis. The development of, for example, stereotyped motor patterns in the chick is precocial, but not in the rat, perhaps accounting for the differences in the distribution of muscarinic receptor between the rat (Rotter et. al., 1979)) and chick cerebellum during development. On the other hand, the development of systems serving to integrate and associate post hatch/post natal experience, eg. the cerebral cortex and perhaps hyperstriatum ventrale of the rat and chick respectively, exhibit a very similar temporal order of development with respect to the appearance of muscarinic antagonist binding sites. This not only speaks for the possibility of homology of certain cortical neuronal populations with those of the hyperstriatum ventrale, but in addition suggests that the cell processes and onset of 'function' underlying adaptive behaviour between these two species of vertebrate, in preparation for and as a consequence of actual experience, are possibly very similar.

In agreement with the view expressed amongst others by Changeux, the observations of the present study suggest that the distribution of muscarinic cholinergic receptor, localised perhaps to *all* cells of the brain during critical stages of development (eg. arrival of afferents), is 'stabilised' as an adult pattern as a result of afferent neuronal activity. In particular, the release of neurotransmitter, the cell synthesis or availability of release are very probably largely a gene expression. The results of this report only describe MAChR antagonist binding sites from 10 days in ovo onwards, but I would suggest, as shown in other vertebrate species, that hindbrain regions are populated by antagonist binding sites before other regions of the brain. The resulting modification of the neuronal activity of the cells in receipt of afferent contacts (perhaps transient) serves to modify the distribution and perhaps class of receptor (see Csaba, 1980) to later forming neuronal contacts of higher brain centres. Of course, the 'stabilisation' of receptor class and perhaps subsequent stabilisation of synaptic contacts is one means of specifying the cellular circuit logic of the brain.

No conclusion can be made with regard to the in ovo transient and developmentally unique patterns of muscarinic antagonist binding sites observed during the latter half of in ovo chick brain ontogenesis.Before understanding what these patterns signify,it is first necessary to know more of the morphogenesis of cell types during chick brain development. In addition, further clarification is required with respect to our current understanding of the 'nature' of ligand labelled muscarinic cholinergic receptor.

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