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The effects of prenatal hypoxia on postnatal cognitive function: a behavioural, pharmacological and structural analysis

by

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I dedicate this thesis to my parents June and Peter.

I would not be half the person I am today without your influence.

A maiden at College, Miss Breeze,
Weighed down by B.A.'s and Ph.D.'s,
Collapsed from the strain.
Said her doctor, "It's plain,
You're killing yourself by degrees."

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Summary

THERE IS NOW compelling evidence that suggests that prenatal compromises can result in postnatal neurological deficits. My aims were to determine the effects of prenatal hypoxia on memory function, the central catecholaminergic system, and brain structure in postnatal chicks.

Gas exchange was restricted in hen's eggs from days 14-18 (W14-18 chicks), or 10-18 (W10-18 chicks) of incubation. This reduced PO_2 and increased hematocrit in both groups. Postnatal memory function was assessed at 1-2 and 5-6 days post-hatching using a discriminated bead task and an aversive wheat task, respectively. Four days of restricted gas exchange from late incubation resulted in chicks having normal labile memory and good learning ability, but an inability to consolidate memory. Eight days of restricted gas exchange from mid incubation left some chicks with poor labile memory, as well as impaired long-term memory. Gas exchange was also restricted in eggs from days 10-14 (W10-14 chicks). In this group, memory consolidation was impaired to a similar degree to that of the W10-18 chicks.

The effects prenatal hypoxia on plasma and brain catecholamine levels was examined in the W10-18 and W14-18 chicks after hatching. Plasma noradrenaline levels were elevated in the W14-18 chicks at 2 days after hatching, adrenaline levels approached

significance. Plasma noradrenaline and adrenaline concentrations tended to be elevated at 2 days in the W10-18 chicks. It is likely that circulating catecholamine levels were increased *in ovo* in response to the hypoxic insult. Analysis of the brain tissue of both groups of prenatally hypoxic chicks showed that noradrenaline concentrations were decreased in the anterior forebrain.

Increased prenatal catecholamine levels induced by hypoxia would have penetrated the cerebral circulation, which may have altered the responsiveness of the central catecholaminergic system after birth. To determine whether the prenatal insult caused an alteration in the state of β-adrenoceptors (ARs) in the brain, β_2- and β_3-AR agonists were administered to chicks following training on the discriminated bead task. Memory consolidation was promoted in the W10-18 chicks by administration of either β_2- or β_3-AR agonists, however, administration of the β_2-AR agonist to the W14-18 chicks did not enhance consolidation, suggesting either desensitization of β_2-ARs or a decrease in receptor numbers. Stimulation of either β_2- or β_3-ARs in the chick brain with adrenaline was shown to consolidate memory in normal chicks; noradrenaline acts in a similar fashion to promote memory consolidation. Thus it is it is possible that increased plasma levels of noradrenaline and adrenaline in response to prenatal hypoxia could cause an alteration in the state of β_2-ARs, which are known to become less effective following excess stimulation.

Morphometric and immunohistochemical techniques were used to assess whether the prenatal hypoxia in the chick leads to alterations in brain structure at 2 days after hatching. There were no overt structural abnormalities in the brains of controls or prenatally hypoxic chicks, furthermore there were no alterations in the ratio of astrocytes/neurons, or the percentage of brain parenchyma occupied by blood vessels. These findings suggest that the cognitive impairment in the prenatally hypoxic chicks is likely to be due to subtle rather than overt perturbations in brain development.

My findings show that prenatal hypoxia from mid-to late gestation can impair memory function after birth. The timing of the prenatal insult, rather than its duration, appears to play a critical role in determining the nature of the memory impairment. My data suggest that the memory impairment is not due to overt brain pathology. Instead, the

inability of the prenatally hypoxic chicks to consolidate memory may be due to a disturbance in the central catecholaminergic system.

Declaration

I, the undersigned, declare that this thesis is less than 100,000 words in length, exclusive of tables, figures and references. Except where due acknowledgement has been made, this thesis comprises original work, no part of which has been submitted for examination for any other degree or diploma at Monash University, or any other university or institution.

Emily J. Camm

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Publications and abstracts arising from this thesis

Publications

E. J. CAMM, M. E. GIBBS, R. HARDING. (2001). Restriction of prenatal gas exchange impairs memory consolidation in the chick. *Devel Brain Res* 132: 141-150.

E. J. CAMM, M. E. GIBBS, R. HARDING. (2002). The role of the central noradrenergic system in memory impairment in prenatally hypoxic chicks. (In preparation, to be submitted to *Neuroscience*).

Abstracts and conferences presentations

E. J. CAMM, M. E. GIBBS, R. HARDING. The effects of prenatal hypoxia on passive avoidance training in the chick. Proceedings of the Australian Neuroscience Society (2000), Melbourne, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Postnatal growth, learning and memory in the chicks: influence of prenatal hypoxia and growth restriction. Plenary session, Perinatal Society of Australia and New Zealand (2000), Brisbane, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Involvement of noradrenaline in memory impairment of growth restricted chicks. Fetal and Neonatal Physiology Workshop (2000), Stradbroke Island, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Hypoxia during late incubation impairs memory consolidation in the postnatal chick. Fetal and Neonatal Physiological Society (2000), Southampton, UK.

E. J. CAMM, M. E. GIBBS, R. HARDING. Learning and memory in the chicks: influence of prenatal hypoxia. Endocrinology and Development (2000), McLaren Vale, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Prenatal hypoxia impairs postnatal brain function in the chick. Perinatal Society of Australia and New Zealand (2001), Canberra, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Effects of pre-hatch hypoxia on plasma catecholamines levels and memory in the chick. Fetal and Neonatal Physiology Workshop (2001), Canberra, Australia.

E. J. CAMM, M. E. GIBBS, R. HARDING. Involvement of the central noradrenergic system in memory impairment in prenatally compromised chicks. Neural Mechanisms of Learning and Memory, IUPS Symposium (2001), Queenstown, NZ.

E. J. CAMM, M. E. GIBBS, R. HARDING. Effects of prehatch hypoxia on plasma catecholamines levels and the involvement of adrenaline on memory formation. IUPS (2001), Christchurch, NZ.

E. J. CAMM, S. M. REES, R. HARDING AND M. E. GIBBS. The effect of prenatal hypoxia on the structure of the chick brain. Fetal and Neonatal Physiology Workshop (2002), Melbourne, Australia.

Abbreviations

ABC	avidin-biotin peroxidase complex
ABR	auditory brainstem response
Ac	nucleus accumbens
ACTH	adrenocorticotrophic hormone
ADHD	attention deficit hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
ANOVA	analysis of variance
AR	adrenoceptor
ATP	adenosine triphosphate
AO	anterior olfactory nucleus
BDNF	brain derived neurotrophic factor
BS	brain stem
BSA	bovine serum albumin
C	cingulate bundle
CC	corpus callosum
Ca^{2+}	calcium
CAM	chorioallantoic membrane
Cb	cerebellum
cAMP	cyclic adenosine monophosphate
CaMKII	calcium/calmodulin-dependent protein kinase II
cm	centimetre
CNS	central nervous system

CREB-1	cAMP response element binding protein-1
CT	central tegmental tract
CTX	cerebral cortex
DA	tractus dorso-archistriaticus
DAB	3,3' diaminobenzidine
DHBA	dihydroxybenzylamine
DHPG	dihydroxyphenylglycol
DIP	nucleus dorsointermedius posterior thalami
DNP	2,4 dinitrophenol
DOPA	dihydroxyphenylalanine
DOPAC	dihydroxyphenylacetic acid
DR	discrimination ratio
DT	dorsal tegmental bundle
E	ectostriatum
EC	external capsule
ERK	extracellular-signal regulated kinase/mitrogen-activated protein kinase
EW	nucleus nervi oculomotorii, pars accessoria
F	fornix
FA	tractus fronto-archistriaticus
g	gram
g	force
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GLUT	glucose transporter
GS	glutamine synthetase
H	hypothalamus
HA	hyperstriatum accessorium
HD	hyperstriatum dorsale
H&E	haematoxylin and eosin
HF	hippocampal formation
Hp	hippocampus
HIS	hyperstriatum intercalatum supremum
HPLC	high-performance liquid chromatography
HV	hyperstriatum ventrale
ICo	nucleus intercollicularis
IGF	insulin-like growth factor
IMHV	intermediate hyperstriatum ventrale
IMT (A)	intermediate memory A
IMT (B)	intermediate memory B
in ovo	in the egg

i.p.	intra-peritoneal
Ipc	nucleus isthmi, pars parvocellularis
K^+	potassium
KA	kainate
LBW	low birth weight
LFS	lamina frontalis superior
LH	lamina hyperstriatica
LLd	nucleus lemnisci lateralis, pars dorsalis
LLi	nucleus lemnisci lateralis, pars intermedia
LMD	lamina medullaris dorsalis
LoC	locus coeruleus
LPO	lobus parolfactorius
LTM	long-term memory
LTP	long-term potentiation
LSD	least significant difference
M	molar
MLd	nucleus mesencephalicus lateralis, pars ventralis
mM	millimolar
MeA	methyl anthranilate
mg	milligram
min	minute
mm	millimetre
ml	millilitre
N	neostriatum
Na^+	sodium
NC	neostriatum caudale
nBOR	nucleus opticus basalis
NGF	nerve growth factor
NMDA	*N*-methyl-D-aspartate
nmol	nanomole
NT	neurotrophin
O_2	oxygen
OB	olfactory bulb
OT	optic tectum
OV	nucleus ovoidalis
PA	paleostriatum augmentatum
pmol	picomole
PCO_2	partial pressure of carbon dioxide
PB	phosphate buffer
PKA	protein kinase A

PL	nucleus pontis lateralis
PNMT	phenylethanolamine-*N*-methyl transferase
PO_2	partial pressure of oxygen
PT	pretectal nuclei
PVCL	plexus choroideus ventriculi lateralis (choroid plexus within lateral ventricle)
QF	tractus quintofrontalis
rpm	revolutions per minute
ROT	nucleus rotundus
RT	reticular formation
S	septum
SAC	striatum album centrale
SCE	stratum cellulare externum
sec	second
SEM	standard error of the mean
SGFS	stratum griseum et fibrosum superficiale
SPL	nucleus spiriformis lateralis
STM	short-term memory
T	tectum
Tn	nucleus taeniae
Th	thalamus
TH	tyrosine hydroxylase
TO	tuberculum olfactorium
TTS	tractus thalamostriaticus
VEGF	vascular endothelial growth factor
VL	ventriculus lateralis
VT	ventriculus teci mesencephali
VIP	vacuum infiltration processor
VMN	nucleus ventromedialis hypothalami
W10-14 chicks	chicks from eggs wrapped from days 10-14
W10-18 chicks	chicks from eggs wrapped from days 10-18
W14-18 chicks	chicks from eggs wrapped from days 14-18
χ^2	chi-squared
µl	microlitre
µm	micrometre
µM	micromolar

Chapter 1

Review of the literature

RECENT EVIDENCE SUGGESTS that adverse prenatal conditions may play a role in the etiology of several neurological disorders which manifest later in life, such as cerebral palsy, mental and physical retardation, and possibly schizophrenia. Hypoxia is a common form of prenatal stress, and has been associated with impaired fetal brain development. In recent years, the majority of the research effort has focussed on the association between adverse intrauterine conditions and severe brain injury, namely cerebral palsy. However, little work has focussed on the more subtle deficits that may occur following prenatal compromise. Learning disabilities (Marlow *et al.*, 1993), Attention Deficit Hyperactivity Disorder (ADHD) (Accardo & Whitman, 1992) and behavioural problems have been suggested to result from adverse prenatal conditions. Such deficits are classified as "minimal brain dysfunction" disorders as they are not characterised by overt brain damage.

Whilst the contribution of adverse prenatal conditions to subtle deficits in neurological function is currently been addressed, little it known about the effects of prenatal hypoxia

on memory function. This thesis addresses the contribution of prenatal hypoxia on memory function after birth. The aims of this thesis will be achieved using the embryo and newborn of the domestic chicken (*Gallus Domesticus*). The chick is an appropriate model to use as the effects of prenatal hypoxia can be assessed independently of perturbations in fetal nutrient supply, as the embryo's nutrient supply from the yolk sac is separated from the area of gas exchange. Furthermore, the effect of prenatal hypoxia on memory function can be readily assessed in the chick, using established behavioural tasks that have been used for 30 years to assess memory formation and consolidation (see review Gibbs & Summers, 2002b). This is of particular advantage since assessing memory in developing postnatal mammals is often difficult.

In this review of the literature, the normal development of the central nervous system (CNS) will be outlined. The relevant literature that pertains to experimental models of prenatal hypoxia will be reviewed, and the advantages of using an avian rather than mammalian species as a model to assess the responses and adaptations to a prenatal insult will be discussed. The cellular mechanisms involved in memory formation in the chick will then be outlined.

1.1 Neural development

The manner in which it the CNS develops is known to be similar in principle from one species to another. However, the stage of brain maturation at birth varies considerably across species, with the rat brain being immature at birth compared to the sheep and chick.

1.1.1 Development of the Neural Tube

The development of the CNS commences at the completion of the gastrula stage of embryogenesis. At this stage, the embryo is composed of three layers of germ cells: an outer ectodermal layer, which forms the nervous system and skin; the middle mesodermal layer, which develops into bone, muscles and connective tissue; and the inner endodermal layer, which forms the gastrointestinal system (see review Pomeroy & Kim, 2000). The first major event in the formation of the CNS occurs when inductive events between the mesodermal and ectodermal layers initiate the formation of the neural plate. Noggin (Lamb *et al.*, 1993), follistatin (Hemmati-Brivanlou *et al.*, 1994),

and chordin (Sasai *et al.*, 1995), released from the mesoderm, are thought to be involved in neural induction. As development continues, the margins of the neural plate gradually become raised and the plate begins to fold along its midline, forming the neural groove. As the neural groove deepens, the lateral edges of the neural plate fuse in both the rostral and caudal direction to form the neural tube. Upon fusion, the neural tube separates from the overlying ectoderm (Fig. 1.1). The lumen of the neural tube ultimately forms the ventricular system of the CNS, and will eventually produce and contain cerebrospinal fluid. The majority of neurons and glia of the future CNS will originate from the neuroepithelial cells within the walls of tube-the ventricular zone. Under the inductive influence of growth factors (see review Hatten, 1999) and genes, for example, sonic hedgehog (Smith, 1994) and HOX genes (see review Pomeroy & Kim, 2000), the anterior portion of the neural tube will give rise to the brain, whereas the posterior portion will form the spinal cord.

1.1.2 Neurogenesis

After the closure of the neural tube, rapid mitotic cell proliferation occurs at the inner surface of the neural tube- the ventricular zone, which consists of a single layer of neuroepithelial cells. Initially, the long processes of the neuroepithelial cells span the entire wall of the neural tube across the ventricular zone to the outer edge of tube- the marginal zone. The nuclei of these cells migrate along the long process towards the marginal zone of the neural tube where they undergo DNA replication. The nucleus then migrates back to the ventricular zone, retracts its processes and divides into two daughter cells. Early in development, both daughter cells re-enter the mitotic cycle. However, after several cycles of cell division, each progenitor cell gives rise to a dissimilar daughter cell (neuroblast), which will leave the mitotic cycle and migrate towards its appropriate position in the developing CNS. This process marks the "birth date" of a neuroblast (see review Pomeroy & Kim, 2000). The "birth date", in conjunction with environmental cues from growth factors, will ultimately determine the final destination and function of the cell in the CNS. During development, most of the CNS regions display an inside-out pattern of cells, with the first born neurons forming the deepest layers, followed by more superficial smaller neurons and finally glia. As migration progresses, the neural tube expands, resulting in the formation of three

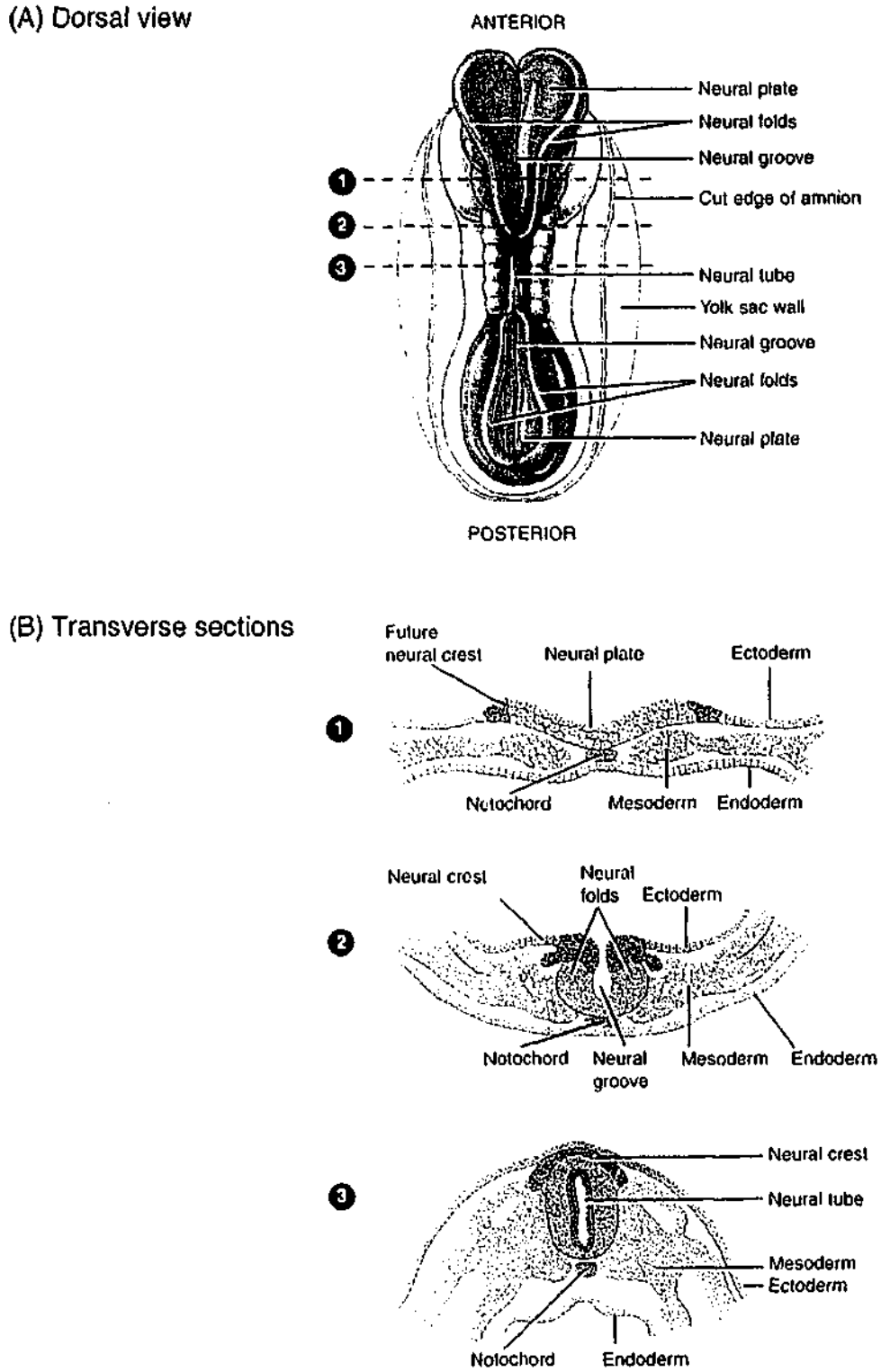


Fig. 1.1. (A) *Dorsal view of the developing nervous system, showing the neural folds which have partly united forming the early neural tube.* (B) *Corresponding transverse sections through the embryo. The first major event in the formation of the CNS occurs when inductive events between the mesodermal and ectodermal layers initiate the formation of the neural plate* (1). *As development continues, the margins of the neural plate gradually become raised and begin to fold along its midline, forming the neural groove* (2). *As the neural groove deepens, the lateral edges of the neural plate fuse in both the rostral and caudal direction to form the neural tube* (3). *Figure modified from Tortora, G. J. and Grabowski, S. R.. (1996). 8th Ed, 'Principles of Anatomy and Physiology'. p 423. Harper Collins College Publishers, New York.*

distinct vesicles; the prosencephalon (future forebrain), the mesencephalon (future midbrain), and the rhombencephalon (future hindbrain).

1.1.3 Neuronal Migration

After several cycles of cell division, the postmitotic neuroblasts leave the proliferative population and migrate away from the ventricular zone towards their definitive location. In the cerebellum, cortex and hippocampus, the immature neurons become apposed to radial glial fibers (see review Hatten, 1999) (Fig. 1.2). These glial processes are derived from the ventricular zone early in neurogenesis, and form a scaffold along which the neuroblasts migrate (Rakic, 1981). The neuroblasts extend a motile, leading process that recognises specific molecular cues on the radial glia, which ultimately aids in the migration of the cell to its definitive location. The movement of neurons along the glial processes is directed by the glycoproteins astroactin (Stitt & Hatten, 1990) and neuregulin (Anton *et al.*, 1997). The glycoprotein reelin, which is secreted by Cajal-Retzius cells in the marginal zone, is involved in signaling to the immature neuron to leave the glial guide and settle in their final destination (Darmanto *et al.*, 2000). Cells in other regions of the CNS, for example, the brainstem, migrate without the assistance of radial glial cells, relying on environmental cues (chemoattractant or repulsive) in the extracellular matrix.

1.1.4 Neuronal differentiation and the growth cone

Once an immature reaches its final position, it begins to send out processes (neurites). At the tip of most growing neurites is a growth cone, which consists of thin-fan shaped structure (lamellipodia) from which finger-like processes extend (filopodia). Collapsin (Messersmith *et al.*, 1995), fibronectin (Pomeroy & Kim, 2000), and laminin (Tessier-Lavigne & Goodman, 1996), which are located in the extracelluar matrix or on cell surfaces, are important environmental cues that govern growth cone advance. Axonal growth can be either attractive or repulsive, and recent evidence suggests that the type of cue is dependent on the on the signalling mechanisms within growth cone itself (Tessier-Lavigne & Goodman, 1996).

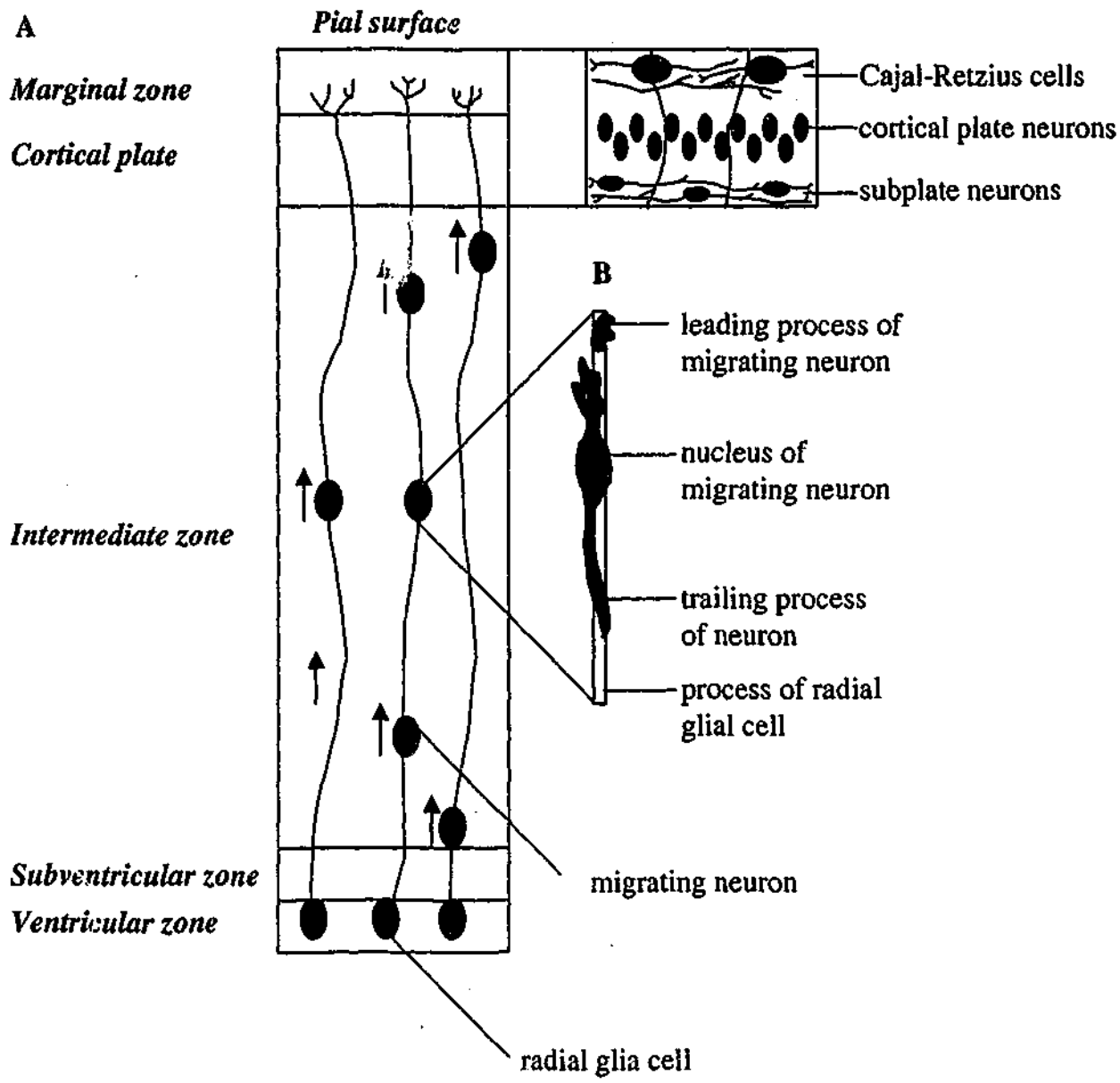


Fig. 1.2. (A) *Diagrammatic representation of the cross-section of the neural tube in the region of the forebrain. Cell proliferation occurs at the ventricular and subventricular zones. Cells migrate through the intermediate zone to the marginal zone. The first cells to arrive form the cortical plate.* (B) *Radial glial cells are derived from the ventricular zone early in neurogenesis, and form a scaffold along which the neuroblasts migrate. The neuroblasts extend a motile, leading process that recognises specific molecular cues on the radial glia, which ultimately aids in the migration of the cell to its definitive location. Figure was modified from Rees S. M. and Walker D. W. (2001). 'Fetal growth and development', p.157. Cambridge University Press, Cambridge.*

1.1.5 Synaptogenesis

When an axon reaches its final destination, a synapse is formed. The development of correct synaptic formation involves an interaction between the presynaptic (the growth cone) and postsynaptic (the target tissue) elements, which appears to involve molecular mechanisms (see review Pomeroy & Kim, 2000).

Once the growth cone contacts its target cell, it expands, accumulates organelles and vesicles, and once mature, develops into a presynaptic bouton which can release transmitter vesicles via exocytosis. The target tissue supplies the neuron with trophic support, thereby ensuring its survival. Trophic support from factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the neurotrophins (NT)-3 and NT-4/5 (Oppenheim, 1989; Maness *et al.*, 1994) is limited, making synaptogenesis a very competitive processes.

1.1.6 Neuronal death

Approximately 50% of the neurons produced during neurogenesis will die during programmed cell death, or apoptosis (Oppenheim, 1991). It is believed that insufficient neurotrophic support from the target tissue alters gene expression, resulting in cell "suicide", where DNA is degraded by an up-regulation of proteases and endonucleases (Oppenheim, 1991). The purpose of apoptosis is two fold: to match a neuronal population to the capacity of its target size; and to correct for errors in migration and projection. Following the phase of programmed cell death, there is an exuberant growth of axons, dendrites and synapses. Some synaptic connections are the pruned back, in order to increase functional efficacy. This selective elimination continues into the postnatal period.

1.1.7 Neuroglial Cells

Following neurogenesis, the production of glial cells (gliogenesis) commences, although there is some overlap between these two processes. Glial cells outnumber neurons by up to 10:1 in some regions, and unlike neurons, can divide and proliferate throughout life (McCarthy & Leblond, 1988). In addition to their supportive role in the CNS, glial cells play an important role in brain development and function, in particular,

transferring substrates and nutrients from the circulation to the neurons. There are two classes of glial cells; macroglia- oligodendrocytes and astrocytes, and microglia.

1.1.7.1 Macroglia

Like neurons, macroglia are derived from the neuroepithelial cells of the ventricular and subventricular zones (see review Rivkin, 2000). Oligodendrocytes are found in the white matter, interspersed throughout the grey matter, and as satellites around neurons. The main function of oligodendrocytes is to form and maintain a myelin sheath around axons in the CNS. Astrocytes can be further classified as fibrous or protoplasmic. Fibrous astrocytes, which contain abundant fibrils in the cytoplasm of their soma and processes, are present in the white matter. Protoplasmic astrocytes, which contain a sparse number of cytoplasmic fibrils, are found predominantly in the grey matter. Astrocytes buffer the potassium (K^+) ion concentration in the extracellular space, and remove chemical transmitters released by neurons during synaptic transmission. Astrocytes, which produce growth factors such as Insulin-like Growth Factors (IGF-1, IGF-2), contribute to the formation of the blood-brain barrier and form glial scars after CNS injury (Landis, 1994). Glial fibrillary acidic protein (GFAP) is a filament protein specific to astrocytes (Eng *et al.*, 1971). Antibodies raised against GFAP are frequently used as a marker for astrocytes; the up-regulation of GFAP indicates that an astrocyte is in a reactive state. Glutamine synthetase is an astrocyte-specific enzyme that converts neuronally released glutamate to glutamine; antibodies raised against GS are also used as a marker for astrocytes.

Radial glia, which are a specialised form of astrocytes, not only act as migrational guides, but recent reports have established that that radial glial cells also generate neurons (Noctor *et al.*, 2001). Compelling evidence also suggests a role for astrocytes in the formation and consolidation of memory in the chick (see Section 1.12).

1.7.1.2 Microglia

Microglia are derived from the yolk sac and/or bone marrow mononuclear leukocytes, and undergo a series of morphological transitions to become microglia (Cuadros & Navascues, 1998). Microglia are the resident immune cells in the brain and play a vital role in mounting defenses against invading micro-organisms and neoplastic cells (Gonzalez-Scarano & Baltuch, 1999). Microglia are involved in CNS inflammatory

responses, as they respond to and produce pro-inflammatory cytokines and growth factors (Aloisi, 2001). Microglia are also involved the phagocytosis of cellular debris, and the removal of waste products and cellular metabolites.

1.2 Neural development in the chick

Basic information about the specific timing of developmental events in the avian embryo is sparse. Most of the following information about the development of the avian brain described below has been obtained from Rogers (1995), unless otherwise stated.

The incubation period of the chick embryo is 21 days. At day 1 of incubation (embryonic day 1: E1), neural folds appear in the chick embryo, and by E2, the neural tube begins to close. By E4, most of the basal forebrain structures have formed. By E6 and E7, respectively, 50% of the neurons of the neostriatum and hyperstriatum ventrale in the forebrain have formed (Fig. 1.3). Neurogenesis in the chick forebrain peaks at day E8 (Sobue & Nakjima, 1978), and at this age, 50% of the neurons of the hyperstriatum accessorium have formed. Neurons in the CNS continue to differentiate from E10 to E17, and by E15, synapse formation peaks (Sedlacek, 1972). In the forebrain as a whole synapse formation is still occurring rapidly at hatching and is not complete until about 1-2 weeks post-hatch.

Neural development of two regions of the chick brain that are involved in memory consolidation (Kossut & Rose, 1984; Rose & Csillag, 1985; Sedman *et al.*, 1991; Gibbs & Summers, 2002b) - the lobus parolfactorius (LPO) and the intermediate hyperstriatum ventrale (IMHV), has also been investigated. From 1 day after hatching until 9 days post-hatch, there is a marked increase in the numerical density of synapses in the IMHV (Curtis *et al.*, 1989) and LPO (Hunter & Stewart, 1989). In the IMHV, the number of neurons per unit volume decreases by approximately 50% from day 16 of incubation until hatching (Curtis *et al.*, 1989). No hemispheric differences in synaptic numerical density or the number of neurons in the IMHV were apparent (Curtis *et al.*, 1989), however, in the LPO, the left hemisphere contained 1.6 times more synapses than the right hemisphere (Hunter & Stewart, 1989). This hemispheric asymmetry in synapses in the LPO may reflect the difference between the functions of the left and right

hemispheres in the processing of memory in the chick (Gibbs *et al.*, 2002) (see Section 1.8).

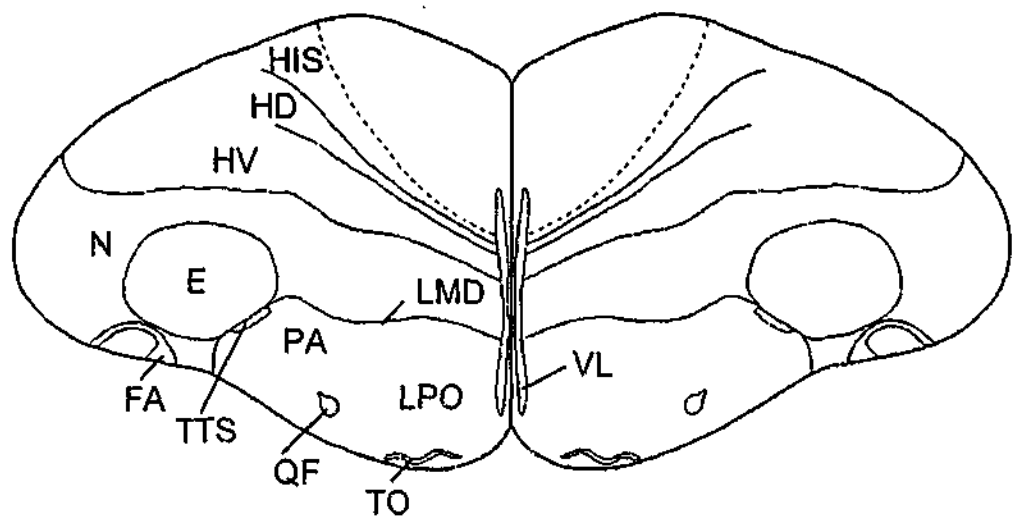


Fig. 1.3. A coronal section of the brain of a two-week old chick. Abbreviations: E, ectostriatum; FA: tractus fronto-archistriaticus; HD: hyperstriatum dorsale; HIS: hyperstriatum intercalatum supremum; HV: hyperstriatum ventrale; LMD: lamina medullaris dorsalis; LPO: lobus paraolfactorius; N: neostriatum; PA: paleostriatum augmentatum; QF: tractus quintofrontalis; TO: tuberculum olfactorium; TTS: tractus thalamostriaticus; VL: ventriculus lateralis. Image adapted from http://jarvis.neuro.duke.edu/nomen/Chicken_Atlas.html. Coronal images of the chick brain on this website were provided by Dr. Wayne Kuenzel.

1.3 Mammalian models of intrauterine compromise

The focus of this thesis is the effects of prenatal hypoxia, in the absence of perturbations in nutrient supply, on postnatal cognitive function. In human pregnancy, hypoxia is a common form of prenatal stress, and can be caused by placental insufficiency, restricted umbilical blood flow, maternal undernutrition or maternal smoking. The long-term consequences of the fetal hypoxemia associated with these insults on human cognitive function remain unknown.

The responses and adaptations to prenatal compromises have been extensively studied in mammalian animal models. The interventions used in these animals remain extrinsic to the fetus and typically involve disrupting uteroplacental vascular function or placental growth. In this section, the effects of prenatal compromises on body growth and brain development will be summarised.

1.3.1 Prenatal compromise in sheep

The use of sheep rather than smaller species to investigate the effects of prenatal compromise is advantageous because the larger size of the fetus allows for catheterisation of the fetus and manipulation of the intrauterine environment. Furthermore, fetal blood gas, endocrine and metabolic status can be closely monitored.

1.3.1.1 Effects on somatic and brain growth, blood gas status and brain structure

Chronic placental insufficiency, induced by reducing placental size by the surgical removal of placentation sites (carunclectomy) prior to conception in the ewe, results in fetal growth restriction. Although the brain was shown to be relatively spared in relation to other organs, brain weights of the growth-restricted fetuses were significantly reduced compared to controls (Rees *et al.*, 1988). Using this technique, fetuses are typically hypoxemic, hypoglycemic and acidemic during the last third of gestation (Robinson *et al.*, 1979). The major effects on brain structure include a reduction in the growth of neuronal processes, in synaptogenesis and in myelination in the visual cortex, hippocampus, and cerebellum (Rees *et al.*, 1988).

Placental insufficiency in the sheep can also be induced by partial embolisation of the umbilico-placental circulation. Twenty days of umbilico-placental embolisation during the last third of gestation has been shown to result in fetal growth restriction, hypoxemia, hypoglycemia, acidemia and hypercapnia (Cock & Harding, 1997). Brain weights, when adjusted for body weight, were significantly increased in the growth-restricted group, indicating that brain sparing has occurred. Umbilico-placental embolisation has been shown to cause abnormalities in brain structure, with the cerebellum being particularly affected. In the cortex, evidence of white mater lesions, a proliferation of astroglia, and an increase in the size of capillaries have been reported (Mallard *et al.*, 1998).

The effect of shorter periods of fetal hypoxemia on somatic growth and brain structure has also been examined in the fetal sheep. In these studies, uterine blood flow was reduced for 6-12 hours near mid-gestation and fetuses were allowed to recover for 7 or 35 days after the hypoxemic insult. Fetuses were growth restricted at 35 days after the prenatal insult, and brain weights in both groups of animals were reduced. During the period of reduced uterine blood flow, fetuses were hypoxemic and there was a tendency

for arterial pH of these fetuses to be lower compared to controls (Rees *et al.*, 1999). The major alterations in brain structure included mild focal damage in the cortical white matter, proliferation of blood vessels, a reduction in the number of Purkinje cells, and a delay in the growth of neuronal process in the cerebellum. The density of pyramidal cells was also reduced in the hippocampus (Rees *et al.*, 1998; Rees *et al.*, 1999).

1.3.1.2 Effects on neurological function

Given the significant damage that has been reported following prenatal hypoxia in the sheep, it would be expected that prenatally hypoxic lambs would show neurological deficits postnatally. Camm *et al.* (2000) investigated the effects of placental insufficiency on learning ability in preterm low birth weight (LBW) lambs. Although the LBW lambs appeared to take longer to perform tasks that assessed their learning ability, and made more errors, the differences were most likely a consequence of preterm birth, as correction for post-conceptional age abolished any differences in learning ability (Camm *et al.*, 2000). As mentioned, the assessment of learning and memory formation in young lambs is often complicated by the fact that most tasks to assess these parameters rely on the offspring's desire to be with its mother as reinforcement.

1.3.2 Prenatal compromise in the guinea pig

1.3.2.1 Effects on somatic and brain growth, blood gas status and brain structure

Placental insufficiency, induced by unilateral artery ligation from mid gestation (30 days) until term (~66 days) results in fetal growth restriction and brain sparing (Lafeber *et al.*, 1984; Nitsos & Rees, 1990; Mallard *et al.*, 1999; Mallard *et al.*, 2000). Growth-restricted fetuses produced under these conditions are typically hypoxemic and hypoglycemic (Jones & Parer, 1983). The major alterations in brain structure following uterine artery ligation include: a delay in the initiation and in the extent of myelination in the spinal cord, corpus callosum and cerebellum; retarded axonal growth in the cortiospinal tract; and proliferation of astrocytes surrounding cortical blood vessels (Nitsos & Rees, 1990). Uterine artery ligation during the second half of gestation has also been shown to result in a reduction in neuronal numbers and reduced growth of neuropile in the cerebellum and hippocampus (Mallard *et al.*, 2000), reduced hippocampal volume and enlarged ventricles (Mallard *et al.*, 1999).

1.3.2.2 Effects on neurological function

Recent studies have examined the effects of placental insufficiency on postnatal neurological function. Electroretinogram deficits have been observed in 8-week old animals following placental insufficiency *in utero* (Bui *et al.*, 2002). Morphologic analysis of the retinas of these animals also revealed significant reductions in all cellular and plexiform (synaptic) layers in both the central and peripheral retina. The outer retina, which contains the photoreceptors and the outer plexiform layer was particularly affected (Bui *et al.*, 2002). Alterations in auditory brainstem responses (ABRs) have also been observed in 8-week old guinea pigs following prenatal compromise (Rehn *et al.*, 2002). The authors suggest that changes in ABRs may reflect alterations in myelination of the auditory brainstem and/or changes in synaptic efficacy, which could have implications for neural conduction.

1.3.3 Prenatal and neonatal compromises in the rat

Experimental studies in the rat have either induced hypoxemia prenatally or in the neonatal period. Fetal hypoxia is usually induced by exposing pregnant rats to daily episodes of hypoxia in a nitrogen chamber for a selected period of intrauterine life. Neonatal hypoxia is frequently used because the rat is functionally immature at birth. Hence, a hypoxemic episode in the neonatal period in rats is considered to be equivalent to an insult in the perinatal period in precocial developers, like the sheep and chick. A common technique to induce neonatal hypoxemia is the modification of the Levine preparation (Levine, 1960) described by Rice *et al.* (1981), which includes a combination of ischemia induced by unilateral occlusion of the carotid artery, followed by exposure to 1½-3 hrs of hypoxia (reduced O_2 in inhaled air) at 7 days of age.

1.3.3.1 Effects of somatic and brain growth, brain structure and neurological function

Prenatal hypoxia (10.5% O_2 in nitrogen) for 4 hrs per day, or continuously, from days 15 to 18 of gestation, does not alter body or brain weights at birth or impair long-term neurological function (Hermans *et al.*, 1992; Longo & Hermans, 1992). Conversely, moderate hypoxia (13% O_2 in nitrogen) for 2 hrs a day during the last 14 days of gestation has been shown to impair maze performance in young rats at 20 days after birth (McCullough & Blackman, 1976). Thirty minutes of hypoxia-ischemia at day 17 of gestation results in impaired learning ability in rats at 28 days of age (Cai *et al.*,

1999). Daily exposure to hypoxia throughout the entire gestational period has been shown to reduce fetal and brain weights at birth and alter brain structure (Brichova, 1986). Glial development was found to be delayed in layers I to III in the motor cortex of rats exposed to prenatal hypoxia; changes in the structure of capillary walls and small blood vessels were also evident. These changes appeared to persist in the growth-restricted rats 90 days after birth (Brichova, 1986)

1.3.3.2 Effects of neonatal hypoxia on neurological function

Given the short period in which rats are exposed to neonatal hypoxia, alterations in somatic and brain growth are not evident. In spite of this, long-lasting alterations in learning and motor behaviour have been reported. Repeated postnatal anoxia on postnatal days 1, 2 and 3 has been shown to impair adult spatial memory performance (Buwalda *et al.*, 1995). Ischemia- hypoxia at 7 days of age using the Levine model has been shown to cause long-lasting learning (Balduini *et al.*, 2000), sensorimotor and locomotor deficits (Jansen & Low, 1996).

1.3.4 Summary

Taken together, the results from experimental studies suggest that prenatal hypoxia, typically induced by disrupting placental blood supply, can decrease body and brain weights at birth. It is evident that growth restricted fetuses resulting from techniques aimed at restricting placental or uteroplacental blood flow are typically hypoxemic, hypoglycemic, acidemic and hypercapnic. Structural analysis of the brains of these fetuses also indicate that prenatal compromise can affect basic neurodevelopmental processes, such as neuronal migration and synaptogenesis, depending on the duration and the stage of development the insult takes place. Hence, prenatal insults during late gestation are expected to affect later developing structures like the cerebellum, more than earlier developing structures, such as the hippocampus. It is important to remember that the techniques that aim to induce fetal hypoxemia also cause fetal hypoglycemia, as well as metabolic, cardiovascular and endocrine perturbations. Therefore, whilst it is likely that both hypoxia and undernutrition contribute to reduced fetal growth and alterations in brain structure, the individual contributions of these factors cannot be evaluated.

1.4 The chick embryo

The chick embryo is now emerging as a valuable animal model to assess responses and adaptations to prenatal hypoxia, and there are several advantages of using the chick rather than mammalian models. As mentioned, the chick embryo can develop independently of its mother, thus there are no confounding effects of maternal and placental physiology, and materno-fetal nutrient transfer. Importantly, nutrient supply from the yolk sac is separated from the area of gas exchange (see Romanoff, 1960), enabling the direct effects of hypoxia to be assessed independently of perturbations in fetal nutrient supply. Due to the characteristics of gas exchange of the chicken egg, exposure to prenatal hypoxia can be made non-invasively by either covering part of the eggshell with a membrane impermeable to O_2 (Metcalfe *et al.*, 1979), or by decreasing ambient O_2 concentration (Mulder *et al.*, 1998 ; Miller *et al.*, 2002).

Assessing learning and memory following a prenatal insult in mammalian models (e.g. young lambs) is often complicated by the fact that most tasks to assess these parameters rely on the offspring's desire to be with its mother as reinforcement (Camm *et al.*, 2000). Learning and memory can be easily assessed in newly hatched chicks using well-established behavioural tasks (Andrew & Rogers, 1972; Gibbs & Ng, 1977b; Gibbs & Summers, 2002b). The one-trial discriminated bead task has been widely used to study memory formation, and involves day-old chicks discriminating between red and blue beads (see review Gibbs & Summers, 2002b). The pebble floor task has been the most extensively used food-based task in chicks, and involves presenting 5-6 day old chicks with food scattered amongst pebbles of similar size and colour (Andrew & Rogers, 1972). An analogous task to discriminated bead task is the aversive wheat task, which involves training 5-8 day old chicks to discriminate between red and yellow crushed wheat (Gibbs & Ng, 1977b). Chicks are ideal for behavioural testing, as they can be readily obtained in large numbers. Furthermore, as chicks are primarily prenatal developers (precocial), they can be taught to perform particular tasks soon after hatching (Gibbs & Summers, 2002b). Given the advantages of using the chick embryo over mammalian models to study the effects of prenatal hypoxia on neurological function, the chick has been used in the experiments outlined in this thesis.

1.4.1 The avian egg

At the time of laying, an avian egg contains all of the nutrients and minerals needed for embryonic development, with the exception of O_2, which is supplied by passive diffusion through the pores of the egg shell (Rahn *et al.*, 1979; Vleck & Vleck, 1987) (Fig. 1.4). The egg requires constant warming and periodic turning in order to prevent the adhesion of the embryo to the shell membranes (Rahn *et al.*, 1979).

Initially, gas exchange takes place in the area vasculosa, which is the vascularised region of the yolk sac. The area vasculosa rapidly grows during the first 5 days of incubation, and spreads around the yolk sac (Ackerman & Rahn, 1981). From around day 6 of incubation, O_2 and carbon dioxide are exchanged through the vascularised membrane situated below the surface of the egg shell - the chorioallantoic membrane (CAM) (Metcalfe & Stock, 1993). Oxygen to the embryo is provided by the chorioallantoic vein, and nutrients are provided through the vitelline circulation from the yolk sac. The CAM is considered to be equivalent to the placenta in mammals, whereas the chorioallantoic artery and vein are equivalent to the umbilical circulation (Metcalfe & Stock, 1993). As gas is exchanged across the CAM, water vapour is continually lost. Since the shell is rigid, gas enters to replace the lost water and forms an air cell at the blunt end of the egg (Rahn *et al.*, 1979). Gas tensions within the air cell are nearly identical with those in the air spaces of the shell membranes, thereby providing a convenient site to sample the gases within the egg (Rahn *et al.*, 1979).

The O_2 consumption of the embryo increases slowly during the first week and a half of incubation (Rahn *et al.*, 1979). Towards the end of the incubation period, O_2 demand exceeds the capacity of gas diffusion for adequate O_2 delivery (Rahn *et al.*, 1974). In the chorioallantoic artery and air cell of the egg, O_2 tension progressively decreases, and carbon dioxide increases, from days 14-17 of incubation (Freeman & Mission, 1970; Rahn *et al.*, 1974). On about days 19-20 of incubation, the chick penetrates the air cell at the blunt end of the egg with its beak, a process called internal pipping (Rahn *et al.*, 1979). The chick then begins to breathe from the air cell, ventilating its previously unused lungs (Rahn *et al.*, 1979).

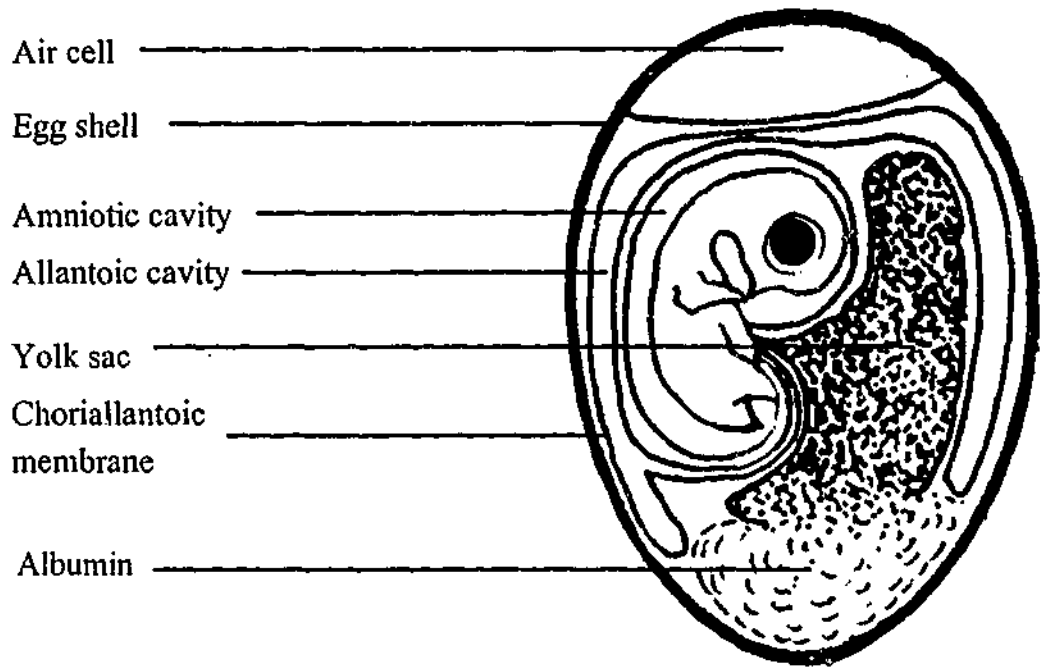


Fig. 1.4. Schematic representation of the components of a fertile chick egg.

1.5 Prenatal hypoxia in the chick embryo

Previous studies examining the effect of *in ovo* hypoxia on development have either induced hypoxia throughout the entire incubation period, or for a brief period, typically during the later third of incubation. Due to the characteristics of gas exchange of the chicken egg, O_2 supply can be restricted by either covering part of the eggshell with a membrane impermeable to O_2, or by reducing incubator O_2 concentration.

1.5.1 Effects on blood gas status

Restricting gas exchange *in ovo* has been shown to alter blood gas status in the embryo. Reducing the shell area available for respiration by coating either the air cell or narrow end of the egg at day 0 of incubation, decreases PO_2 and pH in chorioallantoic venous blood, whilst increasing PCO_2 and hematocrit at mid- to late-incubation (Tazawa *et al.*, 1971b). Covering one half of the eggshell with paraffin wax produces a 23% lowering of PO_2, and a 32% increase in PCO_2 (Roncali *et al.*, 1985).

Chick embryos incubated in 10% O_2 from days 14-18 of incubation have been reported to have a high hematocrit (Xu & Mortola, 1989). Five minutes of acute hypoxemia (induced by replacing incubator gas with 100% nitrogen) during the later stages of

development, has been shown to significantly decrease arterial PO_2 and PCO_2; pH was not altered (Mulder *et al.*, 2000). A study by Dzialowski *et al.* (2002) showed that 6 days of hypoxia from days 6-12 of incubation (induced by incubating eggs in 110 mmHg), increased hematocrit and hemoglobin at day 12 of incubation, however, by day 18, hematocrit and hemoglobin had returned to control levels. Hypoxia from days 1-6 or 12-18 of incubation did not alter hematocrit and hemoglobin levels when examined at day 18 (Dzialowski *et al.*, 2002).

1.5.2 Effects on somatic and organ growth

Body weights of chick embryos exposed to hypoxia from day 0 of incubation have been shown to be reduced at day 18 of incubation (Metcalfe *et al.*, 1981; McCutcheon *et al.*, 1982), and at hatching (Miller *et al.*, 2002). This decrease in body weight at day 18 of incubation has also been observed in chick embryos exposed to varying degrees of hypoxia from days 6-12 (Dzialowski *et al.*, 2002), 12-18 (Dzialowski *et al.*, 2002), 14-18 (Xu & Mortola, 1989), and 16-18 of incubation (Stock & Metcalfe, 1987; Asson-Batres *et al.*, 1989). Along with the changes observed in body growth, organ growth has been shown to be disproportionately affected by changes in O_2 availability. Heart and brain sparing have been observed in chick embryos exposed to hypoxia from days 0-18 of incubation; liver growth, however, was stunted (McCutcheon *et al.*, 1982). In contrast, exposure to hypoxia from days 0-18 of incubation does not alter vital organ weights relative to body weight (Miller *et al.*, 2002). Similarly, exposure to hypoxia from days 16-18 results in a reduction in heart weight (Asson-Batres *et al.*, 1989).

1.5.3 Effects on cardiac output

In chick embryos, the physiological responses of the cardiovascular system to hypoxia appear to parallel those seen in humans and other mammalian species. In response to acute hypoxemia *in ovo*, the chick embryo shows a marked peripheral vasoconstriction, which aids in redirecting cardiac output towards the brain, heart, and adrenal glands (Mulder *et al.*, 1998). In response to acute hypoxemia from mid- to late incubation, the chick embryo is able to increase plasma noradrenaline and adrenaline concentrations at day 13 and 16 of incubation, respectively. This catecholaminergic response appears to develop with time, as evidenced by a progressive increase in plasma noradrenaline and adrenaline concentrations as the embryo approaches hatching (Mulder *et al.*, 2000).

1.5.4 Catecholamine levels throughout incubation

The reason why the chick embryo is only able to mount a significant catecholaminergic response to acute hypoxemia from mid-incubation is most likely due to the fact that sympatho-adrenal development occurs mainly in the second half of gestation in the chick. Noradrenaline and adrenaline can be detected in very small amounts in the allantoic fluid of the fertile chick egg from day 4 of incubation (Boucek & Bourne, 1962). At this early stage of development, catecholamines are accumulated in the yolk sac and are maternal in origin (Ignarro & Shideman, 1967). Innervation of the adrenals commences at day 8 of incubation, and is sympathetically innervated on about day 10 of incubation (Fujita *et al.*, 1976). Whilst very small amounts of adrenaline are present in the adrenals at day 10 of incubation, adrenaline levels remain low until day 15 of incubation, after which there is a marked increase up until hatching (Wassermann & Bernard, 1970). The detection of catecholamines in plasma has been reported at days 10 and 14 of incubation (Epple *et al.*, 1992).

Catecholamine levels have also been assessed in various regions of the chick brain pre- and post-hatching. Adrenaline levels appear to peak at day 19 of incubation in the chick telencephalon, and at 2 days after hatching in the diencephalon, mesencephalon and cerebellum. Noradrenaline levels increase dramatically after hatching in all these structures, and are significantly higher than adrenaline levels (Revilla *et al.*, 2001).

1.5.5 Effects on brain development

Little is know about the effects of prenatal hypoxia on brain structure and development in the chick embryo. A study by Lee *et al.* (2001) investigated the effects of prenatal hypoxia on cerebellar development in the chick embryo. Prenatal hypoxia, induced by sealing half of the air cell with melted wax on day 2 of incubation until days 18-20, resulted in reductions in Purkinje cell size and density, and in dendrite development in the cerebellum (Lee *et al.*, 2001).

The effects of prenatal hypoxia on the optic tectum microvascolature have also been investigated in the chick embryo. In this study, hypoxia was induced by covering one half the eggshell with paraffin wax from day 2 of incubation, until days 8, 14 or 17. Under hypoxic conditions, vessel number increased in the developing optic tectum to a

greater extent compared to controls, and vessel diameter transiently increased. The distance between contiguous neural blood vessels was also measured in the optic tectum, and was found to be much lower in the hypoxic chick embryos than controls (Ribatti *et al.*, 1989). The morphological alterations in the cerebral vasculature reported by Ribatti *et al.* (1989) are most likely due the capacity of the growing neural vascular bed to adapt to O_2 deprivation. The dilatation of pre-existing blood vessels or the formation of new vessels under hypoxic conditions would result in an increase in cerebral blood flow, thereby maintaining oxygenation of the brain tissue.

1.5.6 Effects on brain function

There have been no investigations into the effects of prenatal hypoxia on postnatal neurological function in chicks. Two studies, however, have investigated the effects of postnatal hypoxia and cerebral ischemia, respectively, on memory function (see Section 1.6.1 for details of the task). Willson *et al.* (1994) found that cerebral ischemia, induced in day-old chicks by bilateral carotid artery occlusion, produced amnesia for the one-trial passive avoidance task (with avoidance of the target object being the measure of memory retention) 24 hours after initial training. Seven days after the ischemic insult, the brains of the animals were examined. Neuronal damage, determined qualitatively using a silver impregnation method, was evident in several forebrain regions, including the hippocampus, hyperstriatal regions, and ventral archistriatum (Willson *et al.*, 1994). Allweis *et al.* (1984) also used the discriminated bead task to examine the effect of hypoxia on memory consolidation. Hypoxia was induced in day-old chicks immediately after or 5 min after training on the bead task, by placing the animals into a plastic bag containing 2% O_2 in nitrogen for approximately 15 sec. Hypoxia induced immediately after training did not significantly effect memory, however treatment 5 min after training produced a transient retention loss 30 min after training (Allweis *et al.*, 1984).

1.5.7 Effect of prenatal albumin removal (malnutrition) on chick embryo development

The effect of protein malnutrition on development in the chick embryo has also been examined (Hill, 1993; Miller *et al.*, 2002). The removal of 1, 2, 4, 8, 12 or 16% of albumin from eggs prior to incubation decreased chick size at hatch, and retarded the righting reflex response of chicks in which 2 and 4% of albumin were removed (Hill, 1993). Twenty days following hatch, there was no difference in body weights between

control chicks and chicks which had albumin removed from their eggs prior to incubating, although albumin removal had significant long-term effects on the growth rate of female chicks, whereas growth rates of males were not altered (Hill, 1993).

The removal of 10% of albumin prior to incubation has also been shown to decrease body weight at hatch, with relative sparing of the brain and heart, lungs and kidneys (Miller *et al.*, 2002). Malnutrition, in combination with hypoxia (14% O_2 in nitrogen) from days 10-18 of incubation, resulted in chicks being more growth restricted at hatch compared to chicks subjected to malnutrition alone. Relative sparing of the heart, brain, lungs and kidneys were also evident in the malnourished hypoxic chicks (Miller *et al.*, 2002).

1.5.8 Summary

Taken together, the results from experimental studies in the chick embryo suggest that prenatal hypoxia alters blood gas status, decreases body weight at birth, and disproportionately affects organ growth. The redistribution of cardiac output towards the cerebral, myocardial and adrenal circulations in the chick embryo mimics what is seen in mammalian species (Cohn *et al.*, 1974). The effect of prenatal hypoxia on brain development, particularly neural migration and proliferation, synaptogenesis and myelination, which are typically affected following fetal hypoxemia in the sheep and guinea pig, remain to be determined. Although the hypoxic insult was not induced prenatally, the findings of Willson *et al.* (1994) and Allweis *et al.* (1984) suggest that memory function may be altered following acute hypoxia. Taken together, it is possible that post-hatch function may be impaired in the chick following prenatal hypoxia.

1.6 Memory testing in the chick

As the focus of this thesis is on the chick, the cellular mechanisms involved in memory formation in this species will be outlined below. As mentioned, the chick is an ideal model for the study of the basic cellular processes in memory formation. As chicks are precocial (prenatal developers), they can be taught to perform particular tasks soon after hatching. It is also assumed that the basic memory mechanisms in the chick are similar to those of other species, including mammals.

1.6.1 The single trial passive avoidance task in the neonatal chick

The single trial passive avoidance task was developed by Cherkin (1971) and Mark and Watts (1971). The original paradigm involved training chicks, in pairs, to avoid a small chrome bead (typically red) previously coated in a bitter tasting, non-toxic chemical, methyl anthranilate. Chicks form a lasting memory of the experience, so when they were later presented with a dry red bead, they typically avoided it. The measure of memory retention used by Cherkin (1971) was the proportion of chicks in any one group that avoided the training bead on test (passive avoidance learning).

In 1976, a version of the single trial passive avoidance task using discrimination was introduced (Gibbs & Ng, 1977b), and has since been widely used to study memory formation in the chick. This version of the task also involves training day-old chicks to peck at a red bead that has been dipped in methyl anthranilate, however, on test, chick are presented with a blue bead as well. The number of pecks and the latency to first peck for each chick are recorded, and memory retention is assessed by calculating the ratio of the number of pecks at the blue bead to the total number of pecks at the blue and red beads on test. This index of memory retention is referred to as the discrimination ratio. Percent discrimination, which is the proportion of chicks that avoid the red bead on test but continue to peck the blue bead, is also used as an indicator of memory retention. Chicks that avoid the blue bead on test are excluded from the definition of both of these indices of discrimination memory.

Using the discriminated bead task, a three stage model of memory formation has evolved following refinements to the original experimental design (Gibbs & Ng, 1976), and the effect of weak reinforcers on memory formation has also been investigated (Crowe *et al.*, 1989b).

1.6.2 Tasks used to assess learning and memory formation in older chicks

As it becomes difficult to induce pecking by the presentation of beads in chicks older than three days of age, other tasks that can assess learning and memory in the chick typically involve the presentation of food (see Andrew, 1991).

The "pebble floor task" has been the most extensively used food-based task, and was introduced by Dawkins (1971) and later modified by Andrew and Rogers (1972). In brief, the task involves presenting chicks from one to two weeks of age, with familiar food (typically chick starter crumble) scattered amongst pebbles of similar size and colour (Dawkins, 1971; Andrew & Rogers, 1972). The pebbles, however, are adhered to the floor of testing box. Learning is assessed by the rate at which the number of pecks at pebbles, rather than food, declines over the testing period.

An analogous task to discriminated bead task is the aversive wheat task, which involves training 5-8 day old chicks to peck at crushed wheat that had been coloured red and soaked in quinine and mustard (Gibbs & Ng, 1977b). At testing, chicks are required to discriminate between red and yellow crushed wheat. Memory retention is assessed using the discrimination ratio (as with the discriminated bead task), which is ratio of the number of pecks at the yellow wheat to the total number of pecks at the yellow and red wheat on test.

1.6.3 A model of memory formation

By testing memory at various times after training, it was demonstrated that there were three stages of memory formation in the chick: short-term memory (STM), intermediate memory (A) (ITM A) and (B) (ITM B), and long-term memory (LTM) (Gibbs & Ng, 1976, 1977b) (Fig. 1.5). The "dips" in retention evident at 15 and 55 minutes after training defined the different stages of memory. These stages were further defined on the basis of their vulnerability to interference by different pharmacological agents. By varying the time of injection of different classes of amnesic drugs at a set time after training, intermediate memory was revealed by the inhibition of long-term memory and short-term memory was revealed by the inhibition of intermediate memory (Gibbs & Ng, 1976, 1977a). It therefore became evident that the "dips" in memory retention were representative of the times for the cross over of the different stages of memory (Gibbs & Summers, 2002b).

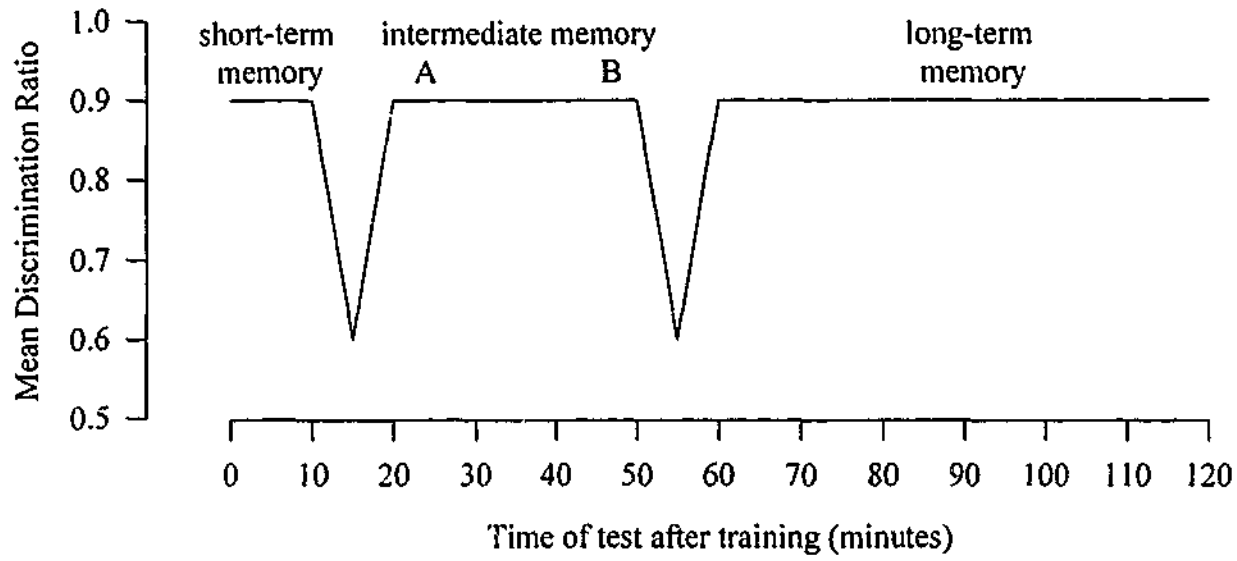


Fig. 1.5. *A schematic representation of the different stages of memory based on performance tested at various times after training. Normal learning is indicated by a discrimination ratio (DR) of ~0.9; complete abolishment of memory is reflected by a DR of 0.5. Short-term memory (STM) occurs between 5 and 10 min after training. Intermediate memory (ITM) is divided into two parts: ITM (A), which occurs between 20-30 min post-training, and ITM (B), which occurs between 30-50 min. Long-term memory (LTM) occurs from 60 min post-training. The "dips" in retention evident at 15 and 55 minutes after training separate the different stages of memory.*

1.6.3.1 Strongly reinforced learning

When separate groups of chicks are trained with a red bead that has been dipped in a strong reinforcer (100% anthranilate), they form a permanent memory of the experience and tend not to peck at red beads up to several weeks or months later (see review Gibbs & Summers, 2002b). Chicks trained on 100% anthranilate show a retention function consisting of the three distinct memory stages, with each stage being separated by transient retention deficits at 15 and 55 minutes post-training (Fig. 1.6).

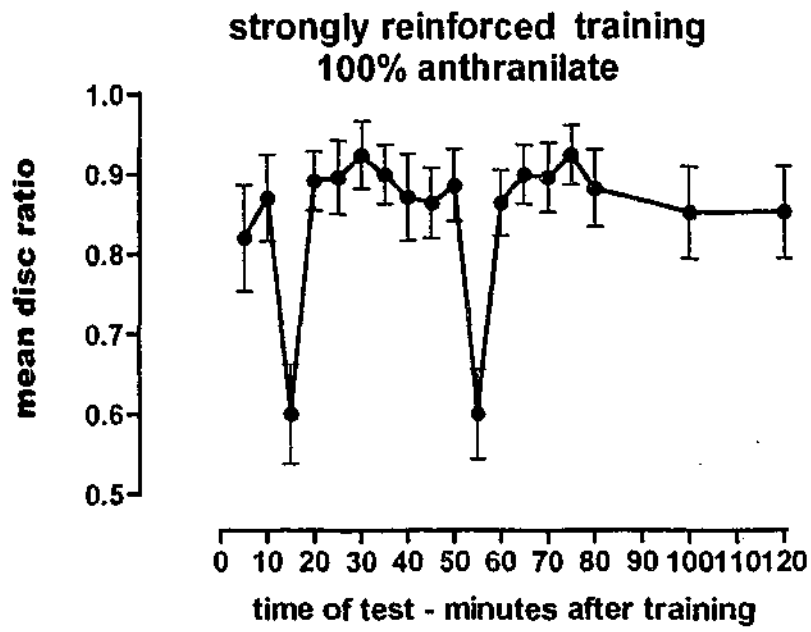


Fig. 1.6. Strongly reinforced training with 100% anthranilate. Memory retention, as measured by the discrimination ratio, remains high at all times of test, except at 15 and 55 minutes after training. Figure obtained from Gibbs, M. E. & Summers, R. J. (2002). Prog Neurobiol, 67: 345-391.

1.7 Cellular mechanisms of memory formation

1.7.1 Dissection of the memory time course in the chick with drugs

As mentioned, the three sequentially dependent stages of memory formation in the chick were revealed by varying the time of injection of different classes of drugs that produced amnesia. A second protocol was then employed in which the time of administration of the inhibitory drugs remained constant, but the chicks were tested at various times after training to identify when memory loss first occurred (see review Gibbs & Summers, 2002b). It was determined from these studies that there was a clear, stage-specific variability in susceptibility to pharmacological agents, suggesting that memory formation during each of the three stages was dependent upon different biological processes.

1.7.1.1 Short-term memory

Many agents can inhibit short-term memory, which occurs between 5 and 10 min after training. These include drugs that block potassium conductance (1-2 mM potassium chloride, Ng & Gibbs, 1991), produce depolarisation (monosodium glutamate, Gibbs & Ng, 1979), or block calcium channels (lanthanum chloride, (Nordstrom *et al.*, 1983). Gamma-aminobutyric acid (GABA) receptor antagonists (e.g. bicuculine) can also

prevent short-term memory formation, although GABA itself has no effect on memory formation (Gibbs, 1991).

1.7.1.2 Intermediate memory (A)

The inhibitors of IMT (A) are less varied than those which inhibit STM (Gibbs, 1991). The first part of ITM, which lasts between 20 and 30 minutes after training, can be prevented by drugs that alter Na^+/K^+ ATPase activity (the sodium pump), such as ouabain and ethacrynic acid (Gibbs & Ng, 1976). The metabolic inhibitor 2,4-dinitrophenol (DNP) also inhibits the expression of ITM (A), possibly by inhibiting ATP production (Gibbs & Ng, 1977b).

1.7.1.3 Intermediate memory (B)

ITM (B) memory, which occurs between 30 and 50 minutes after training, can be inhibited by the noradrenergic antagonists sotalol (Stephenson & Andrew, 1981), propranolol (Crowe *et al.*, 1991b) and cycloheximide (Gibbs & Ng, 1984). Trifluroperazine, a calcium-calmodulin antagonist, can also inhibit phase (B) of ITM (Bennett *et al.*, 1987).

1.7.1.4 Long-term memory

Consistent with the view that protein synthesis underlies permanent memory encoding (Dunn, 1980), is the finding that intracranial administration of protein synthesis inhibitors, including the antibiotics cycloheximide (Gibbs & Ng, 1977b) and anisomycin (Gibbs & Ng, 1976), causes memory loss within 60 min following training. The protein kinase inhibitor A (PKA) lauryl gallate also impairs memory consolidation (see review Gibbs & Summers, 2002b).

1.8 Areas of the chick brain involved in memory consolidation

The forebrain regions involved in passive avoidance training have been identified using a 2-deoxyglucose autoradiographic technique to measure glucose utilisation as a consequence of learning (Kossut & Rose, 1984). To localise the metabolic changes in different regions of the brain, chicks were injected with 2-deoxyglucose prior to being trained with either methylanthranilate or water. Whilst the overall labeling patterns of the autoradiograms were qualitatively the same between the methylanthranilate and water-trained chicks, the optical densities of the brain regions studied showed that there

was altered 2-deoxyglucose labeling in three regions of the methylanthranilate trained chicks- the IMHV, LPO and the paleostriatum augmentatum (PA) (Kossut & Rose, 1984) (see Fig. 1.3). Subsequent experiments used small regions of the chick brain that has been dissected out, instead of whole coronal sections, and confirmed the role of the LPO and IMHV in passive avoidance learning (Rose & Csillag, 1985). However, alterations in 2-deoxyglucose metabolism were not found in the PA. This was attributed to the large amount of tissue sampled in the original autoradiographic study (Kossut & Rose, 1984). The involvement of the IHMV in memory formation has since been confirmed by the work of Davies *et al.* (1988) and Horn (1985), which showed that lesions in the IMHV affect avoidance learning in the chick (Horn, 1985; Davies *et al.*, 1988).

Along with changes in glucose utilisation as a consequence of learning, several studies by Stewart and co-workers have shown that morphological changes occur at the dendritic and synaptic level following learning. Marked morphological changes in the IMHV and LPO following training on the discriminated bead task include: an increase in presynaptic bouton volume, an increase in density of synaptic vesicles, an increase in synaptic number (Stewart *et al.*, 1984) and an increase in spine density (Patel *et al.*, 1988). Interestingly, left-right asymmetries in synaptic parameters were observed in the IMHV and LPO following learning; this lateralisation may reflect the difference between the functions of the left and right hemispheres in the processing of memory in the chick (Gibbs *et al.*, 2002).

1.8.1 Avian versus mammalian brain

Although the avian brain appears structurally different to the mammalian brain, it is becoming apparent that the avian brain has most of the components that are present in the mammalian brain, but they are arranged differently (Dubbeldam, 1991). The IMHV in the avian brain is a multimodal association area, and is considered to be a cortical association area. The LPO is equivalent to the basal ganglia-caudate putamen in mammals. The caudate putamen of the basal ganglia has marked similarities in terms of connectivity and neurotransmitter content to the mammalian system (see review Durstewitz *et al.*, 1999). The hippocampus is involved in the acquisition of spatial memory in both birds and mammals. The avian hippocampus is considered functionally homologous to the mammalian hippocampus, despite differences in cellular structure

(Bolhuis & MacPhail, 2001), and does not appear to play a role in memory for the discriminated bead task.

1.9 Enhancement of memory formation using weakly reinforced learning

The discriminated bead task can be modified by diluting the aversant with alcohol, so that 20% anthranilate is used rather than 100%. This causes chicks to form a labile memory which lasts for only 30 minutes, after which the memory disappears and the chicks peck equally at both the red and blue beads (Crowe *et al.*, 1989b) (Fig. 1.7). This weakly reinforced version of the task has been a beneficial tool for investigating ways of pharmacologically promoting the consolidation of memory (Gibbs & Summers, 2002b). It is possible to alter the labile memory from one that lasts for only 30 minutes to one that lasts for at least 24 hours. This can be achieved by increasing either the number of trials (Crowe *et al.*, 1989a) or concentration of anthranilate (Crowe *et al.*, 1989b) or alternatively, by the administration of the hormones adrenocorticotropic hormone (ACTH), vasopressin and noradrenaline (Crowe *et al.*, 1990).

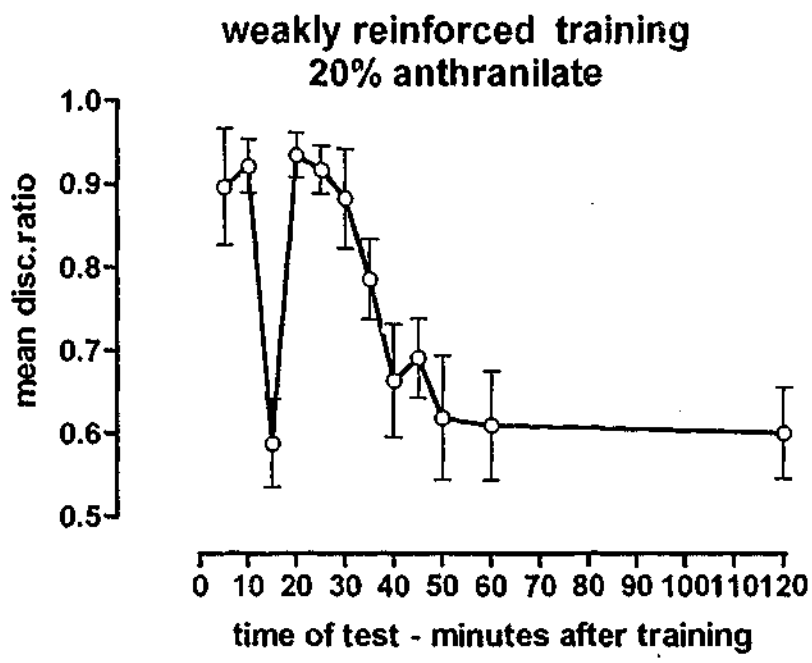


Fig. 1.7. Weakly reinforced training with 20% anthranilate. Unlike with strongly reinforced training, memory disappears 30 min after training. Figure obtained from Gibbs, M. E. & Summers, R. J. (2002). Prog Neurobiol, 67: 345-391.

1.10 The role of noradrenaline in memory formation

1.10.1 Action of noradrenaline at different adrenoceptors

The possibility that the release of noradrenaline from noradrenergic cells in the locus coeruleus and other brain stem centres promotes memory consolidation has been appreciated for some time. Exogenous administration of noradrenaline immediately after training has been shown to promote a labile memory into a permanent one (Crowe *et al.*, 1990; Gibbs, 1991), however, until recently, it was not known which adrenoceptor (AR) subtype noradrenaline was acting on to cause this effect. Noradrenaline can act at any one of 9 adrenoceptor subtypes (Fig. 1.8), all of which are expressed widely in the brain (Summers *et al.*, 1995; Nicholas *et al.*, 1996). By using the weakly reinforced version of discriminated bead task, Gibbs and Summers (2000) were able to show that noradrenaline, acting at different ARs, can have different effects on memory formation and can either consolidate or inhibit memory depending on the dose. Low doses of noradrenaline (100 pmol) appeared to have an action on β_3-ARs, whereas higher concentrations of noradrenaline (1 nmol) acted on β_2-ARs. At even higher doses (3 to 10 nmol), the action of noradrenaline was inhibitory, and appeared to be mediated by α_1-ARs (Fig. 1.9).

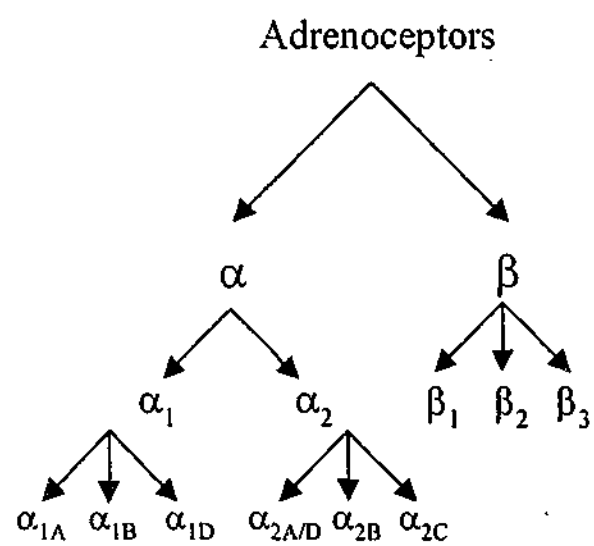


Fig. 1.8. The nine different adrenoceptor subtypes in the brain.

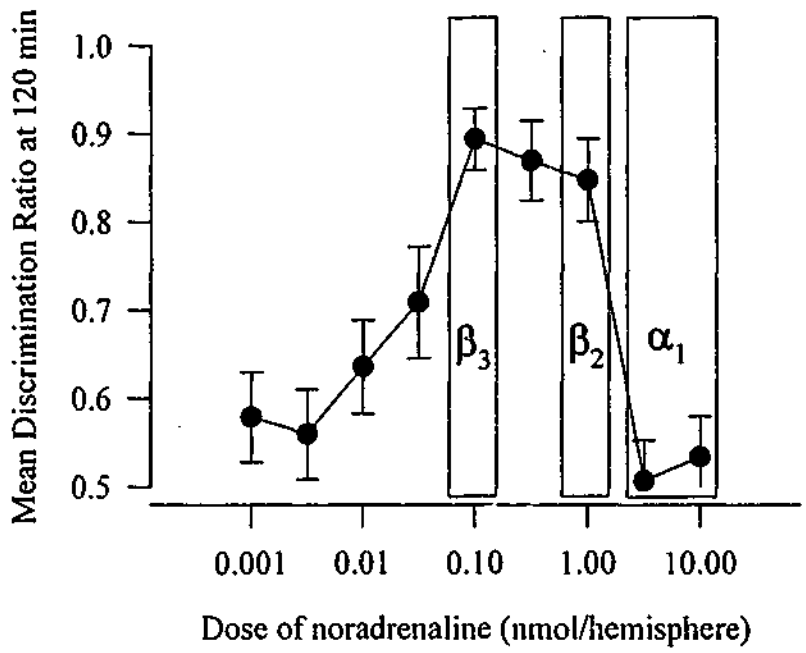


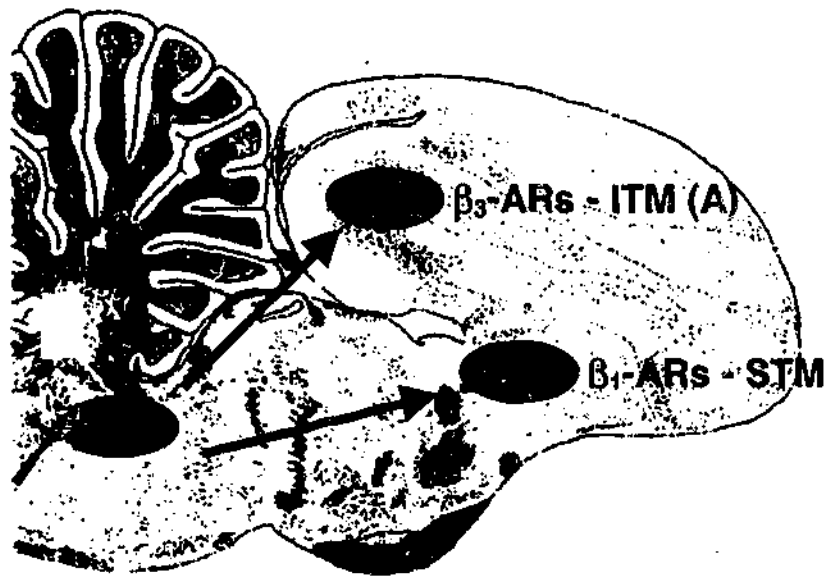
Fig. 1.9. Summary of AR involvement in the enhancement of consolidation of weakly reinforced memory noradrenaline. The promotion of memory consolidation by noradrenaline at low doses is attributable to β_3-ARs, and at intermediate doses to β_2-ARs. Higher doses of noradrenaline inhibit memory consolidation, and this effect appears to be mediated by α_1-ARs. Graph adapted from Gibbs, M. E. & Summers, R. J. (2000). Neuroscience, 95: 913-922.

1.10.2 Hypothesis for the role of noradrenaline in memory consolidation

In the discriminated bead task, consolidation of memory into long-term storage requires the action of noradrenaline on different ARs in at least two different locations in the forebrain: LPO and the IMHV (Gibbs & Summers, 2002b). Whilst noradrenaline is critical for the consolidation of memory, it is also required at the time of learning. The following describes the role of noradrenaline in memory consolidation.

The release of noradrenaline at the time of learning, possibly due to arousal or attentional mechanisms, is necessary for the activation of the β_1-ARs in the LPO, where it is required for the formation of STM (Fig. 1.10). At the ITM (A) phase, β_3-ARs in the IMHV are activated by locus coeruleus induced noradrenaline release. The activation of the β_3-ARs, which appears to be related to the increase in uptake of glucose, stimulates further release of noradrenaline in the LPO, which stimulates the α_2-ARs. The activation of the α_2-ARs in the LPO, which appears to be related to reinforcement, then increases noradrenaline release in the IMHV. β_2-ARs are then activated in the IMHV, the activation of which leads to the consolidation of memory. The role of α_1-ARs in

A



B

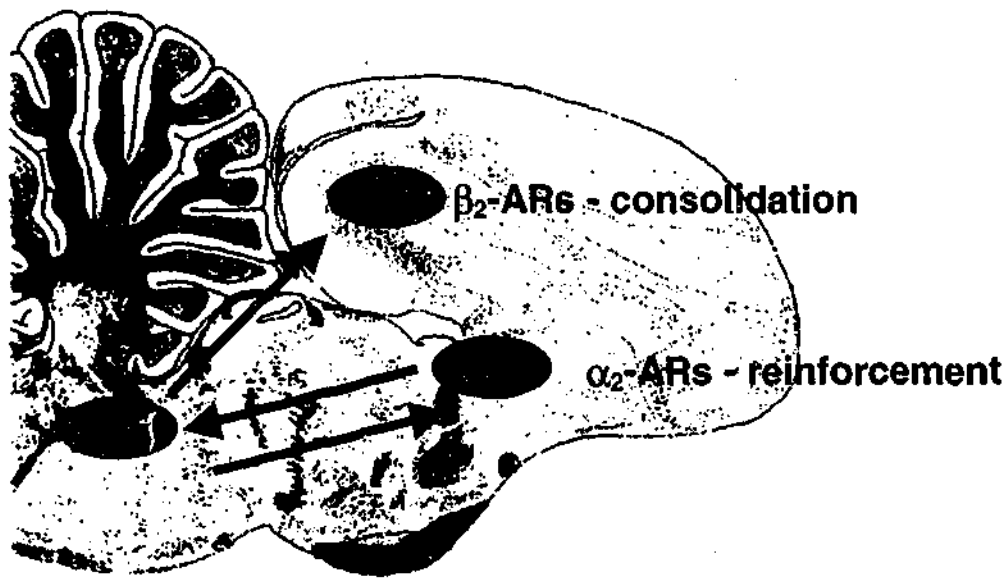


Fig. 1.10. Summary of adrenoceptor (AR) involvement in the enhancement of consolidation of weakly reinforced memory by noradrenaline. (A) *Incoming information from the gustatory and visual systems is responsible for the arousal induced stimulation of the locus coeruleus (LoC) and subsequent noradrenaline release in the lobus parolfactorius (LPO), where the activation of β_1-ARs is required for the formation of short-term memory (STM). At the intermediate memory A (ITM A) stage, β_3-ARs in the intermediate hyperstriatum ventrale (IMHV) are activated by LoC induced noradrenaline release.* (B) *It is not known whether the IMHV and LPO feed back to the LoC directly or via another neurotransmitter system, but there is further noradrenaline release in the LPO, which activates α_2-ARs; the activation of which appears to be related to reinforcement. The activation of α_2-ARs in the LPO increases noradrenaline release in the IMHV, which activates β_2-ARs and leads to the consolidation of memory. Figure adapted from Gibbs, M. E. & Summers, R. J. (2002). Prog Neurobiol, 67: 345-391.*

memory consolidation may be linked to stress, as when levels of noradrenaline are too high, α_1-ARs are activated to inhibit memory consolidation (Gibbs & Summers, 2002b).

1.10.3 Distribution of catecholaminergic fibres and cell bodies

The distribution of catecholaminergic cell bodies in the avian brain appears to resemble that seen in mammals. In avian brain, the catecholaminergic cells groups of the retina and brainstem appear to be predominantly dopaminergic, whereas the catecholaminergic groups of the hindbrain are commonly noradrenergic and adrenergic (Kitt & Brauth, 1986; Reiner *et al.*, 1994). The locus coeruleus sends afferent fibres to nearly all the major brain areas associated with memory such as the amygdala, prefrontal cortex, hippocampus and the caudate putamen as well as to mid and hind brain centres in mammals (Foote *et al.*, 1983; Papadopoulos & Parnavelas, 1991) as well as in birds (Kitt & Brauth, 1986; Reiner *et al.*, 1994) (Fig. 1.11). In the rat, the two main dopaminergic cell groups in the midbrain, which include the substantia nigra pars compacta and the adjacent areas of the midbrain tegmentum, send major ascending dopaminergic inputs to discrete regions in the limbic system and the cortex (Fig. 1.12).

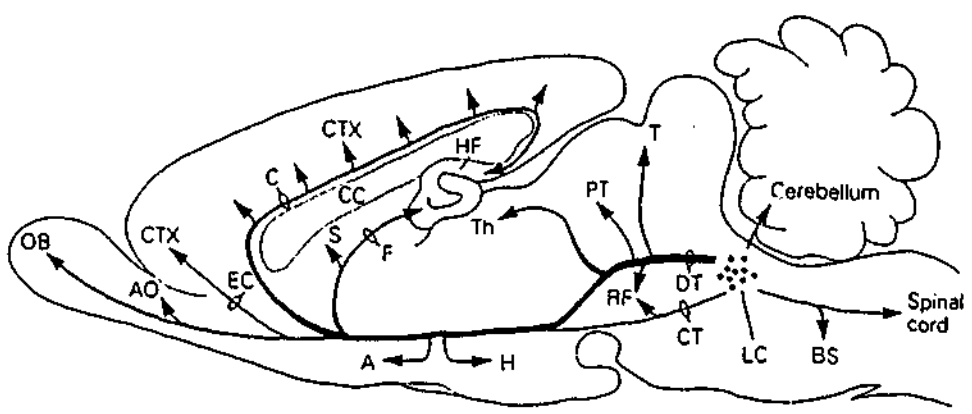


Fig. 1.11. Summary diagram of the noradrenergic projections of the locus coeruleus in the rat. The locus coeruleus provides major ascending output to the thalamus and cerebral cortex, and descending projections to the brain stem, cerebellum and spinal cord. Abbreviations: A: amygdala; AO: anterior olfactory nucleus; BS: brain stem: C: cingulate bundle; CC: corpus callosum; CT: central tegmental tract; CTX: cerebral cortex; DT: dorsal tegmental bundle; EC: external capsule; F: fornix; H: hypothalamus: HF: hippocampal formation; LC: locus coeruleus; OB: olfactory bulb; PT: pretectal nuclei; RT: reticular formation; S: septum; T: tectum; Th: thalamus. Diagram adapted from Kandel, E.R., Schwartz, J. H. and Jessell, T. M. (2000). 'Principles of neural science', 4th Ed., p.891. McGraw-Hill Companies, New York.

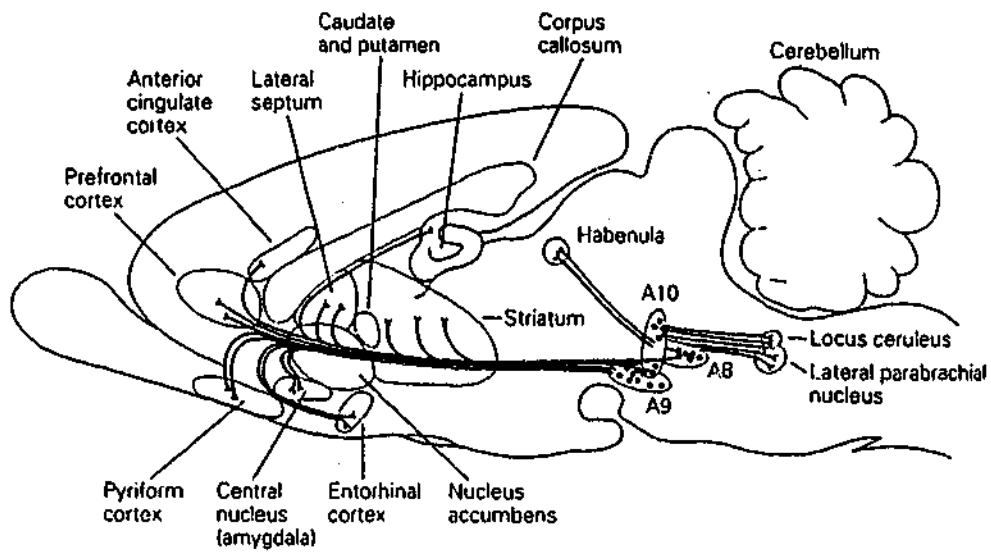


Fig. 1.12. Summary diagram of the dopaminergic cell groups in the brain stem in the rat. The A8-A10 cell groups, which include the substantia nigra pars compacta and the adjacent areas of the midbrain tegmentum, send major ascending dopaminergic inputs to the telencephalon. Abbreviations: A8: retrorubral nucleus; A9: substantia nigra; A10: ventral tegmentum. Diagram adapted from Kandel, E.R., Schwartz, J. H. and Jessell, T. M. (2000). 'Principles of neural science', 4th Ed., p.892. McGraw-Hill Companies, New York.

1.10.4 Distribution of adrenoceptors

Alpha$_1$-, α_2- and β-ARs have been shown to be present in the chick brain (Dermon & Kouvelas, 1988). However, the exact timing of the appearance of these ARs in the embryo brain is not clear. Dermon and Kouvelas (1988) examined the ontogeny of the α_1-, α_2- and β-AR sites in the developing chick brain, and found that that the development of α_2 binding sites preceded that of α_1 and β. Results from this study also suggested the presence of β-ARs in the avian brain from embryonic day 13 (Dermon & Kouvelas, 1988). Conversely, Revilla et al. (1998) found that β_2-ARs were present from embryonic day 17, and the β_1-ARs from embryonic day 18. What is clear, however, is that the β_2 subtype is the predominant AR in the chick brain (Fernandez-Lopez *et al.*, 1997; Revilla *et al.*, 1998), and is more widely distributed than the β_1-ARs, which appear to be limited to the hyperstriatum and cerebellum (Revilla *et al.*, 1998). Alpha$_2$-ARs also appear to be heterogeneously distributed throughout the avian brain (Ball *et al.*, 1989), and it has been suggested that the majority of these ARs are located at the post-synaptic level (Balthazart *et al.*, 1989).

1.11 The role of noradrenaline in brain development

Since neurotransmitters, such as the catecholamines, appear early in the developing mammalian CNS, it has been suggested that they may play a role in neural development (Levitt *et al.*, 1997; Herlenius & Langercrantz, 2001). Kasamatsu and Pettigrew (1976) showed, using electrophysiological methods, that removal of cortical-projecting catecholamine systems by 6-hydroxydopmaine treatment early in development, prevented the ocular dominance shift normally induced by monocular occlusion in kittens (Kasamatsu & Pettigrew, 1976). The findings of Kasamatsu and Pettigrew (1976) suggested a role for catecholamines in the maintenance of cortical plasticity during a critical period of development. Recent studies have shown that noradrenaline plays a role in regulating the development of Cajal-Retzius cells (Naqui *et al.*, 1999), which are the first neurons to be born in the cerebral cortex and have been implicated in neuronal migration and laminar formation. Noradrenaline may also play a role in the differentiation of the cerebral cortex, as depleting the central noradrenergic system around the time of birth has been shown to lead to subtle permanent cortical dendritic changes (Felten *et al.*, 1982; Parnavelas & Blue, 1982). Taken together, the findings from the above studies suggest that the noradrenaline plays a role in neural development. An early insult during a critical period of development could disrupt the timetable of the expression of noradrenaline and its receptors in the CNS and/or affect neuronal plasticity.

1.12 The role of glia in memory

There is compelling evidence for the involvement of glial cells during the formation and consolidation of memory in the chick (O'Dowd *et al.*, 1994a; Gibbs *et al.*, 1996). Before the evidence for the involvement of glial cells in memory is discussed, the role of glial cells in the CNS, and how glutamate and GABA precursors are formed from these cells, will be discussed.

Glial cells are crucial for *de novo* formation of glutamate and GABA precursors from glucose, and for the clearance and metabolism of these transmitters after their release from neurons (Robinson *et al.*, 1998). Glutamate is the most abundant excitatory neuronstransmitter in the CNS, and serves not only as a precursor for the inhibitory neurotransmitter GABA, but performs a number of metabolic roles as well (Fonnum,

1984). After its release from glutamatergic neurons, glutamate is preferentially taken up into adjacent glial cells, particularly astrocytes, where it is partly converted to glutamine via the glial-specific enzyme, glutamine synthetase (Norenburg & Martinez-Hernandez, 1979) (Fig. 1.13). The glutamine produced by the glia is subsequently released into the extracellular fluid, where some is transported back to neurons which convert it to either glutamate or GABA (Ward *et al.*, 1983; Pow & Robinson, 1993). This sequence of events is commonly referred to as the glutamate-glutamine cycle. However, a substantial portion of glutamate that is taken up by the astrocytes is used as a metabolic fuel, thereby producing a net loss of glutamate from the glutamate-glutamine cycle (Hertz *et al.*, 1988). Since glutamate cannot readily cross the blood-brain barrier, *de novo* synthesis of glutamate is produced from glucose through the carboxylation of pyruvate to oxaloacetate in the astrocytes, which can be further oxidatively metabolised by the citric acid cycle intermediate α-ketoglutarate (Yu *et al.*, 1982; Shank *et al.*, 1985).

Evidence suggests that glia play an important role in the establishment of memory in the chick. Administration of methionine sulfoximine, which inhibits the conversion of astrocytically accumulated glutamate to glutamine, has been shown to prevent memory consolidation in chicks trained on the discriminated bead task (Gibbs *et al.*, 1996). Memory loss was observed at the time corresponding to the appearance of IMT (A). The amnesic effect of methionine sulfoximine was successfully ameliorated by the administration of L-glutamine, monosodium glutamate and a combination of α-ketoglutarate and alanine (Gibbs *et al.*, 1996). Taken together, the results from this study suggest that the availability to neurons of astrocytic glutamine is of critical importance to the formation, maintenance and/or expression of IMT (A). Fluroacetate, a gliotoxin which inhibits the astrocytic *de novo* formation of glutamate from glucose, has also been shown to extinguish memory during ITM (A) (O'Dowd *et al.*, 1994a).

Glutamatergic processes may also be involved in the establishment of long-term memory. Administration of idoacetate, a specific inhibitor of glycolysis and therefore of glycogenolysis, has been shown to inhibit ITM (B) formation (O'Dowd *et al.*, 1994b). Results from this study also demonstrated that glycogenolysis occurs in the forebrains of trained chicks between 35 and 55 min after learning-the same period in which ITM (B) occurs (O'Dowd *et al.*, 1994b). Taken together, these findings suggest that the

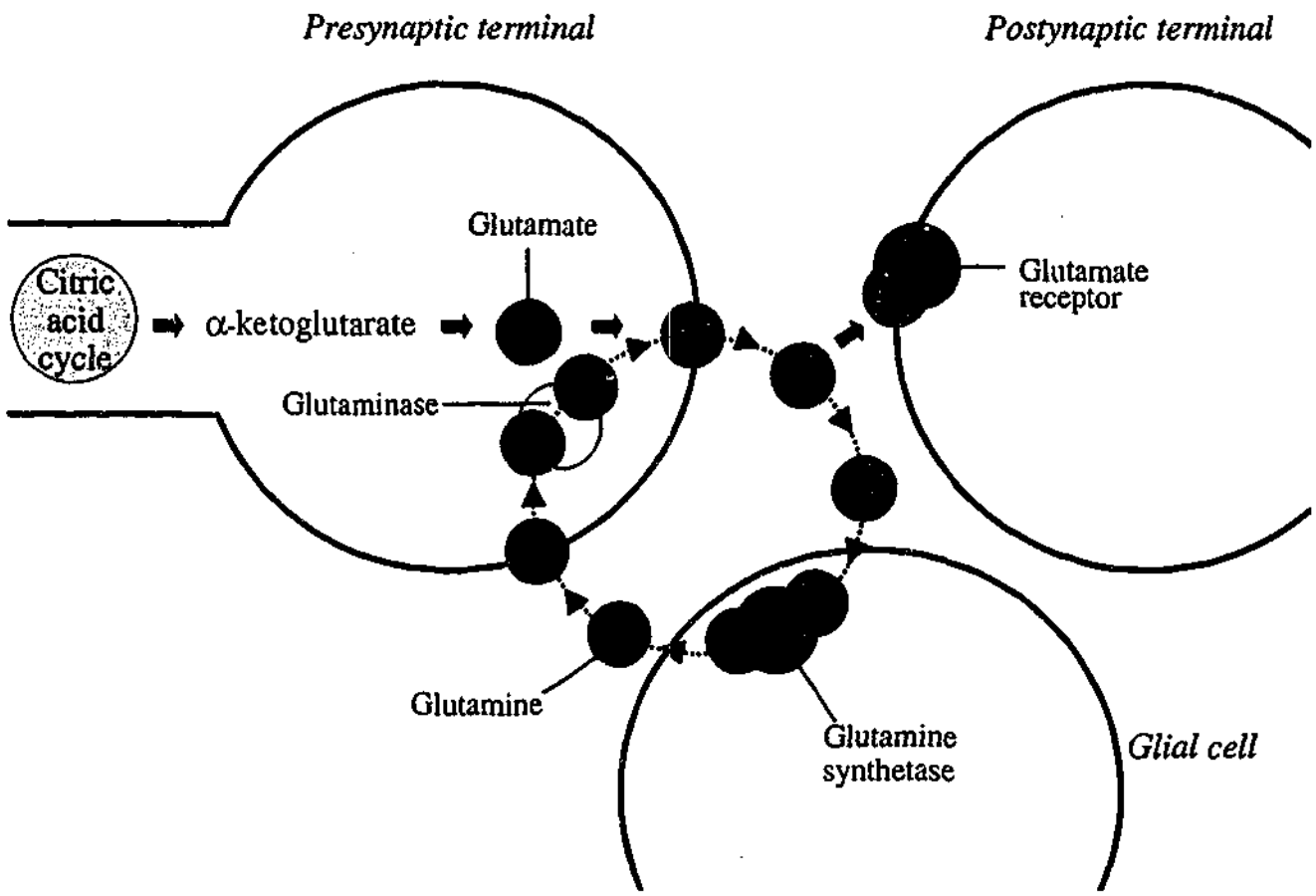


Fig. 1.13. The glutamate-glutamine cycle. Glutamate is synthesized from a product of the citric acid cycle, and is converted to glutamine via glutamine synthetase in glia. The glutamine produced by the glia is subsequently released into the extracellular fluid, where some is transported back to neurons, which convert it to either glutamate or GABA as required. Diagram adapted from Rhoades, R. and Pflanzer, R. (1992). 'Human Physiology', 2nd Edition, p. 274. Saunders College Publishing, Orlando.

energy demands during ITM (B) are met by glycogenolysis, furthermore, since glycogenolysis is virtually restricted to astrocytes, these cells appear to play a critical role in meeting the energy demands for the establishment of ITM (B).

As mentioned in Section 1.8.1, noradrenaline plays a key role in memory consolidation (Gibbs & Summers, 2000), and increases in noradrenaline levels have been found during ITM (B) of the three-stage model of memory (Crowe *et al.*, 1991a). Administration of the β-AR antagonist propranolol leads to extinction of memory during ITM (B) (Crowe *et al.*, 1991b). Given that noradrenaline has been shown to stimulate glycogenolysis (Takagaki *et al.*, 1959), it is likely that a reduction in noradrenaline-induced glycogenolysis is the key factor in producing this memory loss. It appears that β-ARs (Aoki, 1992; Mantyh *et al.*, 1995), and possibly α_1- and α_2-ARs, may be localised on astrocytes. If this is in fact the case, neuronally released noradrenaline following learning may activate astrocytes rather than neuronal receptors.

1.13 Summary of the biological processes involved in memory in the chick

As mentioned in Section 1.5.1, memory formation during each of the three stages of the Gibbs-Ng model of memory is dependent upon different biological processes. The processes that underlie each stage of memory formation, and the agents that can inhibit these stages, are summarised in Fig. 1.14. Short-term memory may be associated with a phase of neuronal hyperpolarisation, arising from changes in potassium (K^+) conductance across neuronal membranes following neural input. Na^+/K^+ ATPase activity appears to play a role in the formation of ITM (A). Phase B of ITM involves the action of noradrenergic neurotransmitters, furthermore, energy demands during ITM (B) appear to be met by glycogenolysis. Protein synthesis seems to be the underlying mechanism for the establishment of permanent memory encoding.

1.14 Other models of memory formation and consolidation

As indicated above, the focus of this thesis is on memory function in the chick. In the section below I will discuss the recent theories of short- and long-term memory storage. It is not my intention to provide a comprehensive overview of the neural mechanisms of memory.

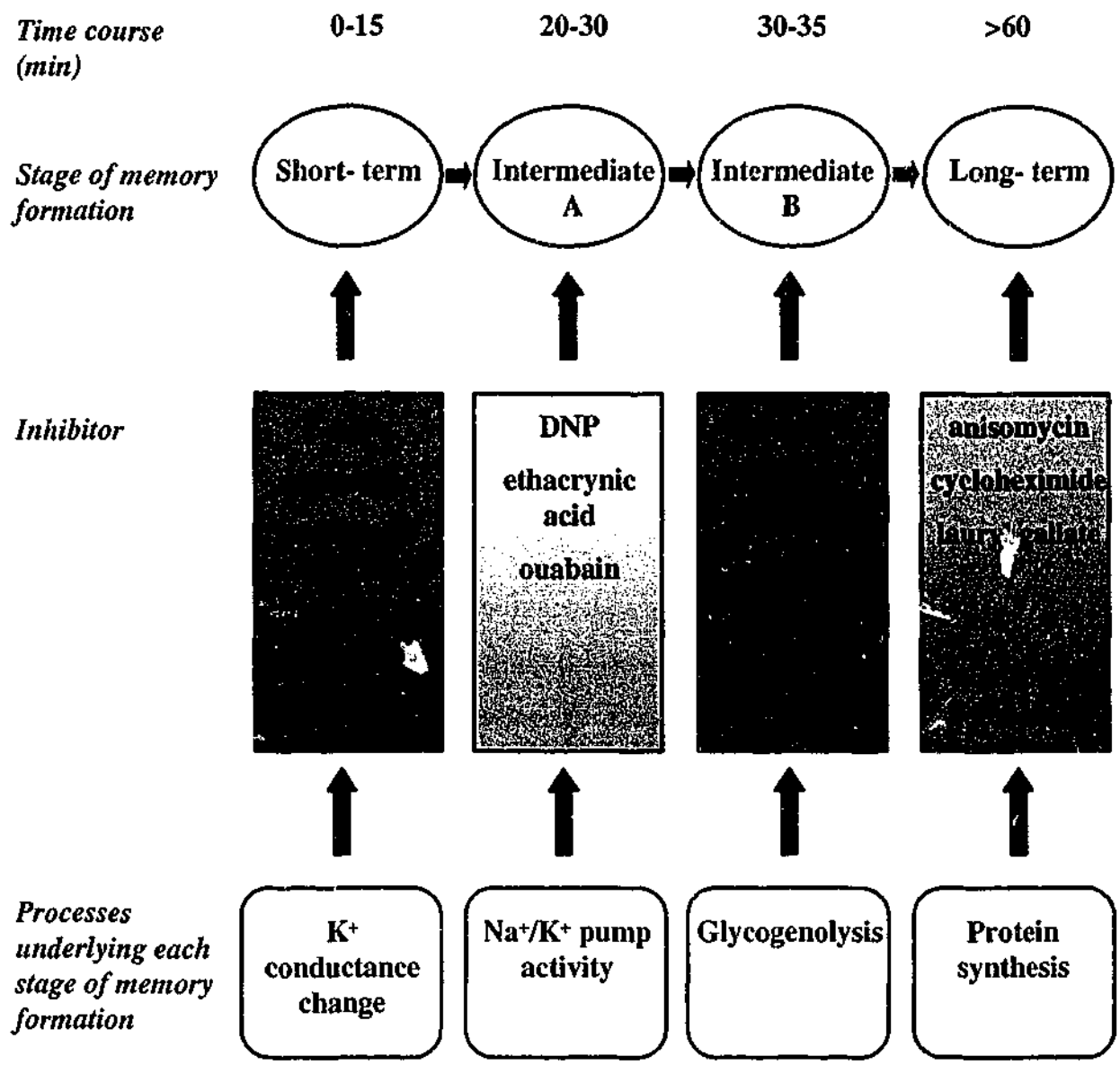


Fig. 1.14. A summary of the processes that underlie each stage of the Gibbs-Ng model of memory, and the agents that can inhibit these stages. There is a stage-specific variability in susceptibility to different inhibitory agents, suggesting that memory formation during each of the stages is dependent upon different biological processes. Abbreviations: DNP: 2,4-dinitrophenol; GABA: γ-aminobutyric acid.

1.14.1 Short-and long-term storage

Information storage in the CNS is widely held to be based upon enduring activity-dependant changes in the number, strength or downstream consequences of synaptic connections between neurons. Studies in *Drosophila* (Lee & O'Dowd, 2000) and *Aplysia* (Fox & Lloyd, 2000) suggest that short-term synaptic plasticity is mediated by a second messenger system such as cyclic adenosine monophasphate (cAMP) (see also reviews Kandel, 2001; Mayford & Kandel, 1999). Increases in cAMP, and subsequent activation of the cAMP-dependant protein kinase (PKA) (Byrne & Kandel, 1996), are thought to be involved in controlling synaptic strength by regulating transmitter release. Whilst short-term synaptic changes involve modification of pre-existing proteins, leading to modifications of pre-existing synaptic connections, long-term synaptic changes involve activation of gene expression, new protein synthesis and the formation of new connections. Long-term memory storage appears to involve a core signalling pathway involving PKA, the mitogen-activated protein kinase (MAPK) and the cAMP response element binding protein–1 (CREB-1) (Sanyal *et al.*, 2002). It is believed that activation of CREB-1 activates immediate response genes (e.g. ApC/EBP and Ap/AF) (Bartsch *et al.*, 2000), which in turn act on down-stream genes to give rise to the growth of new synaptic connections. Long-term synaptic facilitation requires not only activation of memory enhancing genes, but also may involve the inactivation of memory-suppressor genes, such as the transcription factor ApCREB-2 (see review Kandel, 2001).

Not only do neurotransmitters activate second-messenger kinases that are transported to the nucleus where they initiate neuronal growth, but recent work has established that neurotransmitters can "mark" the specific synapses for capture of long-term process and regulate local protein synthesis for stabilisation (Casadio *et al.*, 1999).

1.14.2 Long-term potentiation

In 1973, Bliss and Lomo discovered that a brief tetanic stimulation in the CA1 region mammalian hippocampus produced a long lasting form of synaptic plasticity, referred to as long-term potentiation (LTP) (Bliss & Lomo, 1973). Long-term potentiation is operationally defined as a long-lasting increase in synaptic efficacy between two neurons that follows high-frequency stimulation of afferent fibers. Long-term

potentiation has been a widely used paradigm for long-term synaptic plasticity in a central synapse, although LTP as a cellular model for information storage in the brain is not universally accepted (see Holscher, 1999 and Zamanillo *et al.*, 1999).

Since Bliss and Lomo's original observation, it has been determined that LTP at two of the major synaptic regions in the hippocampus (the dentate gyrus and area CA1) is, in part, dependent on calcium (Ca^{2+}) influx through the N-methyl d-aspartate (NMDA) type of glutamate receptor and channel (Collingridge & Bliss, 1987). Calcium initiates the persistent enhancement of synaptic transmission by activating two Ca^{2+} dependent protein kinases: the Ca^{2+}/ calmodulin kinase and PKC, which are then thought to become persistently active. Whereas the activation the induction of LTP in the CA1 region of the hippocampus appears to depend on postsynaptic depolarization (Ca^{2+} and activation of second messenger systems), the maintenance of synaptic efficacy in LTP requires, in addition, an increase in presynaptic transmitter release.

Changes in dendritic spines, such an enlargements of the spine head and shortenings of the spine neck, have been observed following LTP, suggesting that these morphological changes may also be necessary for the induction or maintenance of LTP (Engert & Bonhoeffer, 1999; see review Yuste & Bonhoeffer, 2001). Recent work has also shown that several protein kinases are critical in the induction and expression of LTP and enhance the efficacy of synaptic transmission. These include the calcium/calmodulin-dependent protein kinase II (CaMKII) (see reviews Fink & Meyer, 2002; Rongo, 2002), the cAMP-dependant protein kinase PKA, PKC (Shobe, 2002), and most recently, the extracellular-signal regulated kinase/mitrogen-activated protein kinase (ERK) (Adams & Sweatt, 2002).

1.15 Relationship between prenatal compromises and postnatal learning and memory deficits

Whilst there is considerable evidence from human populations that there is a link between adverse prenatal development and postnatal neurological deficits, there is no clear understanding as to what kind of neural dysfunction underlies these deficits. It is appreciated that there could be numerous factors that could alter brain function postnatally; the areas of focus/interest in this thesis are addressed below.

1.15.1 Structural alterations in the brain

As described in the preceding sections, prenatal hypoxia in mammals, typically induced by disrupting placental blood supply, can affect basic neurodevelopmental processes, such as neuronal migration, proliferation, and synaptogenesis (Bisignano & Rees, 1988; Rees *et al.*, 1988; Nitsos & Rees, 1990). Delayed or altered development of any one of these processes could therefore potentially affect neural connectivity and subsequently impair postnatal brain function.

Alterations in cognitive function as a consequence of prenatal compromise may not be necessarily due to overt pathologies within specific brain regions. Prenatal insults may lead to disproportionate cell loss or abnormal ratios of glia/neurons, which may produce alterations between interacting neuronal systems that are transmitter specific, or result in the failure of neurons to properly migrate and assemble into functional units, particularly if there is an effect on radial glial cells.

The relationship between spine pathology and cognitive deficits has gained a lot of interest in recent years. As principal sites of synaptic input, spines play a key role in connectivity in the brain. Alterations in spine morphology resulting from hypoxia, for example, could have a significant impact on mental function. Mental retardation has been consistently associated with abnormalities in dendrite structure, and in the shape and density of dendritic spines (Huttenlocher, 1970, 1991; Kaufmann & Moser, 2000). Subtle changes in spine structure or connectivity may also underlie less extreme conditions, such as minimal brain dysfunction disorders and learning disabilities (see Altman, 1986; Morgane *et al.*, 1993).

Prenatal compromises could also have the potential to affect cell function. The disruption of functional and metabolic interactions between neurons and astrocytes has been implicated in several pathological conditions involving behavioural dysfunction (see Hertz *et al.*, 1996). As mentioned in Section 1.10, the inhibition of specific metabolic steps occurring only in glia, can impair memory formation and consolidation in the chick (O'Dowd *et al.*, 1994a; O'Dowd *et al.*, 1994b; Gibbs *et al.*, 1996).

1.15.2 Catecholamine levels and the functioning of the central catecholaminergic system

Prenatal or neonatal hypoxia is known to alter the level of catecholamines in plasma (Cohen *et al.*, 1982; Kitanaka *et al.*, 1989; Hooper *et al.*, 1990; Mulder *et al.*, 2000) and the brain (Chanez *et al.*, 1981; Seidler & Slotkin, 1990; Jensen *et al.*, 1996). There is now considerable evidence that suggests that the level of monoamines are reduced in the brains of patients suffering neurodevelopmental disorders, such as Rett Syndrome, autism and Down Syndrome (Okado *et al.*, 2001). Evidence also suggests that monoamines are reduced in Alzheimer's disease (Hertz, 1989; Dringenberg, 2000). Neurotransmitter disturbances, including noradrenaline imbalance, have also been reported in suffers of schizophrenia, depression, and ADHD (Russell *et al.*, 2000; Bradshaw, 2001).

Since noradrenaline is thought to play a neurotrophic role during brain development (Kasamatsu & Pettigrew, 1976; Berger-Sweeney & Hohmann, 1997; Levitt *et al.*, 1997; Herlenius & Langercrantz, 2001), alterations in the level of catecholamine before birth could have the potential to alter, for example, neuronal differentiation and the formation and maintenance of synapses. It has also been suggested that an early insult or stress, like hypoxia, could affect the timetable of the expression of neurotransmitters and their receptors (Herlenius & Langercrantz, 2001). The noradrenergic system of the CNS appears to be critically linked to cognitive function in the chick (Gibbs & Summers, 2002a), thus alterations in the functioning of the central catecholamine system and/or neuronal plasticity, could potentially impair memory function.

1.16 Aims

The studies comprising this thesis examine the effects of restricting gas exchange *in ovo* on memory function, the central catecholaminergic system and brain structure. The issue of whether it is the timing, duration or severity of the prenatal insult that is important in determining the extent of postnatal neurological impairment will also be addressed. The overall aim of these studies is to not only advance our knowledge of the consequences of adverse prenatal conditions on cognitive function, but to enhance our understanding of what prenatal factors may contribute to neurological deficits after birth.

Chapter 2

Given the paucity of data in relation to the effects of prenatal hypoxia on cognitive function after birth, in particular memory function, Chapter 2 of this thesis will examine the effects of prolonged restrictions in gas exchange on memory function. The **first** aim of this chapter is to determine the effects of restricting prenatal gas exchange, on memory function after 4 and 8 days of hypoxia *in ovo*, commencing at days 14 and 10 of incubation, respectively. Memory function will also be examined in a group of chicks after 4 days of hypoxia, commencing at day 10 of incubation. This group will be studied in order to address the issue of whether the gestational timing, rather than the duration of the prenatal insult, is important in determining the extent of postnatal neurological impairment. The **second** aim of this chapter is to examine the effects of restricting prenatal gas exchange on somatic and organ growth soon after hatching, and at 8 days after hatching.

Chapter 3

Prenatal or neonatal hypoxia is known to alter the level of catecholamines in plasma (Cohen *et al.*, 1982; Kitanaka *et al.*, 1989; Hooper *et al.*, 1990; Mulder *et al.*, 2000) and the brain (Chanez *et al.*, 1981; Seidler & Slotkin, 1990; Jensen *et al.*, 1996). It has been suggested that an early insult or stress (e.g. hypoxia) during critical periods of development could affect neuronal plasticity, as well as the programming of catecholaminergic neurotransmitter and receptor expression (Herlenius & Langercrantz, 2001; Okado *et al.*, 2001; Weinstock, 2001). Since the catecholaminergic systems of the CNS appear to be critically linked to cognitive function in the chick (Gibbs & Summers, 2002a), alterations in catecholamine levels for a prolonged period could lead to alterations in receptor-mediated responses or receptor expression, and consequently impair memory formation and consolidation. Therefore, the aim of Chapter 3 is to examine whether restricting prenatal gas exchange alters noradrenaline and adrenaline concentrations in the blood at 2 and 8 days after hatching, and in brain tissue at 2 days after hatching.

Chapter 4

If circulating catecholamine levels are increased *in* ovo, they will likely enter the brain tissue as the blood brain barrier in the chick is not developed until after hatching (Stewart & Wiley, 1981; Liebner *et al.*, 1997). Increased adrenaline levels could cause

the ARs in the brain to become desensitized or down-regulated, which could have implications for postnatal memory function. Centrally administered noradrenaline acts via β_2- and β_3-ARs in the IMHV of the chick brain to promote memory consolidation (Gibbs & Summers, 2000). It is not known whether adrenaline acts in a similar way to noradrenaline. The aim of Part 1 of this chapter is to determine whether exogenous adrenaline promotes memory consolidation, and the contribution of the different AR subtypes in the action of adrenaline on memory consolidation.

Part 2 of Chapter 4 addresses the issue of whether increased circulating catecholamine levels in response to a prenatal insult cause an alteration in the state of β-ARs. Desensitization of ARs in response to excess stimulation by catecholamines has been described in some detail for the β_2-AR (Bouvier *et al.*, 1989; Carpene *et al.*, 1993; Rang *et al.*, 1995); β_1-ARs can also become desensitized to some degree (Carpene *et al.*, 1993), but β_3-ARs show very little, if any, desensitisation (Carpene *et al.*, 1993; Nantel *et al.*, 1993). The aim of Part 2 of Chapter 4 is to determine whether memory consolidation can be promoted in prenatally hypoxic chicks using β_2- and β_3-AR agonists. If β_2-ARs have become desensitized or down-regulated as a consequences of prolonged exposure *in ovo*, exogenous administration of β_2-AR agonists would be ineffective in promoting memory consolidation in prenatally hypoxic chicks, whereas β_3-AR agonists would be able to consolidate labile memory seen in prenatally compromised chicks.

Chapter 5

Chapter 5 examines the effect of restricting prenatal gas exchange on the structural and neurochemical development of the brain. Examination of the brains of control and prenatally compromised chicks was performed at 2 days after hatching.

The **first** aim of Chapter 5 is to assess the brains of control and prenatally compromised chicks for any gross morphological changes that could be associated with impaired postnatal memory. As changes in the cerebral vasculature have been reported following prenatal hypoxia, the percentage of the brain parenchyma occupied by blood vessels will also examined.

Given the role of astrocytes in memory function in the chick, the **second** aim of Chapter 5 is to assess the immunoreactivity of glutamine synthetase, the astrocyte-specific enzyme, in the IMHV of control and prenatally compromised chicks, to demonstrate an effect of the prenatal insult on glial cell numbers. Since the normal development of glia in the chick embryo brain has not been investigated, the ontogeny of glia in the IMHV and LPO was examined at embryonic days 8, 10, 14, 16, 18.

The level of catecholamines in various brain regions will be examined in Chapter 3 using HPLC. Whilst HPLC analysis may indicate changes in blood or tissue catecholamine concentrations, immunohistochemistry is required to localise specific regions within the various brain regions that may have been affected following the prenatal insult. Therefore, the **third** aim of Chapter 5 was to assess the immunoreactivity for tyrosine hydroxylase (TH), the rate-limiting enzyme involved in the catecholamine synthesizing pathway, in the areas of the chick brain involved in memory function.

Chapter 2

The effect of restricting prenatal gas exchange on memory consolidation and growth in the chick

PRENATAL COMPROMISES THAT reduce O_2 and nutrient supply to the human fetus are thought to play a causal role in postnatal neurological impairments such as minimal brain dysfunction disorders (see Altman, 1986), learning disabilities (Marlow *et al.*, 1993), and intellectual deficits (Gottfried, 1973). It has been established through experimental studies that a period of reduced O_2 and nutrient supply *in utero*, typically induced by restricting placental blood supply to the fetus, can affect the development of specific brain regions, including the cerebral cortex (Bisignano & Rees, 1988), hippocampus (Rees *et al.*, 1988) and cerebellum (Bisignano & Rees, 1988; Rees *et al.*, 1988; Nitsos & Rees, 1990; Mallard *et al.*, 1998). Little is known, however, about how specific insults during gestation can affect cognitive function after birth, and whether it is the timing, duration or severity of the hypoxic insult that is important in determining the extent of postnatal neurological impairment.

The experiments reported in this chapter were undertaken to investigate the hypothesis that restricted gas exchange, in the presence of normal nutrient supply, would impair postnatal neurological function and body growth. Given the paucity of data in relation to the effects of prenatal insults on memory function after birth, the effect of restricted gas exchange on memory consolidation was examined in chicks after 4 and 8 days of hypoxia *in ovo*, commencing at days 14 and 10 of incubation, respectively. Memory consolidation was assessed following hatching using two tasks: a one-trial discriminated bead task (1-2 days after hatching) and a discriminated aversive wheat task (5-6 days after hatching) (Gibbs & Ng, 1977b). Two different tasks were performed on separate days in order to determine whether impairments detected soon after hatching persisted into postnatal life. Memory consolidation was also examined in a group of chicks after 4 days of hypoxia, commencing at day 10 of incubation. This group was studied in order to address the issue of whether the gestational timing, rather that the duration of the prenatal insult, is important in determining the extent of postnatal neurological impairment.

2.1 Materials and Methods

2.1.1 Incubation and hatching

Fertile eggs (White Leghorn-Black Australorp, obtained from Research Poultry Farm Pty. Ltd.) were weighed prior to incubation, and weight matched between the control and experimental groups. In order to restrict gas exchange across the eggshell, approximately one half of each egg was wrapped lengthways with a polyvinyl film (22 μm thick) for 4 or 8 days, commencing at day 14 and day 10 of incubation, respectively. In both groups, the film was removed at day 18 of incubation, as internal pipping, or rupture of the air cell occurs at this age. Chicks from eggs wrapped from days 14-18 are referred to as W14-18 chicks, and chicks from eggs wrapped from days 10-18 are referred to as W10-18 chicks. A third group of eggs were half-wrapped from days 10 to 14 of incubation (W10-14 chicks). Controls eggs were not covered, but were incubated simultaneously with wrapped eggs at 37.6°C, 55-60% humidity, and rotated about their long axis three times a day.

Chicks were allowed to hatch in the incubator, after which they were housed in groups of approximately 10 in communal brooders until postnatal testing commenced. Chicks

were tagged with numbered leg rings and weighed daily following hatching to monitor growth. W10-14 chicks were humanely killed following testing at 1-2 days posthatching using Nembutal (Pentobarbitone Sodium, 60 mg/ml: 0.5ml i.p.); only the behavioural data from this group are presented in this chapter.

At 2 days after hatching, a group of control, W14-18 and W10-18 chicks were deeply anesthetised using Nembutal (Pentobarbitone Sodium, 60 mg/ml: 0.2ml i.p.). Brains were perfused intracardially via the left ventricle of the heart (see Section 5.1.2). Once fixed, the brain, liver and heart were removed and weighed. The remaining chicks were kept until 8 days of age to test their performance in the discriminated wheat task at 5-6 days and to monitor their growth and development. At 8 days after hatching, the same protocol was used to fix and collect brain, liver and heart tissue.

2.1.2 Assessment of the degree of prenatal hypoxia

In order to confirm the presence of hypoxia in embryos with wrapped eggs, hematocrit was measured in a separate group of eggs at day 18 of incubation following 4 and 8 days of wrapping, commencing at days 14 and 10 of incubation, respectively. Embryos were quickly removed from their shells and decapitated; blood was collected in heparinised microhematocrit tubes. The samples were spun in a microhematocrit centrifuge at 11,800 rpm for 5 min and analysed with a microhematocrit reader.

In conjunction with the hematocrit measurements, the partial pressures of O_2 (PO_2) and CO_2 (PCO_2) in the air cell of wrapped and unwrapped eggs were also assessed in another group of eggs. Gas tensions in the air cell are nearly identical with those in the air spaces of the shell membranes (Rahn *et al.*, 1979), thereby providing a means of assessing the effects of wrapping on gas status of the embryo. Gas tensions in the air cell were measured at day 16 of incubation in eggs wrapped from day 10 and controls, and at day 17 for eggs wrapped from day 14. A small hole was drilled into the air cell prior to sampling in order to fill the cell with approximately 3 mls of saline. The hole was then sealed and the egg returned to the incubator for approximately 3 hours in order for the saline to equilibrate with the gases in the air cell; after which 0.2-0.4 mls of the saline was removed for PO_2 and PCO_2 analysis (Radiometer ABL 520).

2.1.3 Memory testing

2.1.3.1 Discriminated bead task (1-2 days after hatching)

In the one-trial discriminated bead task, chicks were trained to peck at a red bead dipped in a chemical aversant. At various times after training, chicks were required to discriminate between a red and a blue bead. If chicks remembered that red beads taste bitter, they would refuse to peck at that bead on subsequent presentations, but would continue to peck at blue beads (Gibbs & Summers, 2000).

Chicks were kept in pairs throughout testing. In order to familiarize the chicks with the presentation of beads into their experimental environment and to encourage them to peck, each pair of chicks was presented twice with a small metal bead (2 mm diameter) dipped in water. Chicks were presented with a red glass bead (4 mm diameter), followed by a blue glass bead of the same size, for 10 sec; both beads had been dipped in water. For the training trial, chicks were presented for 10 sec with an identical red bead that had been dipped with the chemical aversant, methyl anthranilate (MeA), instead of water. Each trial was separated by 30 min. Data from chicks that failed to peck at the bitter-tasting red bead on the training trial were excluded from the analysis at the completion of the experiment. On subsequent tests of memory retention, chicks were presented with a dry red bead and blue bead successively, each for 10 sec. Data from chicks that failed to peck at the blue bead were also excluded from the final analysis, because they were unable to discriminate. Nevertheless, only a maximum of 4 chicks were excluded per group for avoiding the blue bead. The number of pecks and the latency to first peck were recorded for each chick using a hand-held data recorder and later downloaded onto a computer for decoding. Separate groups of 10-15 chicks were tested at 10, 30, 60 or 120 min after training. Separate groups of chicks were required for each time-point as it was not possible to test the animals more than once; re-testing, particularly soon after training, can alter the memory or even restart memory processing (Gibbs, 1991). The times chosen to assess memory retention correspond to distinct stages of memory formation in the chick; short-term memory (10 min), intermediate memory (30 min) and long-term memory (60 min) (Gibbs & Ng, 1977b; Gibbs, 1991). Memory retention was assessed by using a discrimination ratio (DR), defined as the ratio of the number of pecks at the blue bead (non-aversive) to the total number of pecks at the blue and red (aversive) beads on test. If chicks only pecked at the blue bead, but

avoided the red bead (DR=1.0), it was concluded that they had formed a lasting memory of the training experience. However, if they pecked equally at both the red and blue beads (DR=0.5), it was concluded that there was no permanent memory of the training experience. The proportion of chicks avoiding the red bead, over the total number of chicks at each time-point was also assessed for each group of chicks.

2.1.3.2 Discriminated wheat task (5-6 days after hatching)

In the discriminated wheat task, chicks were trained to peck at crushed wheat that had been coloured red and soaked in quinine and mustard. On test, chicks were required to discriminate between red and yellow crushed wheat. If chicks remembered that red wheat tastes bitter, they would refuse to peck at that wheat on subsequent presentations (Gibbs & Ng, 1977b).

On the day before the experiment, chicks were familiarized with the experimental boxes in groups of four. The chicks were deprived of food overnight, but water was freely available. On the morning of the experiment, pairs of chicks were placed for 2 min in the test box containing plain crushed wheat that had been scatted on the floor. After a second pre-training trial, each pair of chicks was placed for 30 sec in a test box which had dry yellow-coloured wheat on the floor, then placed in a test box containing red-coloured wheat which had been soaked in water. This preparation mimicked the colour and texture of the bitter tasting red wheat used for the training trial. Each pre-training experience was separated by an interval of 30 min.

For the training trial, bitter tasting red-coloured wheat that had been soaked in a quinine and mustard mixture (0.25% quinine in 0.1M hydrochloric acid and 20% by volume of mustard powder) was scattered on the floor. Each pair of chicks was placed in the test box for 30 sec. After 5 pairs of chicks had been trained a fresh portion of the aversive red wheat was scattered on a clean floor, in order to maintain the potency, texture and correct distribution of the wheat. Chicks that failed to peck during training were excluded from the analysis at the completion of the experiment.

Retention trials were conducted in separate groups of 10-15 chicks at 30 and 120 min after training using two test boxes. One test box contained red wheat soaked in water. The other test box contained dry yellow coloured wheat. Chicks were placed in the first

box containing red wheat for 20 sec, followed by 20 sec in the second box containing yellow wheat. Chicks were then placed back in the first box containing red wheat for a further 20 sec in order to determine if there was any effect of placing the chick in the red test box first. As with the training trial, fresh wheat was scattered on a clean floor after 5 pairs of chicks had been tested.

For each trial, the number of pecks and the latency to first peck were recorded for each chick. A DR score was calculated for each chick, which was the proportion of pecks at the yellow wheat to the total number of pecks at the yellow and red wheat.

2.1.4 Statistical Analysis

All data are presented as mean ± SEM. Hematocrit and gas status in the air cell were analysed using a one-way analysis of variance (ANOVA). Post hoc analysis was performed using Dunnett's test. Discrimination ratio data from the bead task were analysed using a two-way ANOVA with unweighted means. Simple main effects were used for post hoc analysis. The percentage of chicks avoiding the red bead on test was assessed using χ^2 analysis. All other data were analysed using an unpaired t-test. If data were found to be heterogeneous, square root or log transformations were performed and the most homogenous transformation was used for further analysis. Statistical significance was accepted at the level of $P<0.05$. Note that separate groups of chicks were required for each time point for the discriminated bead and aversive wheat tasks. Furthermore, not all of the chicks represented in the body weight graphs were included in the behavioural data.

2.2 Results

2.2.1 Assessment of the degree of prenatal hypoxia

Analysis of variance (one-way) revealed that there was a significant effect of treatment on hematocrit levels ($F_{2,28}=5.567$, $P=0.009$). Embryos from eggs wrapped from day 14 had increased hematocrit compared to controls ($P=0.045$, Fig. 2.1). This was also the case for the embryos from eggs wrapped from day 10 ($P=0.006$).

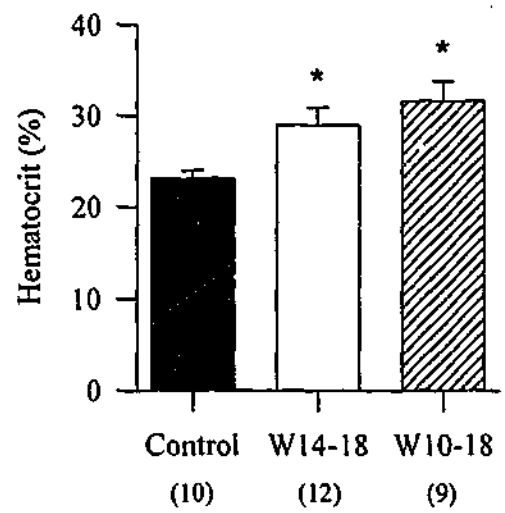


Fig. 2.1. Hematocrit at day 18 of incubation in control embryos, embryos from eggs wrapped from day 14, and embryos from eggs wrapped from day 10. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.

Analysis of variance (one-way) revealed that there was a significant effect of treatment on the partial pressures of O_2 ($F_{2,15}$=12.744, P=0.001) and CO_2 ($F_{2,14}$=7.352, P=0.007) in the air cell. PO_2 was decreased in the eggs wrapped from day 14 compared to controls (P=0.047; Fig. 2.2A), whereas PCO_2 tended to be increased (P=0.086, Fig. 2.2B). PO_2 was also decreased in the eggs wrapped from day 10 (P=0.004) whereas CO_2 was significantly increased (P<0.001).

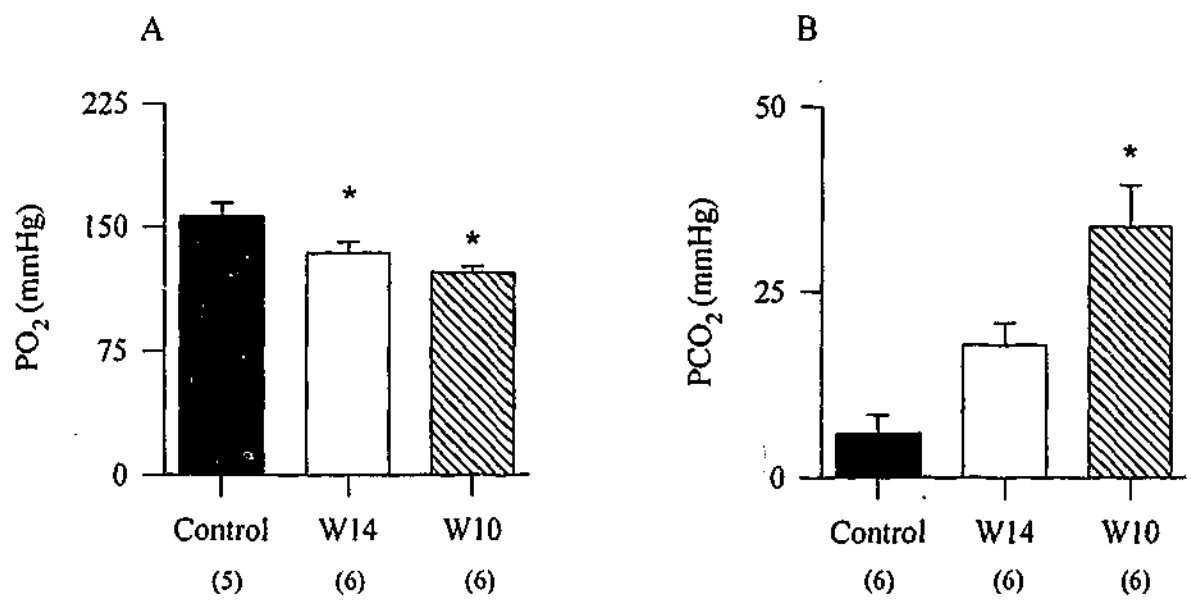


Fig. 2.2. Partial pressures of O_2 (A) and CO_2 (B) in the air cell of control eggs and eggs wrapped from day 10 and 14, respectively. PO_2 was significantly decreased in both groups of wrapped eggs, whereas PCO_2 was increased in the eggs wrapped from day 10. Asterisks (*) indicate values that are significantly different from control values; # indicates values that are significantly different between experimental groups. Animal numbers are in parentheses.

2.2.2 Prenatal growth

2.2.2.1 W14-18 chicks

The average lengths of incubation for control and W14-18 chicks were 22.0±0.0 days and 22.1±0.0 days, respectively (P=0.16). The hatching rates of control chicks and W14-18 chicks were 85±5% and 73±7%, respectively (P=0.3). Hatching weight, which was used as an indicator of prenatal growth, was not different between controls and the W14-18 chicks (P=0.65, Fig. 2.3A). The ratio of hatch-weight: egg-weight also was not altered (Fig. 2.3B). At 2 days post-hatching, brain weights, expressed in relation to body weight, tended to be greater in the W14-18 chicks (P=0.054, Table 2.1), whereas absolute heart weights were decreased in this group.

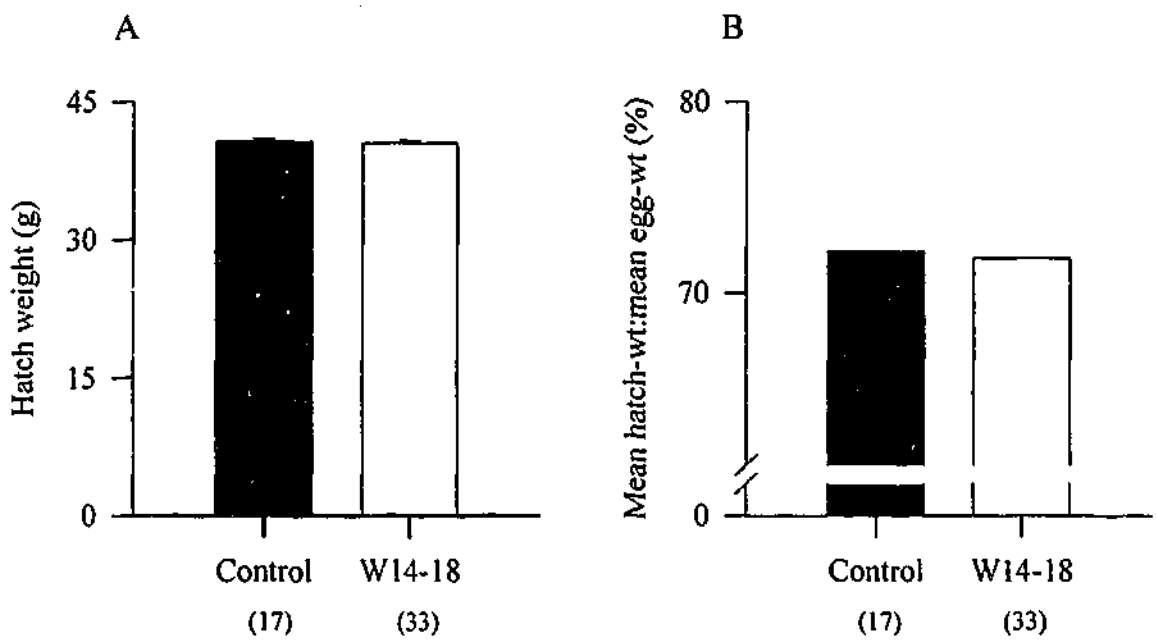


Fig. 2.3. Hatching weights (A) *and the hatch weight: egg weight ratio (%)* (B) *of control and W14-18 chicks. Note: No statistical analysis was performed on the hatch-to-egg weight ratio data as both values were means. Animal numbers are in parentheses.*

Table 2.1. Organ weights of control and W14-18 chicks at 2 days post-hatching. An asterisk (*) indicates value that is significantly different from control value. Animal numbers are in parentheses.

Organ	Control (n=8) mean ±SEM	Wrap days 14-18 (n=10) mean±SEM	P value
Brain (g)	$0.90\pm3.6\times10^{-3}$	0.86±0.02	0.18
Brain/Body wt (g/g)	$0.021\pm7.9\times10^{-5}$	$0.022\pm5.1\times10^{-4}$	0.054
Liver (g)	1.40±0.01	1.36±0.07	0.73
Liver/Body wt (g/g)	$0.03\pm2.5\times10^{-4}$	$0.04\pm1.7\times10^{-3}$	0.18
Heart (g)	$0.39\pm3.0\times10^{-3}$	0.34±0.02*	0.04
Heart/Body wt (g/g)	$9.3\pm6.9\times10^{-5}$	$9.0\pm5.4\times10^{-4}$	0.75

2.2.2.2 W10-18 chicks

The average lengths of incubation for control and W10-18 chicks were 19.1±0.1 days and 19.8±0.1 days, respectively, (P<0.001). The average hatching rate of controls chicks (91±6%) was greater than that of the W10-18 chicks (51±1%, P=0.002). Body weights at hatch were lower in the W10-18 chicks than in controls (Fig. 2.4A), as was the hatch-weight: egg-weight ratio (Fig. 2.4B). Body weights of the W10-18 chicks were also reduced at 1 and 2 days after hatching (P<0.05); absolute brain weights were also reduced in these animals at 2 days (P=0.008, Table 2.2).

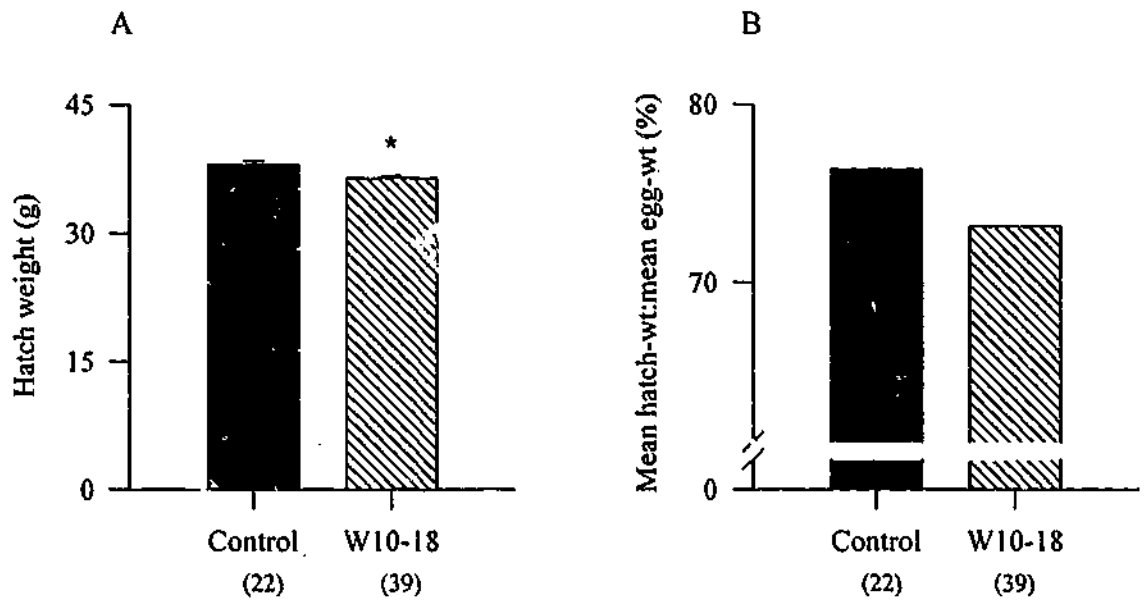


Fig. 2.4. Hatching weights (A) *and the hatch weight: egg weight ratios (%)* (B) *of control and W10-18 chicks. An asterisk* (*) *indicates value that is significantly different from control value. Note: No statistical analysis was performed on the hatch-to-egg weight ratio data as both values were means. This ratio was used as an indicator only. Hence, no asterisk can be placed on the graph to indicate statistical significance. Animal numbers are in parentheses.*

Table 2.2. Organ weights of control and W10-18 chicks at 2 days post-hatching. An asterisk (*) *indicates value that is significantly different from control value. Animal numbers are in parentheses.*

Organ weight	*Control (n=7)* mean±SEM	*Wrap days 10-18 (n=10)* mean±SEM	*P value*
Brain (g)	0.84±0.01	0.77±0.02*	0.008
Brain/Body wt (g/g)	$0.024 \pm 6.2 \times 10^{-4}$	$0.023 \pm 6.8 \times 10^{-4}$	0.52
Liver (g)	1.23±0.03	1.15±0.06	0.29
Liver/Body wt (g/g)	$0.04 \pm 7.2 \times 10^{-4}$	$0.03 \pm 1.7 \times 10^{-3}$	0.78
Heart (g)	0.27±0.02	0.29±0.01	0.45
Heart/Body wt (g/g)	$7.7 \times 10^{-3} \pm 5.3 \times 10^{-4}$	$8.7 \times 10^{-3} \pm 3.4 \times 10^{-4}$	0.12

2.2.3 Postnatal growth

2.2.3.1 W14-18 chicks

The W14-18 chicks had lower body weights compared to controls from 2 to 8 days after hatching (P<0.005), except at day 7 (Fig. 2.5). There was no difference in daily growth rates (g/day) between the two groups (P>0.05), however, absolute brain weights were reduced in the chicks from wrapped eggs (P=0.009, Table 2.3).

2.2.3.2 W10-18 chicks

The body weights of the W10-18 chicks were lower compared to controls throughout the study period (P<0.005; Fig. 2.6). There was no difference in growth rates between the two groups (P>0.05). At 8 days, absolute brain, liver and heart weights were reduced compared to control chicks (P<0.05, Table 2.4). Brain weights, expressed in relation to body weight, were greater in the W10-18 chicks (P=0.002), whereas relative heart weights were reduced (P=0.008).

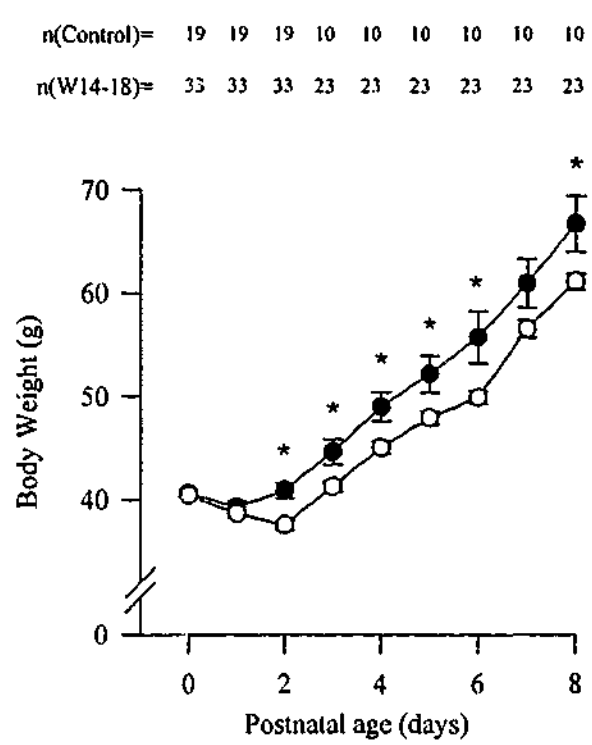


Fig. 2.5. Body weights of control (●) and W14-18 chicks (O) from hatching to 8 days after hatching. Body weights of the W14-18 chicks were reduced from 2 days after hatching, except at day 7. Asterisks (*) indicate values that are significantly different from control values. Animal numbers for each time point are presented above each symbol.

Table 2.3. Organ weights of control and W14-18 chicks at 8 days post-hatching. An asterisk (*) indicate value that is significantly different from control value. Animal numbers are in parentheses.

Organ	Control (n=8) mean±SEM	Wrap days 14-18 (n=11) mean±SEM	P value
Brain (g)	$1.05 \pm 4.8 \times 10^{-3}$	0.96±0.03*	0.009
Brain/Body wt (g/g)	$0.015 \pm 9.7 \times 10^{-5}$	$0.016 \pm 4.8 \times 10^{-4}$	0.7
Liver (g)	2.70±0.02	2.45±0.09	0.16
Liver/Body wt (g/g)	$0.039 \pm 2.0 \times 10^{-4}$	$0.040 \pm 1.3 \times 10^{-3}$	0.47
Heart (g)	0.53±0.01	0.48±0.02	0.16
Heart/Body wt (g/g)	$7.7 \times 10^{-3} \pm 3.9 \times 10^{-5}$	$7.9 \times 10^{-3} \pm 3.6 \times 10^{-4}$	0.60

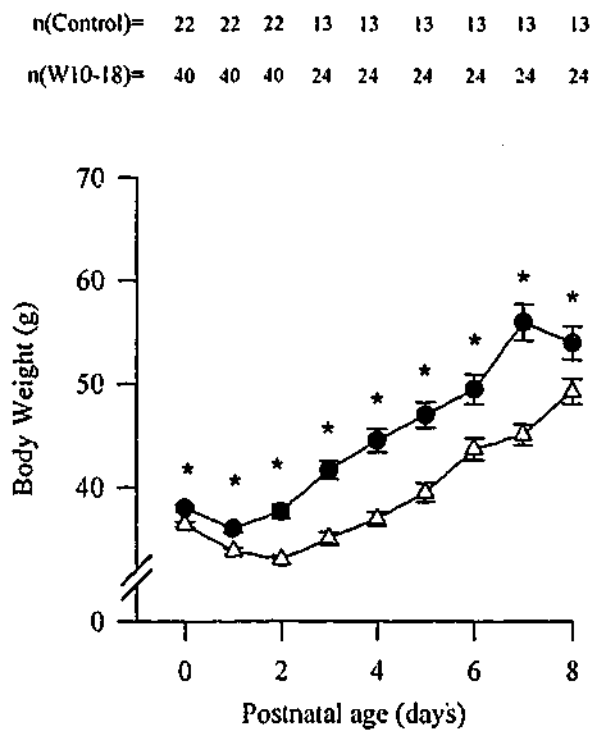


Fig. 2.6. Body weights of control (●) and W10-18 chicks (△) from hatching to 8 days after hatching. Body weights of the W10-18 chicks were reduced from day 0 (hatching) until day 8. Asterisks (*) indicate values that are significantly different from control values. Animal numbers for each time point are presented above each symbol.

Table 2.4. Organ weights of control chicks and W10-18 chicks at 8 days post-hatching. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.

Organ	*Control (n=10)* mean±SEM	*Wrap days 10-18 (n=8)* mean±SEM	*P value*
Brain (g)	1.03±0.02	0.96±0.01*	0.02
Brain/Body wt (g/g)	$0.016 \pm 6.7 \times 10^{-4}$	$0.019 \pm 6.3 \times 10^{-4}$*	0.002
Liver (g)	2.52±0.10	1.96±0.09*	<0.001
Liver/Body wt (g/g)	$0.039 \pm 1.3 \times 10^{-3}$	$0.039 \pm 1.9 \times 10^{-3}$	0.98
Heart (g)	0.60±0.03	0.38±0.03*	<0.001
Heart/Body wt (g/g)	$9.3 \times 10^{-3} \pm 3.9 \times 10^{-4}$	$7.6 \times 10^{-3} \pm 3.8 \times 10^{-4}$*	0.008

2.2.4 Discriminated bead task (1-2 days after hatching)

2.2.4.1 W14-18 chicks

Although the W14-18 chicks learned to discriminate between the red and blue beads and remembered for 30 min, they were unable to consolidate memory ($F_{1,103}$=13.28, P<0.001; Fig. 2.7). Analysis of variance revealed that there was a significant effect of time of test ($F_{3,103}$=5.83, P=0.001), and there was a significant interaction ($F_{3,103}$=3.47, P=0.019). Simple main effects analysis confirmed that retention levels of the W14-18 chicks were not different from those of controls at 10 and 30 min post-training, but were significantly lower at 60 and 120 min post-training (P<0.05).

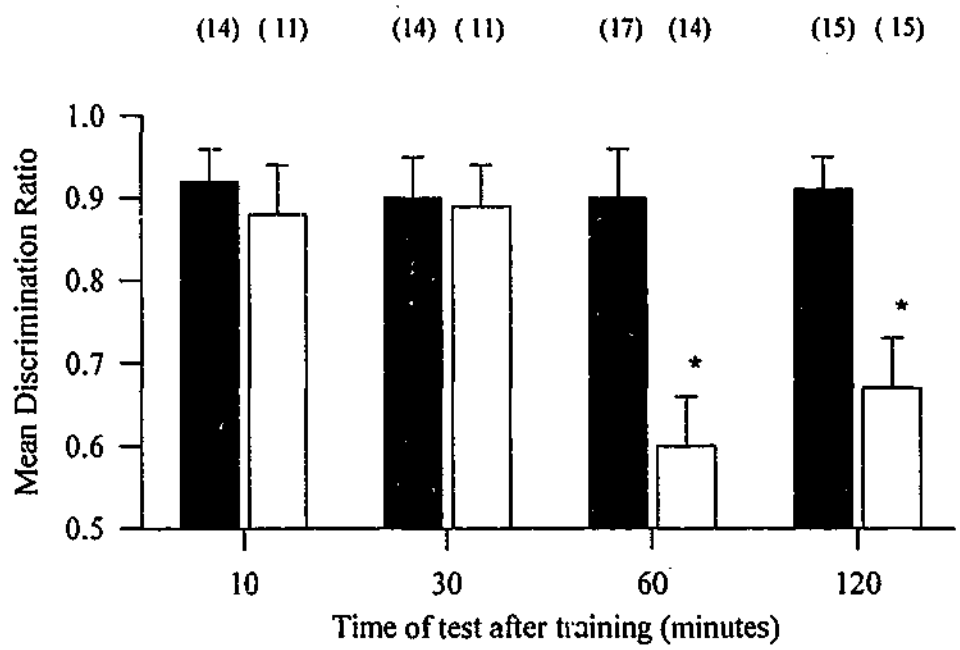


Fig. 2.7. Discriminated bead task. *Mean discrimination ratio of control (■) and W14-18 chicks (□). The W14-18 chicks had reduced retention levels at 60 and 120 min after training. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

At 60 and 120 min after training, the proportion of W14-18 chicks avoiding the red bead whilst continuing to peck at the blue bead had decreased significantly ($\chi^2_{3,\ 61}$=8.05, P<0.045, Fig. 2.8).

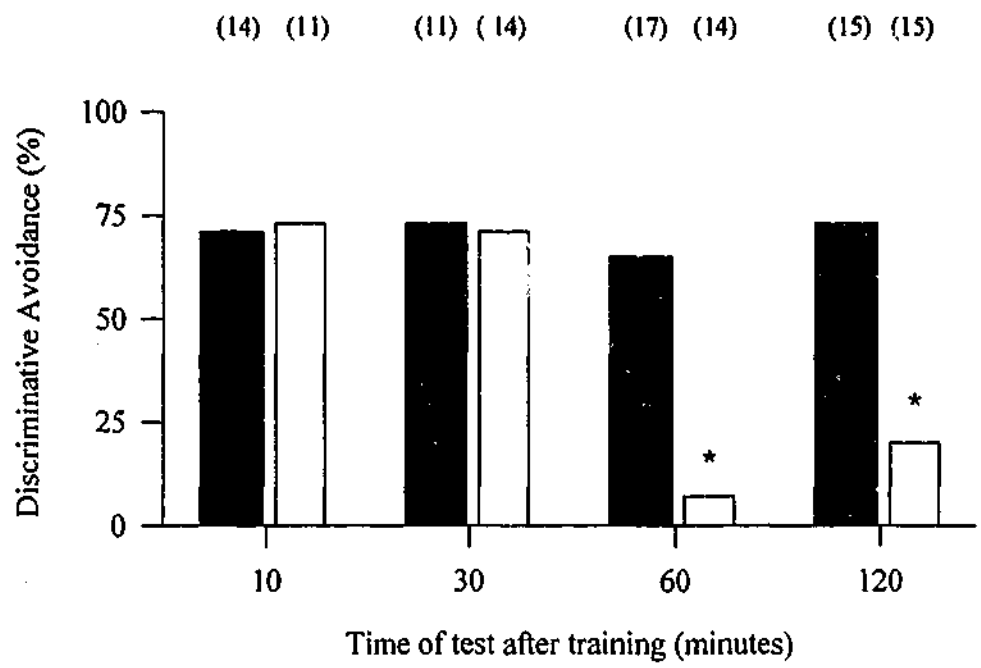


Fig. 2.8. Discriminated bead task. *Percentage discriminated avoidance of control (■) and W14-18 chicks (□). The proportion of W14-18 chicks avoiding the red bead whilst continuing to peck at the blue bead decreased significantly by 60 and 120 min after training. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

It was considered possible that the pecking performance (latency to first peck, number of pecks) of the chicks from wrapped eggs may have been impaired by wrapping. There was no difference in pecking performance between the control and W14-18 chicks on training or at test when pecking at the blue bead; therefore, data were combined. The mean latency to first peck and the number of pecks made on training for both control and W14-18 chicks was 3.3±0.3 sec and 1.7±0.1 pecks/10 sec, respectively. The mean latency to first peck and the number of pecks made at the blue bead on test for both groups of chicks was 3.0±0.2 sec and 2.9±0.2 pecks/10 sec, respectively. As reflected by the DR scores described above, the W14-18 chicks in the 60 min and 120 min groups were quicker to first peck at the red bead on test compared to controls (60 min: $P<0.0001$; 120 min: $P=0.02$, Table 2.5), and pecked more frequently at the red bead (60 min: $P=0.004$; 120 min: $P=0.01$).

2.2.4.2 W10-18 Chicks

Analysis of variance revealed that W10-18 chicks had impaired memory retention at all times of test compared to controls ($F_{1,83}=13.74$, $P<0.001$; Fig. 2.9). There was no

Table 2.5. Discriminated bead task. *Latency to first peck and number of pecks for control and W14-18 chicks. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

		Time of test after training (minutes)							
		10		30		60		120	
Trial	**Group**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**
Test Red Bead	Control	8.1±1.0 (n=14)	0.6±0.3	8.4±1.0 (n=11)	0.3±0.1	7.8±0.8 (n=17)	0.6±0.2	8.3±0.8 (n=15)	0.4±0.2
	W14-18	8.4±1.1 (n=11)	0.5±0.3	8.8±0.7 (n=14)	0.4±0.2	2.6±0.7* (n=14)	2±0*	5.3±0.9* (n=15)	1±0*
Test Blue Bead	Control	3.1±0.6	3±1	2.5±0.5	3±0	2.3±0.4	3±0	3.2±0.7	3±0
	W14-18	4.2±0.9	3±1	2.6±0.5	4±1	3.6±0.6	3±0	2.9±0.4	2±0

significant effect of time of test ($F_{3,83}$=0.54, P=0.66) and there was no interaction ($F_{3,83}$=0.63, P=0.60).

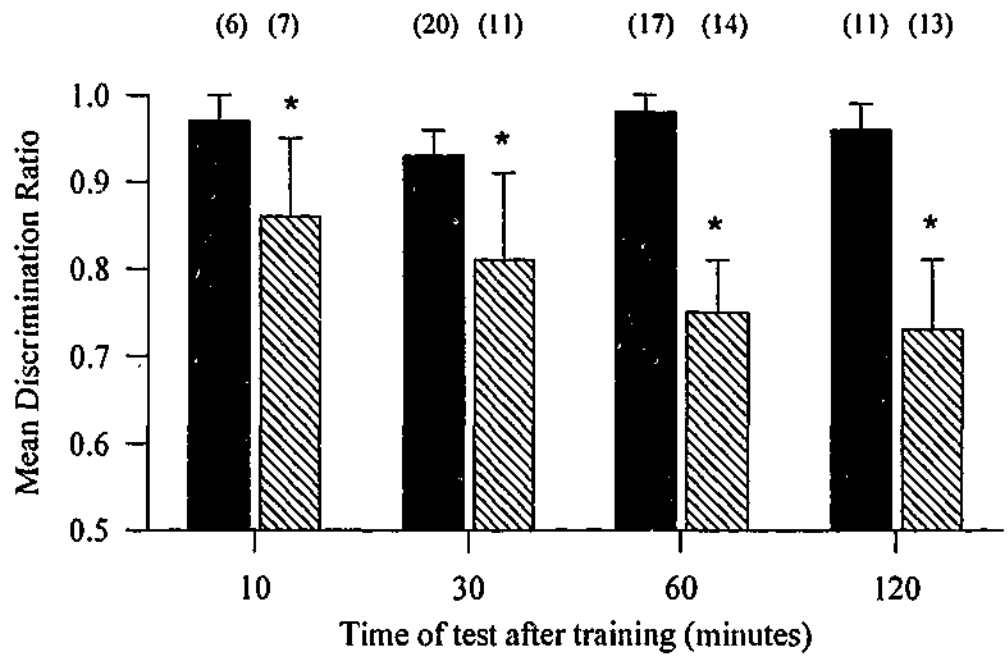


Fig. 2.9. Discriminated bead task. *Mean discrimination ratio of control (■) and W10-18 chicks (▨). The W10-18 chicks had impaired memory retention at all times of test compared to controls. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

The proportion of W10-18 chicks avoiding the red bead tended to be lower than in controls at all times after training, however the difference was not significant ($\chi^2_{3,55}$=3.8, P=0.2, Fig. 2.10).

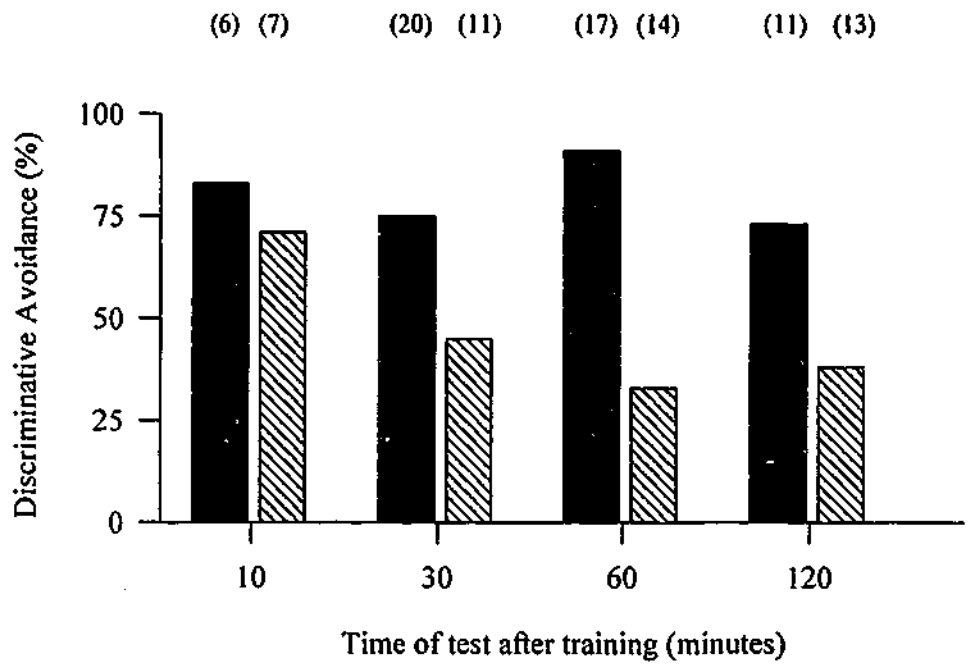


Fig. 2.10. Discriminated bead task. *Percentage discriminated avoidance of control (■) and W10-18 chicks (▨). Although the proportion of W10-18 chicks avoiding the red bead tended to be lower than in controls at all times of test, the proportion of W10-18 chicks avoiding the red bead difference was not different from controls. Animal numbers are in parentheses.*

The W10-18 chicks in the 30 min group took longer to first peck at the blue bead compared to controls during pre-training (5.6±1.2 sec vs. 2.5±0.4 sec, P=0.008), and pecked less frequently (3.0±1.1 vs. 5.9±0.5 pecks/10 sec, P=0.01). As there was no difference in pecking performance between control W10-18 chicks on training or at test when pecking at the blue bead, data were combined. The mean latency to first peck and the number of pecks made on training for both control and W10-18 chicks was 3.0±0.2 sec and 2.6±0.2 pecks/10 sec, respectively. The mean latency to first peck and the number of pecks made at the blue bead on test for both groups of chicks was 2.9±0.2 sec and 3.1±0.3 pecks/10 sec, respectively. As reflected in the DR scores, the W10-18 chicks in the 60 min test group were quicker to first peck at the red bead on test compared to controls (P=0.003, Table 2.6), and pecked more frequently (P=0.007). The controls in the 120 min test group were also quicker to first peck at the red bead on test compared to the W10-18 chicks (P=0.02).

Table 2.6. Discriminated bead task. *Latency to first peck and number of pecks for control and W10-18 chicks wrapped from 10-18. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

		Time of test after training (minutes)							
		10		30		60		120	
Trial	**Group**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**
Test Red Bead	Control	8.6±1.4 (n=6)	0.2±0.2	8.8±0.6 (n=20)	1±0	9.7±0.3 (n=17)	0.1±0.1	2.5±1.2 (n=11)	0.4±0.2
	W10-18	9.6±0.5 (n=7)	0.3±0.2	6.5±1.2 (n=11)	1±0	6.4±0.8* (n=14)	1±0*	6.5±1.0* (n=13)	2±1
Test Blue Bead	Control	7.1±1.8	3±1	3.0±0.5	4±0	2.7±0.5	4±1	2.8±0.5	5±1
	W10-18	3.3±1.2	3±1	2.4±0.3	4±0	2.2±0.2	3±0	2.3±0.3	4±1

2.2.4.3 W10-14 chicks

Analysis of variance revealed that the W10-18 chicks had impaired memory retention at all times of test compared to controls ($F_{1,74}$=37.18, P<0.001; Fig. 2.11). There was no significant effect of time of test ($F_{2,74}$=1.38, P=0.3), however, there was a significant interaction ($F_{2,74}$=3.34, P=0.04).

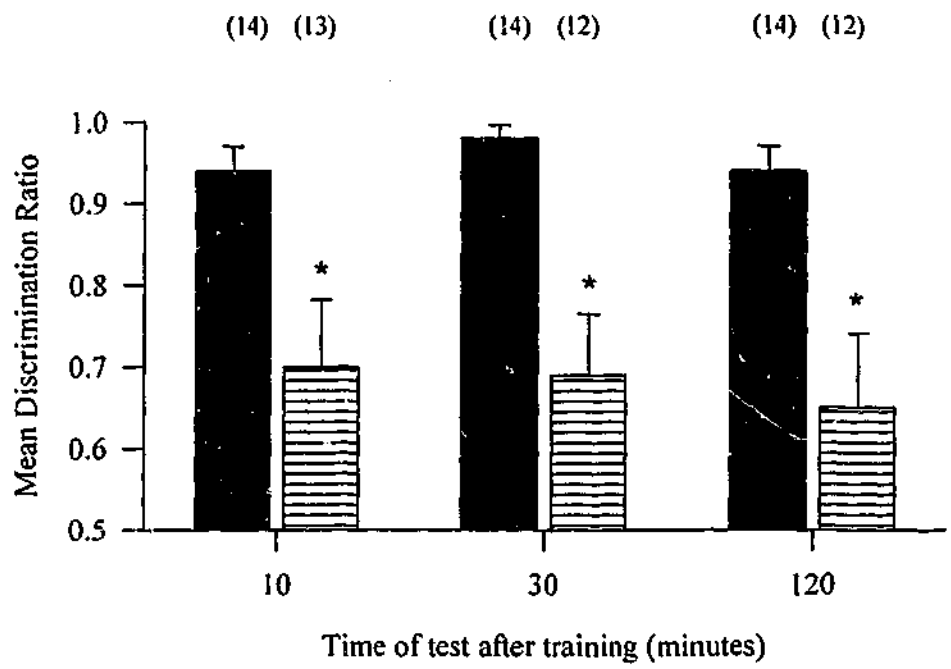


Fig. 2.11. Discriminated bead task. *Mean discrimination ratio of control (■) and W10-14 chicks (⊟). The W10-18 chicks had impaired memory retention at all times of test compared to controls. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

The proportion of W10-14 chicks avoiding the red bead tended to be lower than in controls at all times after training, however the difference was not significant ($\chi^2_{2,50}$=0.44, P=0.8, Fig. 2.12). Four days of restricted gas exchange from mid-incubation appeared to impair memory consolidation to a similar degree to that of the W10-18 chicks.

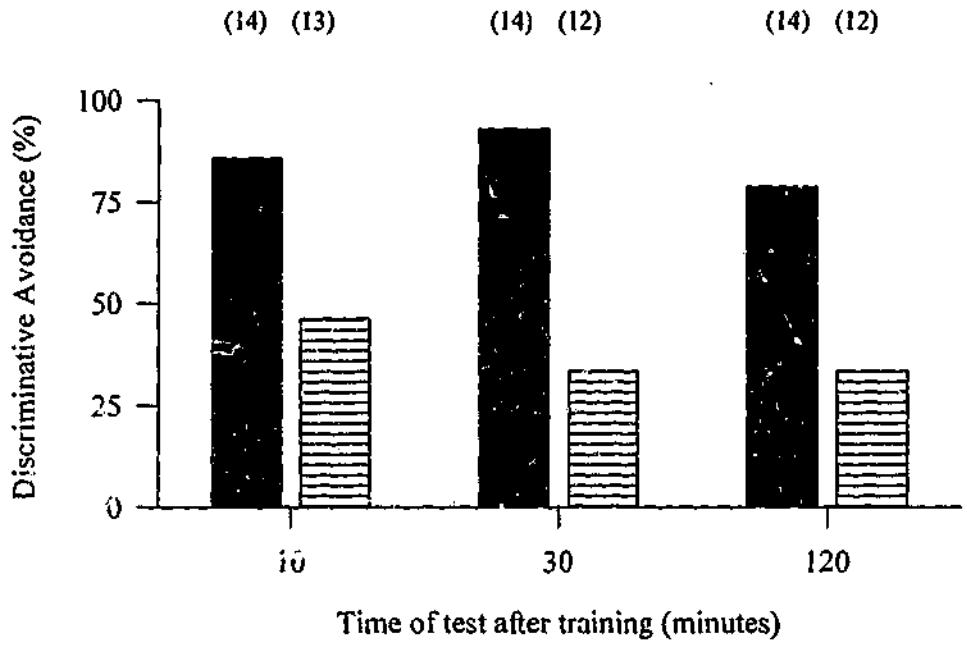


Fig. 2.12. Discriminated bead task. Percentage discriminated avoidance of control (■) and W10-14 chicks (⊟). The proportion of W10-14 chicks avoiding the red bead tended to be lower than in controls at all times after training, however the difference was not significant. Animal numbers are in parentheses.

There was no difference in pecking performance between the control and W10-14 chicks on training or at test when pecking at the blue bead, hence, data were combined. The mean latency to first peck and the number of pecks made on training for both control and W10-14 chicks was 3.1±0.4 sec and 2.1±0.1 pecks/10 sec, respectively. The mean latency to first peck and the number of pecks made at the blue bead on test for both groups of chicks was 3.6±0.5 sec and 2.9±0.2 pecks/10 sec, respectively. The W10-14 chicks in the 120 min group were quicker to first peck at the red bead on test compared to controls (P=0.01, Table 2.7). The W10-14 chicks in the 10 and 120 min groups pecked more frequently at the red bead on test compared to controls (10 min: P=0.05; 120 min: P=0.01). The W10-14 chicks in the 30 min group tended to peck more frequently at the red bead on test compared to controls, however the difference was not significant (P=0.053).

Table 2.7. ***Discriminated bead task.*** *Latency to first peck and number of pecks for control and W10-14 chicks. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

		Time of test after training (minutes)					
		10		30		120	
Trial	**Group**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**	**Latency (sec)**	**Pecks/ 10 sec**
Test Red Bead	Control	9.0±0.7 (n=14)	0.4±0.2	9.4±0.6 (n=14)	0.3±0.3	9.0±0.6 (n=14)	0.2±0.1
	W10-14	6.6±1.0 (n=16)	1.4±0.4*	6.2±1.1 (n=14)	1.4±0.5	6.1±1.0* (n=12)	1.8±0.5*
Test Blue Bead	Control	3.0±0.5	4.1±0.8	3.0±0.6	3.6±0.7	3.6±0.7	3.0±0.6
	W10-14	3.1±0.6	2.8±0.5	2.2±0.3	2±0.3	3.6±0.6	2.0±0.3

2.2.5 Discriminated wheat task (5-6 days after hatching)

Both control and W14-18 chicks learned the task, as evidenced by good discrimination at 30 min after training (Fig. 2.13A). However, at 120 min after training, retention levels of both groups of chicks from wrapped eggs were reduced compared to controls (P<0.05, Fig. 2.13A,B).

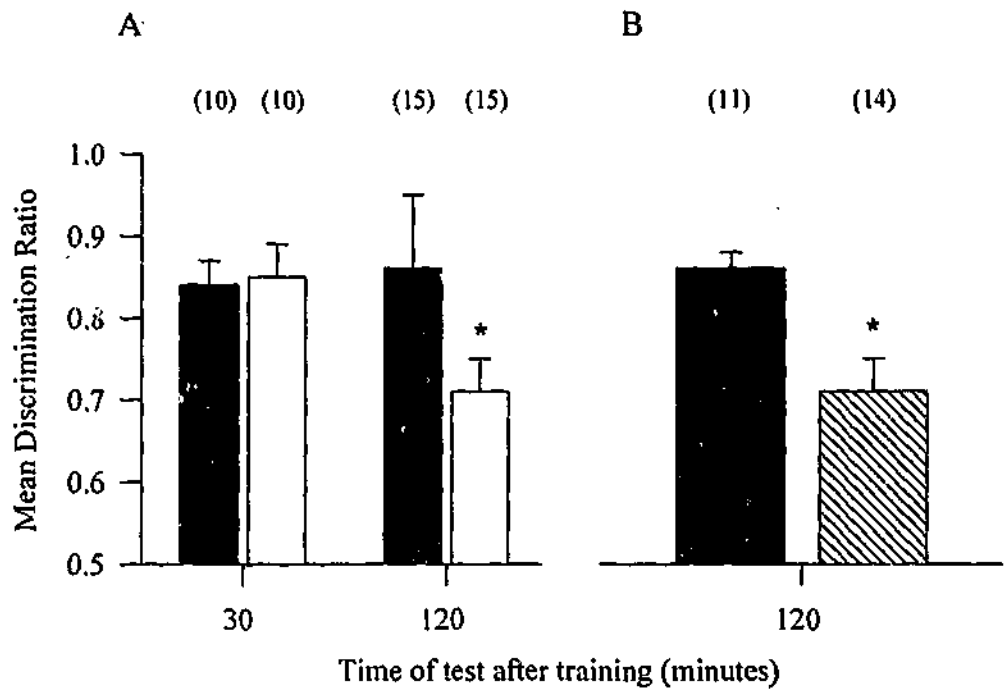


Fig. 2.13. Discriminated wheat task. (A) *Mean discrimination ratio of control* (■) *and W14-18 chicks* (□). (B) *Mean discrimination ratio of control* (■) *and W10-18 chicks* (▨). *At 120 min after training, retention levels of both groups of chicks from wrapped eggs were reduced compared to controls. Note: The 30 min time point for the W10-18 chicks was not obtained due to limited numbers of chicks. Asterisks* (*) *indicate values that are significantly different from control values. Animal numbers are in parentheses.*

There were no differences in both the latency to first peck and the number of pecks between the first presentation of the red wheat and the second for both groups of chicks from wrapped eggs (data not presented).

The W14-18 chicks in the 120 min test group were quicker to take their first peck at the red wheat on test compared to controls (P=0.04, Table 2.8), and pecked more frequently at the red wheat (P=0.02).

Table 2.8. Discriminated wheat task. *Latency to first peck and number of pecks for control and W14-18 chicks. Asterisks (*) indicate values that are significantly different from control values. Animal numbers are in parentheses.*

		Time of test after training (minutes)			
		30		120	
Trial	**Group**	**Latency (sec)**	**Pecks/20 sec**	**Latency (sec)**	**Pecks/20 sec**
Test red wheat	Control	10.4±2.2 (10)	3±1	10.3±2.5 (15)	3±1
	W14-18	9.5±2.4 (10)	4±1	6.8±1.9* (15)	8±2*
Test yellow wheat	Control	3.6±0.7	15±2	1.8±1.9	17±4
	W14-18	2.0±0.4	21±1	1.9±0.3	17±2

The W10-18 chicks pecked less frequently at the yellow wheat during pre-training (12±1 vs. 16±1 Pecks/20 sec, P=0.01), and took longer to take their first peck during training, compared to controls (9.8±1.4 sec vs. 3.7±0.6 sec, P=0.003). The W10-18 chicks also pecked less frequently at the yellow wheat on test (P<0.001, Table 2.9).

Table 2.9. Discriminated wheat task. *Latency to first peck and number of pecks for control and W10-18 chicks. An asterisk (*) indicates value that is significantly different from control value. Animal numbers are in parentheses.*

		Time of test after training (minutes)	
		120	
Trial	**Group**	**Latency (sec)**	**Pecks/20 sec**
Test red wheat	Control	7.1±1.0 (11)	4±1
	W10-18	6.7±0.8 (14)	6±1
Test yellow wheat	Control	1.4±0.1	24±1
	W10-18	3.2±0.8	15±1*

2.3 Discussion

The data in Chapter 2 show that restricting gas exchange across the eggshell from either day 10 or 14 of incubation until day 18 impairs the ability of chicks to consolidate memories into long-term storage for at least 5 days after hatching. Four days of

restricted gas exchange late in the incubation period resulted in chicks having normal labile memory and good learning ability, but an inability to consolidate memory. Eight days of reduced O_2 availability mid-incubation left some chicks with poor labile memory that may have resulted from an inability to learn the task or deficient short-term memory, as well as impaired long-term memory consolidation. Four days of restricted gas exchange from mid-incubation impaired memory consolidation to a similar degree to that of the W10-18 chicks.

2.3.1 Effect of wrapping on the gas status of the embryo

It is pertinent to question the degree to which wrapping half of the eggshell alters the blood gas status of the embryo. The technique we used to restrict prenatal gas exchange was based on previous studies by Metcalfe *et al.* (1979, 1981) and McCutcheon *et al.* (1982) in which half of the eggshell was covered lengthways with a "Neoprene" membrane. Metcalfe *et al.* (1979) showed that half-covering eggs with this membrane decreased the diffusing capacity of the entire shell by 20%, and resulted in chicks being smaller compared to uncovered eggs at day 18 of incubation (Metcalfe *et al.*, 1981). Tazawa *et al.* (1971b) found that reducing the shell area available for respiration altered blood gas status; in that study gas exchange across the eggshell was blocked by coating approximately one fourth of the eggshell with epoxy cement prior to incubation. Hematocrit and PCO_2 were found to be increased in the chorioallantoic venous blood of the embryos from the coated group compared to controls, whilst PO_2 and pH were decreased. Covering one half of the eggshell with paraffin wax has also been shown to lower PO_2, and increase PCO_2 (Roncali *et al.*, 1985). The results reported in this chapter suggest that partial wrapping results in an increase in hematocrit, which supports the findings of Tazawa *et al.* (1971b). In conjunction with these findings, PO_2 was found to be decreased in the air cell of eggs wrapped from day 10 and 14, whilst PCO_2 was increased in the air cell of eggs wrapped from day 10. Hence, it is likely that that covering one half of the eggshell from mid- to late-incubation induces hypoxia, hypercapnia and acidemia in the embryo.

2.3.2 Effect of wrapping on pre- and post-hatch growth

Body weights of the W10-18 chicks were reduced at hatching, whereas the body weights of the W14-18 chicks were not. These results suggest that limiting gas

exchange across the eggshell from mid-incubation slows embryonic growth; however, reducing gas exchange from late-incubation does not.

In ovo, oxygen is necessary for metabolic provision of energy and to synthesize protein. Therefore, it likely that oxygen restriction during incubation could reduce energy availability and impair protein synthesis, resulting in reduced growth of the embryo. It is also possible that the metabolism of nutrients from the yolk sac may have been inhibited or reduced in the embryos from eggs wrapped from days 10-18, which may have contributed to the reduction in hatching weight.

Body weights of both groups of chicks from wrapped eggs were reduced from 2 days after hatching, with the longer period of reduced gas exchange decreasing postnatal body weight to a greater extent. Relative brain weights of the W10-18 were increased at 8 days after hatching, indicating that brain sparing had occurred, most likely due to the redistribution of cardiac output favouring the brain (Mulder *et al.*, 1998). There was an absence of brain sparing in the W14-18 chicks at 8 days, which is contrary to previous findings (McCutcheon *et al.*, 1982; Asson-Batres *et al.*, 1989). The limitation on post-hatch growth in both groups could have been caused by reduced appetite or by altered gastrointestinal development, which has been observed in the ovine fetus (Avila *et al.*, 1989) and chick embryo (Van Golde *et al.*, 2001) following prenatal hypoxia.

It is puzzling why the W10-18 chicks had a shorter gestational length compared to the W14-18 chicks. Several batches of eggs have since been incubated in which gas exchange was restricted from days 10-18, and in these experiments, chicks hatched at 20-21 days and showed the same impairment in memory consolidation as reported in this chapter (E. J. Camm, *unpublished observation*).

2.3.3 Memory testing

2.3.3.1 Discriminated bead task (1-2 days after hatching)

The W14-18 chicks discriminated as well as controls at 10 and 30 min after training; in contrast, chicks wrapped from day 10 were impaired in their ability to learn the task, suggesting that learning ability or short-term memory was impaired by the longer prenatal insult. Neither group of chicks remembered to avoid the red bead on test at 60

and 120 min post-training, indicating that long-term memory consolidation was impaired as a result of the prenatal insult. W10-14 chicks had impaired memory from 10 min post-training, which was similar to that of the W10-18 chicks.

It was anticipated that the ability of the chicks from wrapped eggs to peck at a bead may have been altered by the prenatal hypoxic insult. The measure of memory retention that was used, the discrimination ratio, takes into account the number of pecks made on both the red (aversive) and blue (non-aversive) bead. If pecking performance of the chicks from wrapped eggs contributed to the results that were obtained, the number of pecks and latency to first peck at the non-aversive bead would also be affected. However, as this was not the case, it can be concluded that the pecking ability of the chicks from wrapped eggs was not altered by the prenatal insult and did not contribute to their inability to remember the aversive bead.

For the W14-18 chicks, the memory impairment which was observed was unlikely to have been due to impaired sensory function, as the chicks discriminated well at 10 and 30 min post-training. W10-14 chicks and W10-18 chicks, however, did not discriminate well 10 min post-training. It cannot be ascertained from these results whether the W10-14 chicks and the W10-18 chicks had impaired sensory function, however, their performance on training was similar to the W14-18. Therefore, restricting gas exchange *in ovo* from day 10 had a greater debilitating effect on the ability to form memories than restricting gas from day 14. Of note, retention levels of the W10-18 chicks were not as poor compared to the W10-14 chicks and W14-18 chicks, indicating that the duration of the prenatal insult may also be of importance. The data therefore suggest that the severity and duration of the prenatal insult may both play a role in determining the degree to which memory is impaired.

2.3.3.2 Discriminated wheat task (5-6 days after hatching)

For the discriminated wheat task, chicks from both groups of wrapped eggs showed poor retention at 120 min after training, indicating that their ability to consolidate memories was impaired as with the bead task at 1-2 days post hatch.. These data show that the ability to consolidate memories was impaired soon after hatching and persisted for at least up to 5 days after hatching, indicating that brain function may have been permanently altered following the prenatal compromise.

2.3.4 Evaluation of the tasks to assess learning ability and memory formation

The learning tasks used in this study have several advantages. Both tasks rely on the innate exploratory behaviour of young chicks and their ability to learn to discriminate between foreign objects and food. As mentioned above, the measure of memory retention takes into account pecking performance with both the red and blue beads; hence any effect of prenatal compromise on pecking performance is avoided. The wheat task was used to assess memory formation at 5-6 days of age, in order to determine whether alterations in memory function persisted. Although a different task was used, this did not appear to affect the results obtained from the wheat task, which were consistent with the findings from the bead task.

2.3.5 Potential mechanisms

It is possible that the impaired ability of the chicks from wrapped eggs to consolidate memories may be due to altered brain development incurred by restricted prenatal gas exchange. Prenatal insults to fetal sheep, such as chronic placental insufficiency or umbilical cord occlusion, have been shown to impair neural migration and proliferation, as well inhibiting the growth of neural processes, synaptogenesis and myelination. (Mallard *et al.*, 1992; Mallard *et al.*, 1995; Mallard *et al.*, 1998). Hypoxic or hypoxic/ischemic insults at or soon after birth in rats have been shown to cause deficits in discrimination and spatial learning behaviours (Nyakas *et al.*, 1996), and long-lasting learning deficits (Lun *et al.*, 1990; Balduini *et al.*, 2000). Other insults, such as thyroid hormone deprivation (Hamburgh, 1969) or starvation (Cragg, 1972) have been shown to alter both neuronal connectivity and behaviour in young rats. These data suggest that an insult to the brain during a critical period of development could interrupt a particular developmental process (e.g. neurogenesis, synaptogenesis) which may lead to a permanent alteration in brain structure. Neurogenesis in the chick forebrain peaks at day E8 (Sobue & Nakjima, 1978), whereas synaptogenesis peaks later, at day E15 (Sedlacek, 1972). Therefore it is possible that a reduction in gas exchange from days 14-18 or from days 10-18 of incubation could interfere with either of these processes, which may potentially affect neural connectivity and subsequently impair postnatal memory function.

An alternative explanation for the impaired ability of the W14-18 chicks to consolidate memories is that the cellular events leading to permanent memory storage may not have been triggered. The excitatory neurotransmitter, glutamate, has been implicated in brain injury caused by hypoxia-ischemia (Espinoza & Parer, 1991). Acute increases in cerebral glutamate and GABA concentrations can disturb short-term memory formation (Gibbs, 1991), however, it is unlikely that high levels of glutamate and GABA at the time of learning produced the memory deficits that were observed. Noradrenaline has been shown to be necessary for memory consolidation in the chick (Crowe & Shaw, 1997; Gibbs & Summers, 2000), and it is possible that the inability of chicks from wrapped eggs to consolidate memories may be caused by disturbances in the functioning of the central noradrenergic system. Plasma noradrenaline and adrenaline concentrations have been shown to be increased following prenatal hypoxia in the chick (Mulder *et al.*, 2000). As the period of maturation of the blood brain barrier in the chick may extend into or beyond the period of hatching (Stewart & Wiley, 1981; Liebner *et al.*, 1997), the blood brain barrier would be poorly developed at the time in which the prenatal insult occurred. Therefore, increased levels of circulating noradrenaline and adrenaline during incubation or at hatching could affect the functioning of the central noradrenergic system post-hatching, which could affect memory consolidation. The W10-14 and W10-18 chicks also appeared to be poorer in their ability to form memories, and the nature of the memory impairment may be different to that seen in the chicks exposed to a shorter duration of reduced gas exchange *in ovo* later in gestation.

2.3.6 The effect of gender on postnatal growth and memory function

It is possible that gender may have differentially affected postnatal growth or memory function of the chicks. Hill (1993) examined the effects of albumin removal prior to incubation on postnatal chick development, and found that albumin removal had significant long-term effects on the growth rate of female chicks, whereas growth rates of males were not altered. A sex-dependant effect on growth rates may have occurred following the restriction of respiratory gases. However, as the chicks were not sexed, it was not possible to determine whether the prenatal insult utilised in this study also differentially affected post-hatch growth. Similarly, it is important to consider the gender of the subject when performing cognitive tests. However, previous studies in the chick have found that the basic memory processes for the discriminated bead task are

the same in females as males (M.E.Gibbs, *unpublished observation*), suggesting that gender did not affect our findings.

2.3.7 Conclusions

The results reported in this chapter demonstrate that prenatal restriction of respiratory gases can impair long-term memory consolidation and pre- and postnatal growth. The timing of the prenatal insult plays a major role in determining the nature of the memory impairment; the duration of the insult may also be of importance. The impairment in memory consolidation evident in chicks from wrapped eggs at 1-2 days persisted until at least 5-6 days after hatching, indicating that brain function may be permanently altered following a period of prenatal hypoxia. The data show that, not only is the avian embryo ideal to study the effects of a reduction in prenatal gas exchange in the presence of normal nutrient supply, but cognitive function can easily be assessed using well established memory tasks. Knowledge from this study may ultimately assist in our understanding of how adverse prenatal conditions can affect memory function.

Chapter 3

The effect of prenatal hypoxia on plasma and brain catecholamine concentrations

PRENATAL OR NEONATAL hypoxia is known to alter the level of catecholamines in plasma (Cohen *et al.*, 1982; Kitanaka *et al.*, 1989; Hooper *et al.*, 1990; Mulder *et al.*, 2000) and the brain (Chanez *et al.*, 1981; Seidler & Slotkin, 1990; Jensen *et al.*, 1996). It has been suggested that an early insult or stress (e.g. hypoxia) during critical periods of development could affect neuronal plasticity (Kasamatsu & Pettigrew, 1976; Naqui *et al.*, 1999) or the programming of catecholaminergic neurotransmitter and receptor expression (Herlenius & Langercrantz, 2001) and cause long-term behavioural effects (Herlenius & Langercrantz, 2001; Okado *et al.*, 2001; Weinstock, 2001). Since the noradrenergic systems of the central nervous system (CNS) appear to be critically linked to cognitive function in the chick (Gibbs & Summers, 2002a), alterations in catecholamine levels for a prolonged period could, for example, lead to alterations in receptor-mediated responses or receptor expression, and consequently impair memory formation and consolidation. The present study was undertaken to investigate whether

prenatal hypoxia in our experiments alters noradrenaline and adrenaline concentrations in the blood and in brain tissue after hatching.

Increases in plasma noradrenaline and adrenaline levels have been reported in response to acute and chronic episodes of prenatal hypoxia. The sheep fetus (Cohn *et al.*, 1974) and chick embryo (Mulder *et al.*, 1998) have been shown to respond to acute hypoxemia by producing a marked peripheral vasoconstriction that aids in redirecting cardiac output towards the brain, heart, and adrenal glands (Reuss & Rudolph, 1980). In the sheep fetus, the peripheral vasoconstriction in response to hypoxemia is triggered by a carotid chemoreflex (Giussani *et al.*, 1993), and is maintained by the release of catecholamines into the fetal circulation. Consequently, fetal plasma concentrations of noradrenaline and adrenaline increase (Cohen *et al.*, 1982). Prolonged hypoxemia in the sheep fetus results in an initial increase in fetal plasma noradrenaline and adrenaline concentrations (Kitanaka *et al.*, 1989; Hooper *et al.*, 1990). After several days of hypoxemia, plasma noradrenaline concentrations remain elevated, whereas adrenaline concentrations appear to return to basal levels (Kitanaka *et al.*, 1989).

Prenatal and neonatal hypoxia has been shown to alter catecholamine turnover and concentrations in the brain, and several studies have reported regionally specific changes in levels of catecholamines. In neonatal rats, exposure to hypoxia at one-day post-partum increased noradrenaline and dopamine turnover in the midbrain and brainstem, with little or no effect in the cerebral cortex or cerebellum. This increase in turnover did not resolve in adulthood, suggesting that neonatal hypoxia has long lasting effects on the central catecholaminergic system (Seidler & Slotkin, 1990). Restriction of maternal blood flow for the last 5 days of pregnancy in the rat has been shown to increase noradrenaline and dopamine concentrations in the forebrain following birth (Chanez *et al.*, 1981). Chronic placental insufficiency in the guinea pig from days 30-64 of gestation (term ~ 68 days) has been shown to increase noradrenaline and dopamine adrenaline levels in the caudate nucleus and brainstem, respectively, (Jensen *et al.*, 1996).

Taken together, results from the experimental studies suggest that it is likely that the prenatal insult used in our studies (wrapping) would alter noradrenaline and adrenaline concentrations in plasma and the brain, although there is no clear indication as to which

brain region would be affected. A recent report by Dent *et al.* (2001) suggested that a prenatal insult can either amplify or blunt the neural stress response, depending upon the stress-responsive system examined, and the developmental age which it is induced (Dent *et al.*, 2001).

The aim of this chapter is to determine whether restricting gas exchange *in ovo* causes changes in noradrenaline and adrenaline concentrations in plasma and the brain in the postnatal chick. Prenatal gas exchange was restricted by covering one half of the eggshell lengthways with a polyvinyl film for 4 or 8 days, commencing at day 14 and day 10 of incubation, respectively. At 2 and 8 days post-hatching, plasma was collected from the two experimental groups and controls for the measurement of noradrenaline and adrenaline concentrations by high-performance liquid chromatography (HPLC). At 2 days post-hatching, the brains of the two experimental groups and controls were removed and the brain regions containing the basal ganglia (lobus parolfactorius- LPO), association cortex (intermediate hyperstriatum ventrale-IMHV), and locus coeruleus (LoC) were dissected and collected for catecholamine analysis using HPLC. The brain regions containing the IMHV and LPO were collected since both areas play a role in discrimination memory in the chick (Gibbs & Summers, 2002b). The hindbrain, containing the LoC, was also of interest as it is the site where the noradrenergic axons innervating the neocortex, hippocampus and cerebellum originate (Foote *et al.*, 1983). I was primarily interested in alterations in the level of catecholamines in the brain, as an indicator of the availability of noradrenaline and adrenaline, rather than changes in metabolites or turnover. It was hypothesised that prenatal hypoxia *in ovo* would result in a prolonged change in plasma catecholamine levels, which would still be detectable 2 days post-hatching, and that brain catecholamine levels will also be altered.

3.1 Materials and Methods

3.1.1 Incubation and hatching

Fertile eggs were half-wrapped lengthways with a polyvinyl film (22 μm thick) for 4 or 8 days, commencing at day 14 and day 10 of incubation, respectively. In both groups, the film was removed at day 18 of incubation. Wrapped eggs were incubated with control eggs (uncovered) under the same conditions reported in Section 2.1.1.

3.1.2 Plasma and brain catecholamine determination

3.1.2.1 Collection of plasma and brain samples

At 2 and 8 days post-hatching, chicks were quickly decapitated and blood was collected in pre-heparinised tubes. Samples were centrifuged for 15 min (8°C, 2400 *g*) and plasma stored at –20°C. Blood was collected at the same time of day at each age sampled in order to avoid time-of-day effects on catecholamine concentrations.

At 2 days post-hatching, the brains of the two experiential groups and controls were quickly removed and rapidly dissected out over dry ice into three regions: anterior forebrain, posterior forebrain and hindbrain, which included the LPO, IMHV and LoC, respectively (Fig. 3.1). Care was taken to ensure that these regions were uniformly dissected out (see Table 3.1 for brain weights). There was a significant difference in total brain weights between the three groups (F=4.043, P=0.033), with the W10-18 chicks having smaller total brain weights compared to controls (P=0.022). The brain regions were frozen in liquid nitrogen and stored at –70°C for subsequent neurochemical determination. The whole procedure took no longer than 30 sec to perform. The optic tectum from both hemispheres and the cerebellum were not collected for neurochemical analysis, as these regions do not appear to play a role in discrimination memory in the chick.

Table 3.1. *The weights (in grams) of the three brain regions collected for neurochemical analysis from control, W14-18, and W10-18 chicks at 2 days post-hatching. Animal numbers are in parentheses.*

Region	***Control (n=9)*** **mean±SEM**	***W14-18 (n=7)*** **mean±SEM**	***W10-18 (n=8)*** **mean±SEM**
anterior forebrain	0.187±0.007	0.159±0.010	0.148±0.009
posterior forebrain	0.258±0.009	0.254±0.011	0.270±0.007
hindbrain	0.163±0.005	0.150±0.012	0.128±0.014

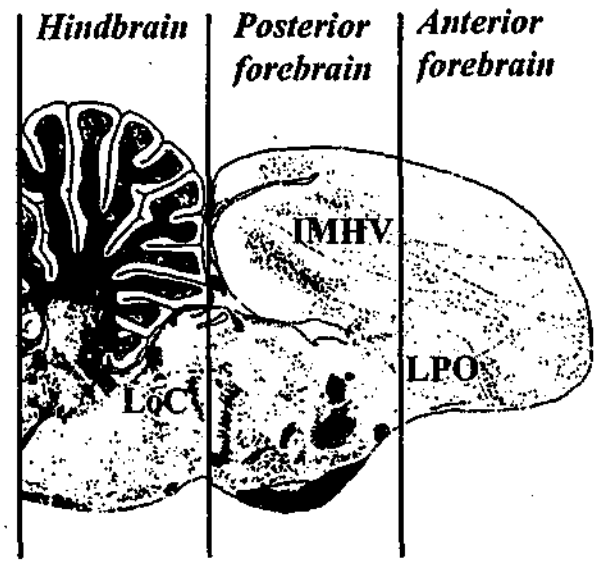


Fig. 3.1. Diagrammatic representation of the brain regions examined for neurochemical analysis. The brain was dissected into three regions, which are indicated by the black lines. These three regions included the intermediate hyperstriatum ventrale (IMHV), *the lobus parolfactorius* (LPO) *and the locus coeruleus* (LoC). *The optic tectum (not shown) and the cerebellum were not collected for neurochemical analysis. Diagram adapted from Kuenzel, W.J. and Masson, M. (1988). 'A stereotaxic atlas of the brain of the chick (Gallus domesticus)', p.140. The Johns Hopkins University Press, Baltimore.*

3.1.2.2 Extraction of catecholamines from plasma

The catechols noradrenaline, adrenaline, dopamine, dihydroxyphenylalanine (DOPA), dihydroxyphenylacetic acid (DOPAC) and dihydroxyphenylglycol (DHPG) were extracted from varying amounts of plasma, depending on the age of the animal, using the method previously described by Lambert and Jonsdottir (1998). Dihydroxybenzylamine (DHBA; 20 µl), Tris/EDTA (pH 8.6; 400 µl) and acid washed alumina (100 mg) were added to 100 (2 day-old chicks) or 10 µl (8 day-old chicks) of plasma. Samples were briefly vortexed, then placed on an orbital shaker for 30 min. The samples were centrifuged for 30-60 sec to pack down the alumina, after which the plasma-Tris effluent was aspirated. The alumina particles were then washed with 1ml of 0.2 M sodium bicarbonate, followed by two washes with 1 ml of Milli Q water. The samples were briefly vortexed and the effluent discarded after the addition of each of these solutions. The catechols were then eluted by the addition of 150 µl of a 10% 0.2 M phosphoric acid in 0.2 M acetic acid solution. The samples were shaken at full speed for 5-10 min after the addition of the acid and then centrifuged for 1 min. The elutant was transferred into numbered microsample vials ready for HPLC analysis (Lambert & Jonsdottir, 1998).

3.1.2.3 Extraction of catecholamines from brain tissue

0.4 M perchloric acid containing 0.01% EDTA (500 µl) was added to 0.1-0.3 g of brain tissue and homogenised over ice. The homogenate was centrifuged for 1 min, after which the supernatant was carefully removed and stored at –70°C for subsequent neurochemical analysis. DHBA (20 µl), Tris/EDTA (pH 8.6; 400 µl) and acid washed alumina (100 mg) were added to 10 (anterior forebrain), 20 (posterior forebrain) or 10 µl (hindbrain) of extracted brain tissue. Following this, the treatment of the brain tissue was the same as for the plasma (see Section 3.1.1.2).

3.1.2.4 Reagents and HPLC conditions

The catechol standards (noradrenaline, adrenaline, DOPA, dopamine, DOPAC and DHPG), and the internal standard DHBA were obtained from Sigma (Sigma Chemical Company, St Louis, USA). The chromatography system consisted of a 25 cm Altex Ultrasphere column (ODS 4.6 mm x 25 cm, 5 µm particle size, Beckman Instruments), model Gina autosampler, model STH 585 column oven, model 480 high precision pump, Chromeleon 3.03 chromatography data system (Dionex, Germergin, Germany), and a 5100A colourometric detector fitted with a model 5021 conditioning cell and a model 5011 analytical cell (Environmental Sciences Associates). The injection volume was 130 µl. The operating potentials were set at +0.35 V for the guard cell and –0.35 and +0.29 V for detectors 1 and 2, respectively. All measurements were made using the oxidizing potential applied at detector 2. Noradrenaline and adrenaline concentrations in plasma and brain extracts were calculated by comparing the sample peak area with that of a known standard which was injected prior to each experiment (Lambert & Jonsdottir, 1998).

3.1.3 Statistical Analysis

All data are presented as mean ± SEM. Catecholamine concentrations in plasma and brain were analysed using a one-way analysis of variance (ANOVA). Post hoc analysis was performed using Dunnett's test. If data were found to be heterogeneous, square root or log transformations were performed and the most homogenous transformation was used for further analysis. Statistical significance was accepted at the level of $P<0.05$.

3.2 Results

3.2.1 Noradrenaline and adrenaline concentrations in plasma

3.2.1.1 Two days post-hatching

Analysis of variance (one-way) revealed that there was a significant difference in plasma noradrenaline ($F_{2,30}$=3.665, P=0.038) concentrations between the groups. Although there was an apparent increase in adrenaline levels, these did not reach significance ($F_{2,30}$=3.127, P=0.058). Post hoc analysis revealed that plasma noradrenaline concentrations were increased in the W14-18 chicks compared to controls (P=0.023; Fig. 3.2). Although there was a tendency for plasma noradrenaline and adrenaline concentrations to be elevated in the W10-18, they were not significantly different from controls (P=0.0.199 and 0.577, respectively).

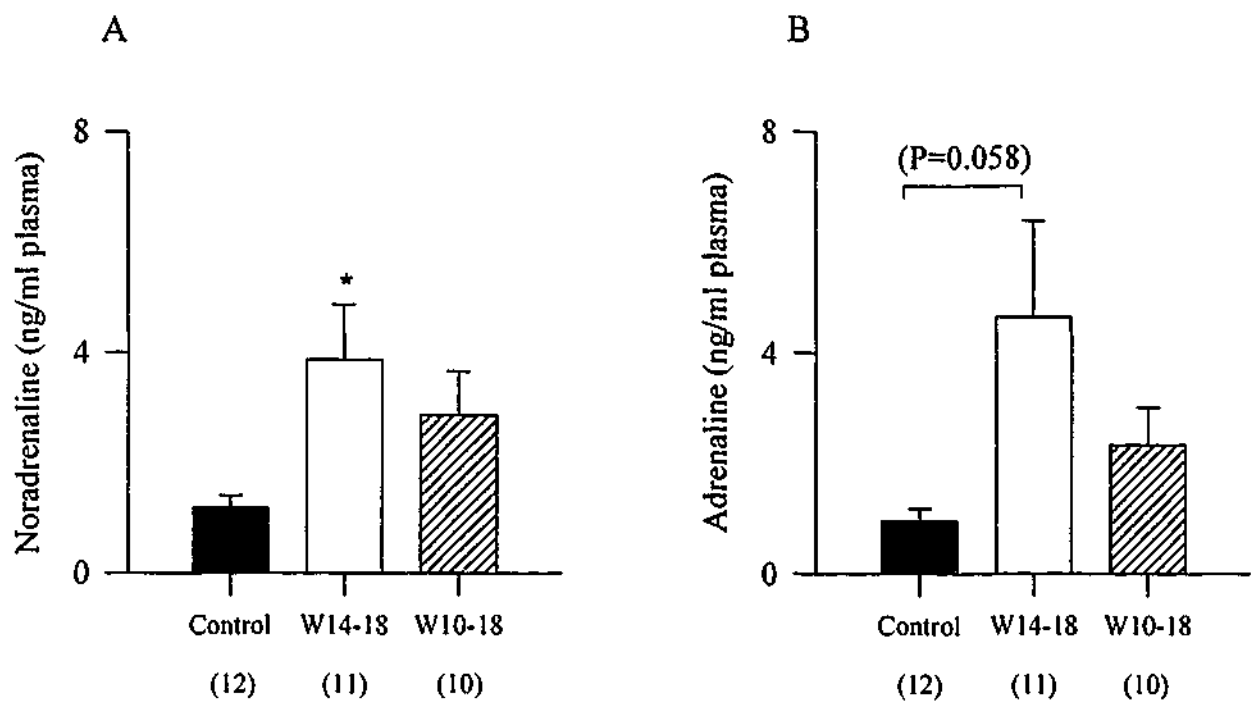


Fig. 3.2. Plasma noradrenaline (A) *and adrenaline* (B) *concentrations at 2 days post-hatching in control and prenatally hypoxic chicks. Plasma noradrenaline levels were significantly elevated in the W14-18 chicks Although there was an apparent increase in adrenaline levels, these did not reach significance (P=0.058). Asterisks* (*) *indicate values that are significantly different from control values. Animal numbers are in parentheses.*

3.2.1.2 Eight days post-hatching

At 8 days post-hatching, there was no significant difference in plasma noradrenaline concentrations between the groups ($F_{2,35}$=0.802, P=0.456; Fig. 3.4). Plasma adrenaline levels were also not significant between the three groups ($F_{2,35}$=2.073, P=0.141).

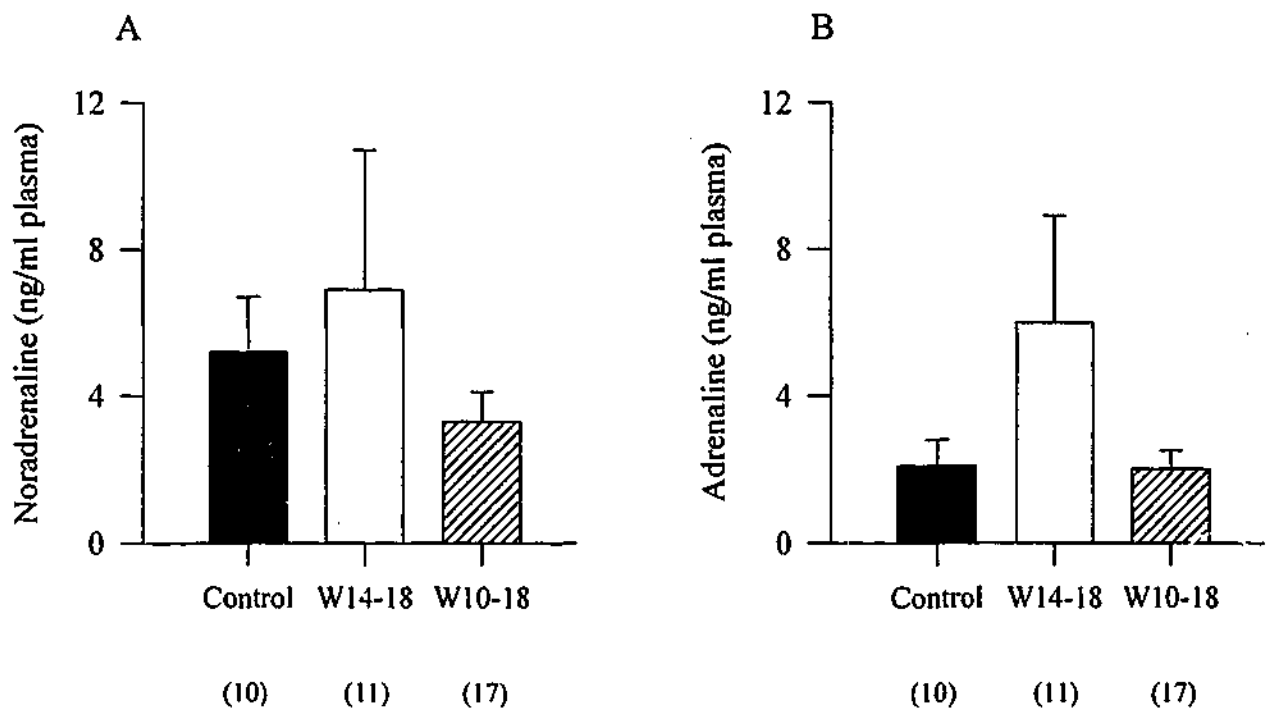


Fig. 3.4. Plasma noradrenaline (A) *and adrenaline* (B) *concentrations at 8 days post-hatching in control and prenatally hypoxic chicks. There was no significant difference in plasma noradrenaline and adrenaline levels between the three groups. Animal numbers are in parentheses.*

3.2.2 Catecholamine concentrations in the brain

Analysis of variance (one-way) revealed that noradrenaline levels were altered in the anterior forebrain ($F_{2,20}$=53.688, P<0.001; Fig. 3.5A). Post hoc analysis revealed that both the W14-18 and W10-18 chicks had reduced levels of noradrenaline compared to controls in this region (both P<0.001). Noradrenaline levels in the prenatally hypoxic chicks were not altered in the posterior forebrain ($F_{2,21}$=0.87, P=0.917), or in the hindbrain ($F_{2,21}$=1.393, P=0.270). Adrenaline levels were not significantly different between the three groups in the posterior forebrain ($F_{2,21}$=1.117, P=0.346; Fig. 3.5B), or hindbrain ($F_{2,21}$=1.699, P=0.207). Adrenaline was not detected in the anterior forebrain. There was also no significant difference between the groups in dopamine levels in each of the regions examined (anterior forebrain: $F_{2,19}$=0.562, P=0.579; posterior forebrain: $F_{2,21}$=0.538, P=0.592; hindbrain: $F_{2,21}$=1.051, P=0.367; Fig. 3.5C). It was apparent that noradrenaline and adrenaline levels were greater in the hindbrain compared to the posterior and anterior forebrain. Conversely, there appeared to be relatively more dopamine in the anterior forebrain compared to the posterior forebrain and hindbrain regions.

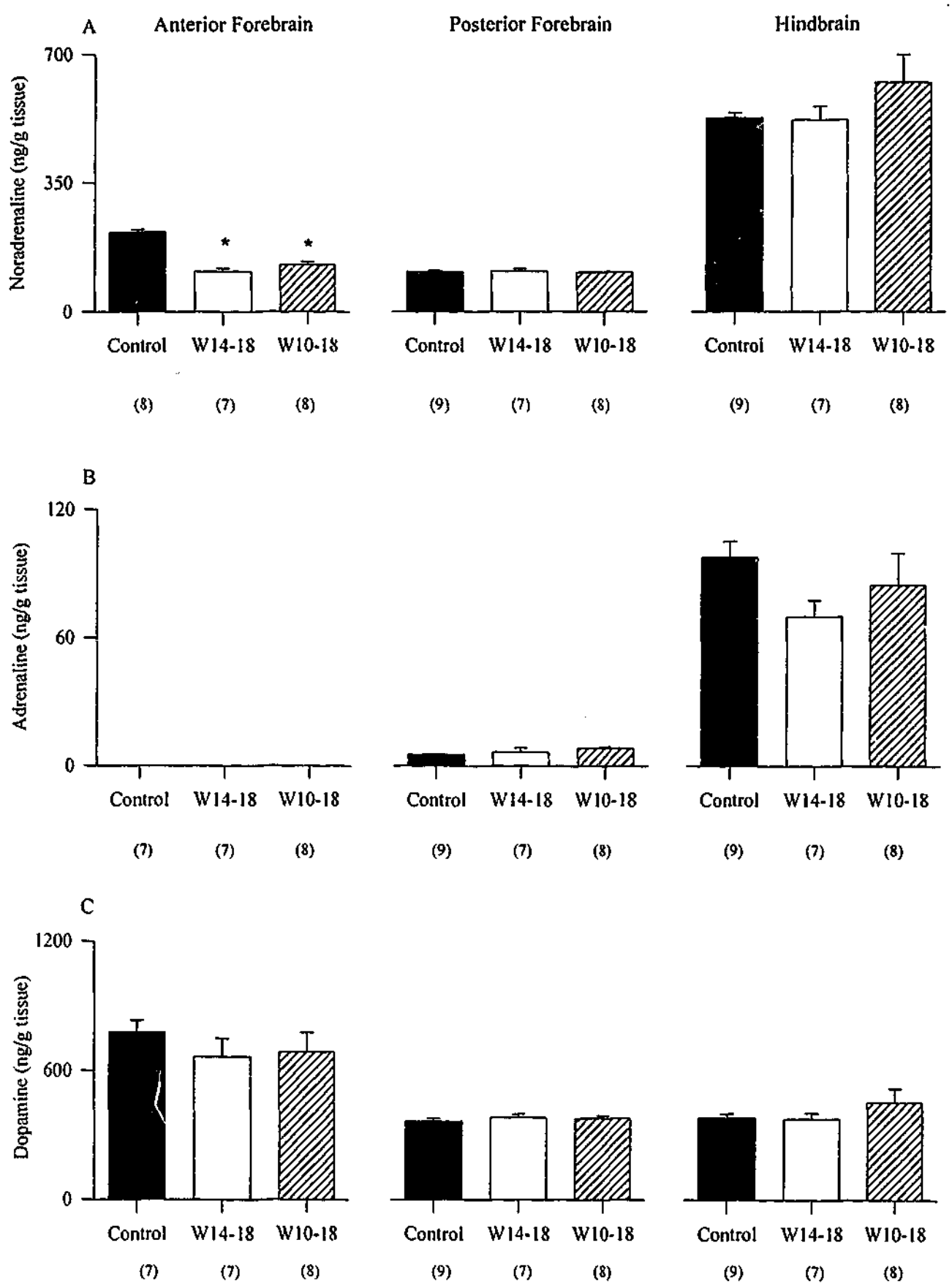


Fig. 3.5. Noradrenaline (A), *adrenaline* (B) *and dopamine* (C) *concentrations in the anterior forebrain, posterior forebrain, and hindbrain. The concentration of noradrenaline in the anterior forebrain was reduced in both groups of chicks from wrapped eggs. Asterisks* (*) *indicate values that are significantly different from control values. Note: Adrenaline was not detected in the anterior forebrain. Animal numbers are in parentheses.*

3.3 Discussion

The data reported in this chapter demonstrates that prenatal hypoxia, induced during the later stages of development, can alter plasma catecholamine levels. Four days of restricted gas exchange from day 14 of incubation resulted in an increase in plasma noradrenaline 2 days after hatching, while adrenaline concentrations approached significance (P=0.058). Plasma noradrenaline and adrenaline concentrations tended to be elevated at 2 days after hatching in the W10-18 chicks. Conversely, both four and eight days of prenatal hypoxia resulted in decreased noradrenaline concentrations in the anterior forebrain.

3.3.1 Prenatal hypoxia and plasma noradrenaline and adrenaline concentrations

Increases in the level of catecholamines in plasma have been reported in response to acute and chronic episodes of prenatal hypoxia in the sheep fetus (Cohen *et al.*, 1982; Hooper *et al.*, 1990) and chick embryo (Mulder *et al.*, 2000). Increases in circulating catecholamines in response to hypoxia is thought to contribute to peripheral vasoconstriction (Cohen *et al.*, 1982), which aids redirecting fetal cardiac output towards the brain, heart, and adrenal glands (Reuss & Rudolph, 1980). In the chick embryo, 5 min of acute hypoxemia *in ovo* has been shown to cause an increase in plasma noradrenaline and adrenaline concentrations at day 13 and day 16 of incubation, respectively. This catecholaminergic response developed with age, as evidenced by a progressive increase in plasma noradrenaline and adrenaline concentrations as the embryo approached hatching (Mulder *et al.*, 2000). In light of these findings, and those of Kitanaka *et al.* (1989), who found that after several days of hypoxemia, plasma noradrenaline concentrations were still significantly elevated, it is likely that catecholamine levels in both groups of chicks from wrapped eggs were elevated *in ovo*. The increase in plasma noradrenaline levels 2 days after hatching in the W14-18 chicks suggests a prolonged effect of prenatal hypoxia on catecholamine levels. Alternatively, there could have been an increase in sympathetic nerve activity or an alteration in brain development and/or neuronal processing leading to the observed increase in circulating noradrenaline levels. The increase in plasma catecholamine concentrations in the W14-18 chicks at 2 days may have been elevated from the stress of hatching. However, if this were the case, you would expect plasma catecholamine levels in the controls at 2 days to be elevated as well. The chicks utilsed in this study did not perform the discriminated bead task, hence, the increase in plasma noradrenaline and adrenaline concentrations in

the W14-18 chicks at 2 days is not due to arousal or reinforcement as a consequence of training or testing on the discriminated bead task.

The significant elevation in plasma noradrenaline concentrations evident 2 days after hatching in the W14-18 chicks did not persist at 8 days, although there was a tendency for plasma catecholamine levels to be elevated at 8 days in these animals. Of note was the large variation in the standard error of the mean of plasma noradrenaline and adrenaline levels, in particular, in the W14-18 chicks at 2 and 8 days post-hatching. Since the differences in noradrenaline and adrenaline concentrations between control and W14-18 chicks at 2 days was large, this variability does not present a problem in the interpretation of the results. The variability in our data may reflect individual differences, with some animals showing a marked increase in plasma noradrenaline and adrenaline concentrations.

3.3.2 Source of catecholamines

In the chick embryo, the major sources of peripheral catecholamines are the adrenal glands, para-aortic chromaffin tissue and sympathetic nerve terminals (Epple *et al.*, 1992). Studies in neonatal rats (Seidler & Slotkin, 1985) and fetal sheep (Cheung, 1990) have shown that catecholamine release in response to stress (e.g. hypoxia) can occur prior to the innervation of the adrenal glands. This response has been shown to be attributed to the direct effect of hypoxia on chromaffin cells (Rychkov *et al.*, 1998). Following the innervation of the adrenal glands, which in the chick is thought to be complete on about day 10 of incubation (Fujita *et al.*, 1976), catecholamine release in response to hypoxia is more dependent on neural sympathetic stimulation, although the adrenals remain sensitive to the direct effect of hypoxia (Mulder *et al.*, 2002).

3.3.3 Collection of plasma samples for HPLC analysis

It is pertinent to question whether decapitation was the most suitable method to obtain blood from the chicks, since decapitation is often accompanied by an increase in noradrenaline and adrenaline levels (Berkowitz & Head, 1978; Hart *et al.*, 1989). Blood samples can be obtained from wing vein punctures in birds (Johnson, 1981; Noirault *et al.*, 1999). However, due the amount of handling required, and the small amount of blood that can be obtained at the one time, this technique was not suitable for the

purposes of this study. Blood samples can also be collected by cardiac puncture; but this technique requires the chick to be anesthetised prior to sampling, which could alter plasma catecholamine concentrations. Hence, decapitation was the most appropriate method for blood collection in this study.

Blood samples can also be collected from the chorioallantoic artery of chick embryos. This procedure is performed inside a clinical infant incubator, in which the correct temperature, humidity and oxygen concentration are carefully maintained (Mulder *et al.*, 2000). Due to the method in which prenatal hypoxia was induced in the present study (wrapping), the technique used by Mulder *et al.* (2000) to sample blood could not be used. Thus, I limited the study to postnatal chicks.

3.3.4 Noradrenaline and adrenaline concentrations in the brain following prenatal hypoxia

The concentration of noradrenaline was found be decreased in the anterior forebrain of both groups of prenatally hypoxic chicks. This decrease in noradrenaline may reflect a stress-induced depletion of noradrenaline as a consequence of the prenatal hypoxic insult. Alternatively, the hypoxic insult may have caused a marked reduction in neuronal activity or neuronal plasticity. Immunohistochemical analysis is required to localise specific regions of the anterior forebrain that may have been affected following the prenatal insult. In Chapter 5, an attempt has been made to examine the immunoreactivity of tyrosine hydroxylase (TH)- the rate-limiting enzyme in the catecholamine synthesizing pathway, in the anterior forebrain.

The distribution pattern of catecholamines in the chick brain reported in this chapter is in agreement with previous findings. Reiner *et al.* (1994) reported that the catecholaminergic cell groups of the hindbrain are typically noradrenergic or adrenergic. The avian forebrain is abundant in dopaminergic fibres but noradrenergic fibres are less common (Reiner *et al.*, 1994), which may be reflected in the higher dopamine levels reported in the forebrain in this study. The range of noradrenaline and adrenaline levels detected in the brain also appear to be in agreement with previous studies (Revilla *et al.*, 2001).

The results reported in this chapter give an indication of the catecholamine concentrations in the areas of the brain containing the IMHV, LPO and LoC. A more accurate indicator of the level of catecholamines in these regions could have been obtained if they were dissected out. Future studies could use *in vivo* microdialysis in order to determine more accurately the extracellular concentrations of catecholamines and their metabolites in the IMHV, LPO and LoC of the chick brain (Parent *et al.*, 2001).

3.3.5 Implications of altered catecholamine levels in the plasma and brain

The release of noradrenaline from noradrenergic neurons is essential for learning and memory consolidation (Gibbs & Summers, 2002b). Noradrenaline can have different actions on learning and memory consolidation, depending on which adrenoceptor (AR) subtypes in the brain is activated. Recent findings have shown that stimulation of either the β_2- or β_3-ARs in the IMHV of the chick brain consolidates a weakly reinforced memory, which would otherwise not progress beyond 30 minutes (Gibbs & Summers, 2000). If circulating catecholamine levels were increased *in ovo* in response to the hypoxic insult, which is what I suspect is occurring given the results reported in this chapter and those of previous studies in the chick (Mulder *et al.*, 2000), the catecholamines would have penetrated the cerebral circulation, as the blood brain barrier in the chick is not developed until after hatching (Stewart & Wiley, 1981; Liebner *et al.*, 1997). Prolonged exposure to catecholamines is known to lead to a loss of receptor-mediated responses (desensitization) or a decrease in receptor numbers (down-regulation; Carpene *et al.*, 1993 and Nantel *et al.*, 1993). Desensitization and down-regulation of ARs has been described in some detail for the β_2-AR (Bouvier *et al.*, 1989; Carpene *et al.*, 1993); β_1-ARs can also desensitise to some degree (Carpene *et al.*, 1993), but β_3-ARs show very little, if any, desensitization (Carpene *et al.*, 1993; Nantel *et al.*, 1993). Thus, given the involvement of β_2-ARs in consolidating memory (Gibbs & Summers, 2000), desensitization and down-regulation of β_2-ARs in the chick brain as a consequence of the prenatal insult could potentially impair memory consolidation. Chapter 4 examines whether β-AR function is altered following prenatal hypoxia.

The anterior forebrain, which was found to have reduced levels of noradrenaline, contained the LPO, an area of the chick brain which has been shown to be involved in

memory formation and consolidation (Kossut & Rose, 1984; Rose & Csillag, 1985). The release of noradrenaline at the time of learning, possibly due to arousal or attentional mechanisms, has been shown to be necessary for the activation of the β_1-ARs in the LPO, where it is required for the formation of short-term memory (STM). If there is sufficient activation of α_2-ARs in the LPO from 10 minutes after training, memory will be consolidated; the activation of the α_2-ARs appears to be related to the level of reinforcement (Gibbs & Summers, 2002b). Hence, if there is a reduction in noradrenaline in the LPO, this may mean that there is less for subsequent reinforcement, and thus, memory will not be consolidated.

The reduction in noradrenaline in the anterior forebrain may not only affect neurotransmission, but given the neurotrophic role that noradrenaline is thought to play during brain development, alterations in the level of this catecholamine *in ovo* could have altered neuronal differentiation and synaptic plasticity (Felten *et al.*, 1982; Lipton & Kater, 1989).

3.3.6 Conclusions

The results reported in this chapter demonstrate that prenatal hypoxia can alter plasma and brain catecholamine concentrations after hatching in the chick. The increase in plasma noradrenaline concentrations evident at 2 days in the embryos exposed to 4 days of hypoxia did not persist at 8 days. The increase in plasma noradrenaline levels 2 days after hatching in the W14-18 chicks suggests a prolonged effect of prenatal hypoxia on catecholamine levels. Alternatively, there could have been an increase in sympathetic nerve activity or an alteration in brain development and/or neuronal processing leading to the observed increase in circulating noradrenaline. Although there was a tendency for plasma catecholamine concentrations to be increased at 2 days in the embryos exposed to 8 days of hypoxia, the increase was not significant. The data also show that noradrenaline concentrations were reduced in the anterior forebrain of both groups of prenatally hypoxic chicks, which may reflect a stress-induced depletion of noradrenaline, or a marked reduction in neuronal activity or neuronal plasticity, as a result of the prenatal hypoxic insult. It is possible that prolonged intrauterine stress (e.g. hypoxia) may not only alter the responsiveness of the central catecholaminergic system

in the postnatal period, but the response to noradrenaline release as a consequence of learning may also be impaired.

Chapter 4

The role of the central noradrenergic system in memory impairment in prenatally hypoxic chicks

THIS CHAPTER COMPRISES two different studies. The first study (Part 1 of this chapter) examines whether adrenaline promotes memory consolidation in a dose-dependant manner by acting via different adrenoceptor (AR) subtypes. The second study (Part 2) investigates whether prenatal hypoxia effects the functioning of central ARs in the postnatal chick.

As reported in Chapter 3, adrenaline levels approached significance (P=0.058) in the W14-18 chicks at 2 days after hatching; plasma adrenaline levels tended to be increased in the W10-18 chicks. Plasma adrenaline levels have previously been shown to increase following acute hypoxemia *in ovo* (Mulder *et al.*, 2000), hence it is likely that adrenaline levels were increased in both groups of chicks from wrapped eggs during the prenatal hypoxic insult induced in Chapters 2 and 3. Circulating adrenaline in the developing embryo will enter the cerebral circulation, as the blood-brain barrier in the chick is not developed until after hatching (Stewart & Wiley, 1981; Liebner *et al.*, 1997). Increased adrenaline levels could be detrimental to the functioning and

development of the central catecholaminergic system. Centrally administered noradrenaline acts via β_2- and β_3-ARs in the intermediate hyperstriatum ventrale (IMHV-association cortex) of the chick brain to promote memory consolidation (Gibbs & Summers, 2000). It is not known whether adrenaline acts in a similar way to noradrenaline. The aim of this chapter is to determine whether exogenous adrenaline promotes memory consolidation, and the contribution of the different AR subtypes in the action of adrenaline on memory consolidation. If adrenaline is found to act via β-ARs to promote memory consolidation, it is possible that the elevated plasma adrenaline levels reported in the W14-18 chicks (Chapter 3) may have caused an alteration in the state of β-ARs, for example, desensitization and down-regulation.

The issue of whether increased circulating noradrenaline and adrenaline levels in response to a prenatal insult causes an alteration in the state of β-ARs is addressed in Part 2 of this chapter. Long-term exposure to circulating catecholamines could lead to a loss of receptor-mediated responses (desensitization) or a decrease in receptor numbers (down-regulation) (Carpene *et al.*, 1993; Nantel *et al.*, 1993; Rang *et al.*, 1995). Desensitization of ARs in response to excess stimulation by catecholamines has been described in some detail for the β_2-AR (Bouvier *et al.*, 1989; Carpene *et al.*, 1993); β_1-ARs can also desensitise to some degree (Carpene *et al.*, 1993), but β_3-ARs show very little, if any, desensitization (Carpene *et al.*, 1993; Nantel *et al.*, 1993). Since the stimulation of β_2-ARs in the chick brain is required for memory consolidation (Gibbs & Summers, 2000), it is possible that the memory impairment in prenatally hypoxic chicks could be due to the β_2-ARs being desensitized or down-regulated by prenatal exposure to circulating catecholamines. In order to support this hypothesis, it would be necessary to show that stimulation of β_2-ARs by the exogenous administration of β_2-AR agonists would be ineffective in promoting memory consolidation in prenatally hypoxic chicks, whereas β_3-AR agonists would be able to consolidate labile memory seen in prenatally compromised chicks. Hence, the aim of Part 2 of this chapter was to determine whether memory consolidation can be promoted in prenatally hypoxic chicks using β_2- and β_3-AR agonists. The ability of the β-AR agonists to promote memory consolidation was assessed using a one-trial discriminated bead task, as described in Section 2.1.3.1. Three groups of chicks were examined; chicks from eggs wrapped from days 14-18, 10-18, and 10-14. Each group consisted of 3 sub-groups, which received an intracranial

injection of the β_2-agonist zinterol, the β_3-agonist CL316243, or saline. Given the difference in the nature of the memory impairment between the three groups of prenatally hypoxic chicks (Chapter 2), and the plasma catecholamine levels of the W14-18 and W10-18 chicks 2 days after hatching (Chapter 3), it is possible that the responses of the prenatally hypoxic chicks to the β-AR agonists may differ.

4.1 Methods: Part 1, The action of adrenaline in memory consolidation

The aim of Part 1 of this chapter was to examine whether adrenaline promotes memory consolidation in the neonatal chick, and determine the roles played by different AR subtypes in the action of adrenaline on memory consolidation.

4.1.1 Discriminated bead task

The discriminated bead task, as described in detail in Section 2.1.3.1, can be altered by diluting the aversant on the red bead to a 20% solution of anthranilate. With the weaker aversant, chicks will remember the bead for about 30 minutes, but the memory disappears and on later tests the chicks will peck at the red bead nearly as much as the blue bead (Crowe *et al.*, 1989b). This weakly reinforced version of the task has been a beneficial tool for investigating ways of pharmacologically promoting the consolidation of memory (Gibbs & Summers, 2002b). It is possible to alter the labile memory from one that lasts for only 30 minutes to one that lasts for at least 2 hours after training by exogenous administration of noradrenaline, β_2-AR agonists, and β_3-AR agonists (Gibbs & Summers, 2000).

The weakly reinforced version of the discriminated bead task was used to determine whether exogenous adrenaline promoted the consolidation of memory. Day-old male chicks were trained on 20% anthranilate and administered a range of doses of adrenaline 20 minutes after training. In a separate experiment, adrenaline (10 μl, 100 pmol/ hemisphere) was administered at different times before or after the training trial to determine the time at which adrenaline is able to promote consolidation. The central action of noradrenaline on memory has been attributed to actions on different ARs depending on the level of noradrenaline administered. To determine whether adrenaline acts in the same manner as noradrenaline, the effects of adrenaline on memory consolidation were challenged by selective AR antagonists. In separate groups of

chicks, saline, or the AR antagonists propranolol (β_{1+2}), SR59230 (β_3), or prazosin (α_1), were administered subcutaneously 5 min after training; adrenaline was given intracranially 20 min after weakly reinforced training. Although propranolol inhibits both β_1- and β_2-ARs, β_1-ARs are not involved in memory consolidation at 20-30 min after training (Gibbs & Summers, 2002b). Retention testing was assessed 120 minutes following training. If the chicks remember that the red bead has tasted bitter on the training trial, then they will avoid the bead or peck only a small number of times compared to the number of pecks they make at the blue bead (Discrimination ratio (DR) score of ~0.9). If the chicks forget the training trial, then they will peck at the red bead the same number of times as at blue bead (DR score approaching 0.5).

The results of the experiments outlined above were compared with those previously obtained using noradrenaline (Gibbs & Summers, 2000).

4.1.2 Drugs and injections

The subcutaneous injections (100 µl) of the AR antagonists were given 5 min after training into a fold of the skin, ventrally over the rib cage (propranolol: 100 nmol; SR59230: 30 pmol; prazosin: 1 nmol). As the blood-brain barrier in the day-old chick is poorly developed, systemically injected drugs rapidly diffuse into the brain. The bilateral intracranial injections of adrenaline were made with a 27-gauge needle (5 or 10 µl, 0.1 µM-1 mM; doses: 0.001-10.0 nmol/hemisphere) into the IMHV region of the chick brain (Fig. 4.1), an area considered to be involved in memory formation (Kossut & Rose, 1984; Rose & Csillag, 1985; Sedman *et al.*, 1991; Gibbs & Summers, 2002b). The intracranial injections were made into loosely restrained, conscious chicks. As the skull of the neonatal chick is unossified, intracranial injections can be made freehand with little disturbance. The injection site was 2 mm from the midline and 3 mm from the suture between the forebrain and the cerebellum (Gibbs & Summers, 2000). A sleeve was placed on the needle to limit the depth of the injection to 3.5 mm.

The following drugs used in this study were obtained from the Sigma Chemical Company (St. Louis, USA): (-)-epinephrine bitartrate, (-)-propranolol, prazosin hydrochloride. SR59230A (3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronapth-1-

ylamino]-2S-2-propanol oxalate) was kindly donated by Dr. Luciano Manara (SANOFI-MIDY S.p.A. Research Centre, Milan, Italy).

4.1.3 Statistical Analysis

All data are presented as mean ± SEM. The number of chicks in each group that were included in statistical analyses varied between 15 and 20. Data from the dose-response relationship and time of injection of adrenaline were analysed using a one-way analysis of variance (ANOVA) with unweighted means. Dunnett's test was used for post hoc analysis. Data from the AR antagonist challenge studies were analysed using a two-way ANOVA with unweighted means. For the two-way ANOVA, post hoc analysis was simple main effects. If data were found to be heterogeneous, square root or log transformations were performed and the most homogenous transformation was used for further analysis. Statistical significance was accepted at the level of $P<0.05$.

4.2 Results: Part 1, The action of adrenaline in memory consolidation

4.2.1 Involvement of adrenaline in promoting memory consolidation

4.2.1.1 Adrenaline dose-response relationship

When adrenaline was administered into the IMHV 20 min after weakly reinforced training (20% anthranilate), there was a dose-dependant effect on memory consolidation ($F_{5,107}=13.859$, $P<0.001$; Fig. 4.2). Post hoc analysis revealed that administration of 0.01 and 0.1 nmol (both $P<0.001$) of adrenaline/hemisphere promoted the consolidation of a labile memory when compared to saline, whereas doses below and above these values did not produce a significant increase in retention levels ($P>0.05$).

4.2.1.2 Time of injection of adrenaline

The dose of adrenaline that optimally promoted memory consolidation (0.1 nmol, Fig. 4.2) was administered at selected times before and after weakly reinforced training to identify when memory loss first occurred. Varying the time of injection revealed five time periods at which adrenaline was effective in promoting memory ($F_{6,116}=11.577$, $P<0.001$; Fig. 4.3). Post hoc analysis revealed that adrenaline consolidated memory when administered from 10 min prior to training up until 30 min after ($P<0.05$), but not when injected after the expiry of labile memory at 40 minutes post training ($P=0.997$).

The results from this study identified a time frame in which memory was susceptible to modulation by adrenaline.

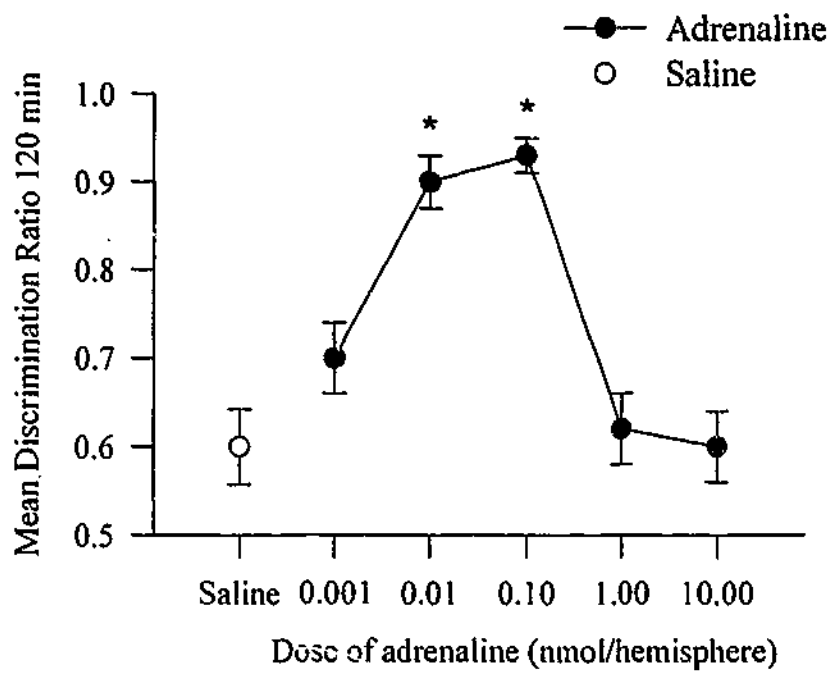


Fig. 4.2. Dose-response relationship for adrenaline at 120 minutes after weakly reinforced training. Administration of 0.01 and 0.1 nmol of adrenaline/hemisphere promoted memory consolidation, whereas doses below and above these values did not produce a significant increase in retention levels. Asterisks (*) indicate values that are significantly different from saline value.

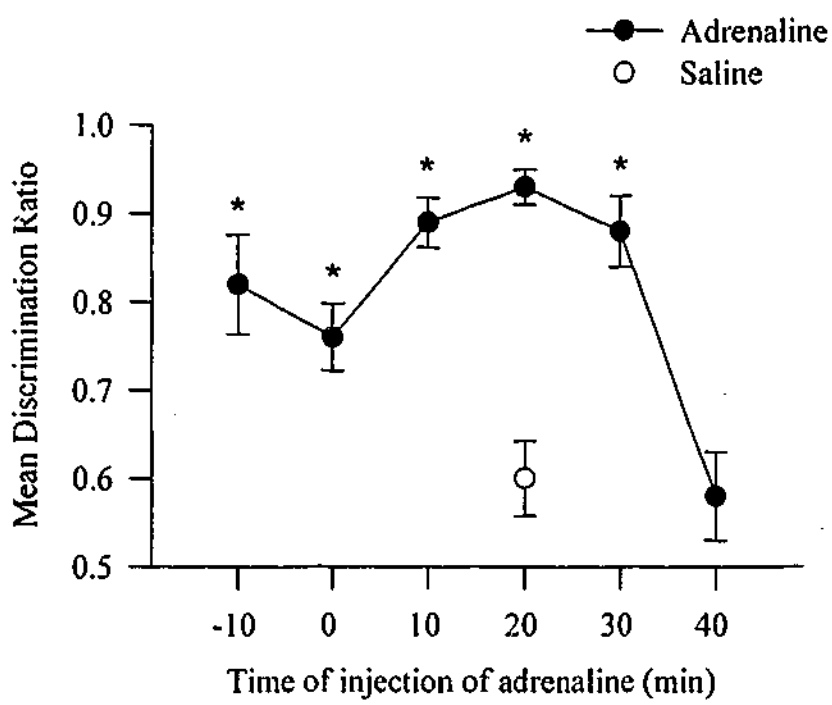


Fig. 4.3. The effect of administering 0.1 nmol of adrenaline/hemisphere before and after weakly reinforced. When adrenaline is administered 10 min prior to training up until 30 min after training, memory consolidation is enhanced. Asterisks (*) indicate values that are significantly different from saline value.

4.2.2 Adrenoceptor involvement in the action of adrenaline on memory consolidation

4.2.2.1 Challenge by β_{1+2} adrenoceptor antagonist

Administration of the β_{1+2}-AR antagonist, propranolol (100 nmol), 5 min after weakly reinforced training, prevented intermediate doses of adrenaline from promoting memory consolidation. Analysis of variance (two-way) revealed a significant difference between saline and propranolol pretreatment ($F_{1,139}$=14.93, P<0.001; Fig. 4.4), and a significant interaction ($F_{3,139}$=4.61, P=0.004). Simple main effects analysis revealed that inhibition of β_{1+2}-ARs by propranolol prevented adrenaline at doses of 0.03 nmol ($F_{1,142}$=8.85, P=0.003) and 0.1 nmol of adrenaline/hemisphere ($F_{1,142}$=18.06, P<0.001) from consolidating memory.

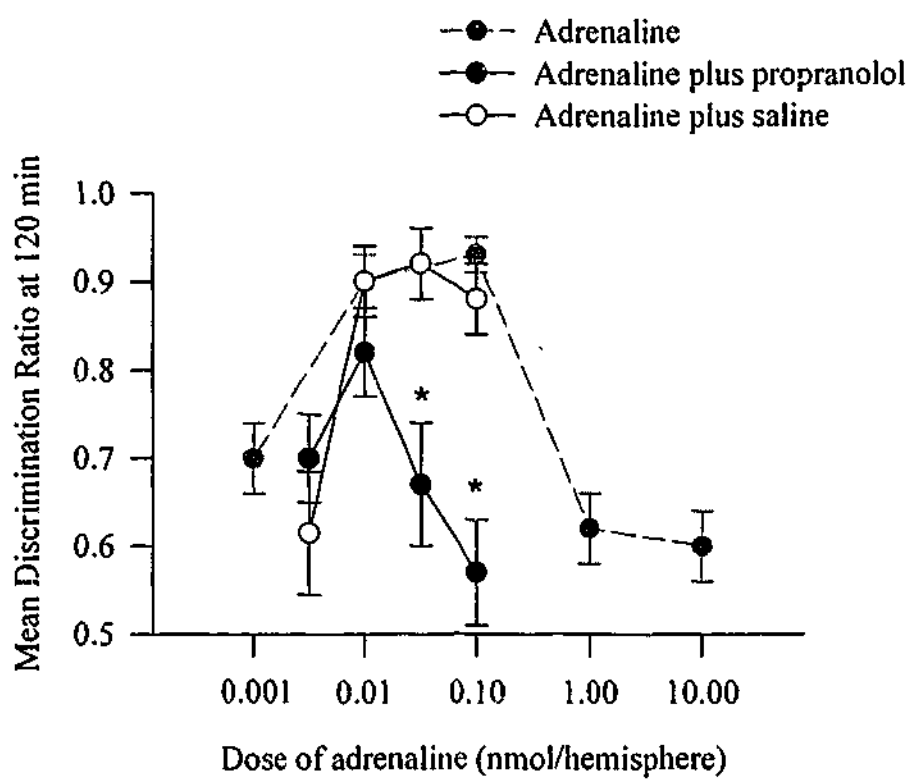


Fig. 4.4. The effect of adrenaline on memory consolidation in the presence of the β_{1+2}-AR antagonist propranolol (100 nmol). At intermediate doses of adrenaline (0.03 and 0.1 nmol), propranolol prevented memory consolidation, suggesting that at these doses, adrenaline acts at β_2-ARs to promote memory consolidation. Asterisks (*) indicate values that are significantly different from adrenaline plus saline values.

4.2.2.2 Challenge by β_3 adrenoceptor antagonist

When β_3-ARs were inhibited with SR59230 (30 pmol), the dose-response curve for the lower doses of adrenaline showed a shift to the right. Analysis of variance (two-way) revealed a significant difference between saline and SR590230 pretreatment ($F_{1,142}$=10.10, P=0.002; Fig. 4.5), and a significant interaction ($F_{3,142}$=6.54, P<0.001). Simple main effects analysis revealed that SR59230 successfully prevented 0.01 nmol of adrenaline/hemisphere from promoting memory consolidation ($F_{1,145}$=23.82, P<0.001).

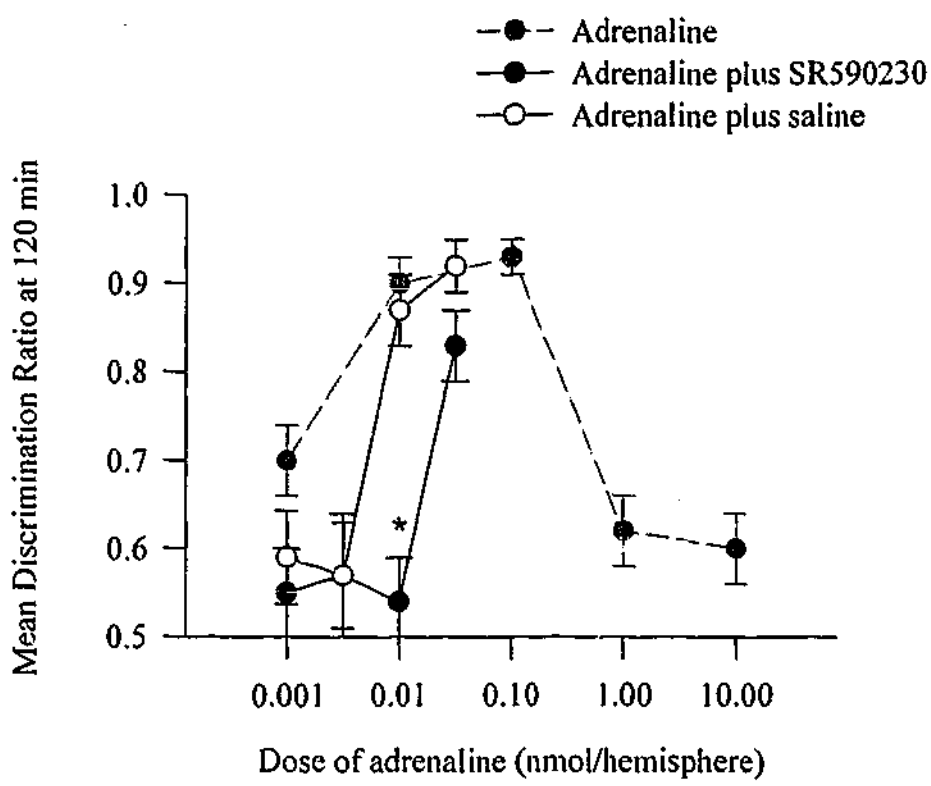


Fig. 4.5. The effect of adrenaline on memory consolidation in the presence of the β_3-AR antagonist SR59230 (30 pmol). At low doses of adrenaline (0.01), SR59230 prevented memory consolidation, indicating that at this dose, adrenaline has its action at β_3-ARs to promote memory consolidation. Asterisk (*) indicates value that is significantly different from adrenaline plus saline value.

4.2.2.3 Challenge by α_1- adrenoceptor antagonist

Administration of the α_1-AR antagonist, prazosin (1 nmol), 5 min after weakly reinforced training, prevented high doses of adrenaline from inhibiting memory consolidation. Analysis of variance (two-way) revealed a significant difference between saline and prazosin pretreatment ($F_{1,144}$=58.88, P<0.001; Fig. 4.6), at doses of adrenaline between 0.3 and 10 nmol of adrenaline/hemisphere. (0.3: $F_{1,147}$=7.16; 1.0: $F_{1,147}$=16.69; 3.0: $F_{1,147}$=17.94 and 10.0: $F_{1,147}$=19.73; P<0.05).

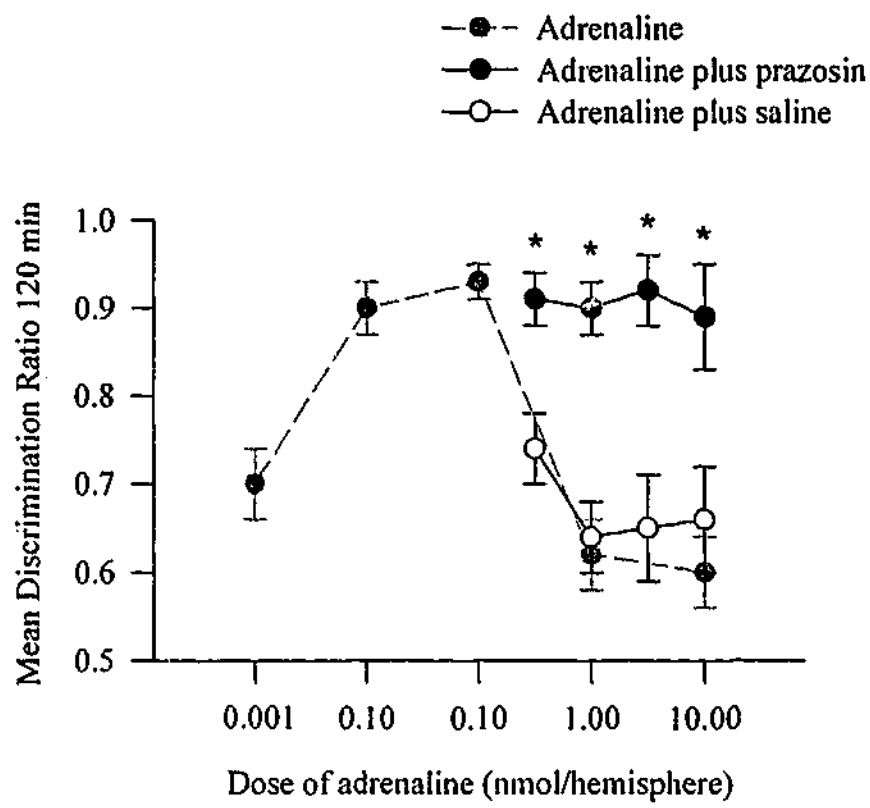


Fig. 4.6. The effect of adrenaline on memory consolidation in the presence of the α_1-AR antagonist, prazosin (1 nmol). Prazosin successfully prevented high doses of adrenaline (0.3-10.0 nmol) from inhibiting memory consolidation, suggesting that at these doses, adrenaline acts at α_1-ARs to prevent memory consolidation. Asterisks (*) indicate values that are significantly different from adrenaline plus saline values.

4.2.3 Summary of the action of adrenaline on memory consolidation

When adrenaline was administered alone by bilateral intracranial injection into the IMHV 20 min after training on 20% anthranilate, a dose-dependant effect on memory retention was observed. Doses of 0.01 and 0.1 nmol of adrenaline/hemisphere significantly enhanced the consolidation of the labile memory, whereas higher doses of adrenaline (1.0 and 10.0 nmol of adrenaline/hemisphere) did not promote consolidation. Adrenaline was found to promote consolidation if administered from 10 min prior to weakly reinforced training, and up until 30 min after. Blockade of either the β_2-ARs by propranolol, or the β_3-ARs by SR59230 revealed that, at low doses, adrenaline was acting via β_3-ARs, and at higher doses through β_2-ARs. At even higher doses, adrenaline appears to inhibit memory by acting at α_1- ARs.

4.2.4 Comparison of the action of adrenaline to noradrenaline in memory consolidation

The promotion of memory consolidation by both adrenaline and noradrenaline at low doses appears to be attributable to β_3-ARs, and at intermediate doses to β_2-ARs (Fig. 4.7). Higher doses of adrenaline and noradrenaline inhibit memory consolidation, and this effect appears to be mediated by α_1- ARs. Comparing between adrenaline and noradrenaline, it appears that lower doses of adrenaline are more effective in promoting memory consolidation than noradrenaline, supporting the fact that adrenaline has a higher potency in eliciting β-ARs mediated effects than noradrenaline (Rang *et al.*, 1995).

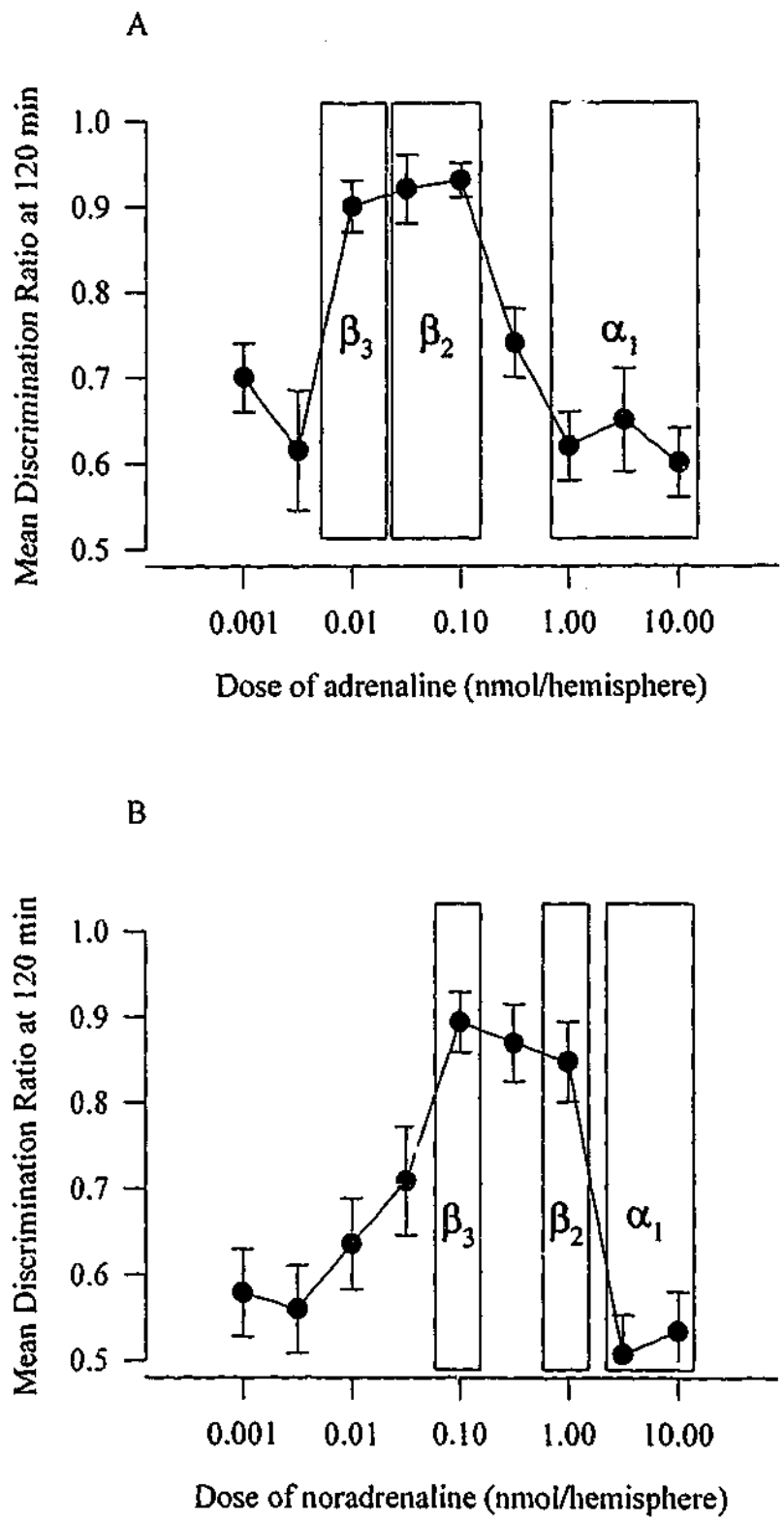


Fig. 4.7. Summary of adrenoceptor (AR) involvement in the enhancement of consolidation of weakly reinforced memory by adrenaline (A) *and noradrenaline* (B) *(Figure 4.7B adapted from Gibbs, M. E. & Summers, R. J. (2000). Neuroscience, 95: 913-922). The promotion of memory consolidation by both adrenaline and noradrenaline at low doses is attributable to β_3-ARs, and at intermediate doses to β_2-ARs. Higher doses of adrenaline and noradrenaline inhibit memory consolidation, and this effect appears to be mediated by α_1-ARs. Comparing the effects of adrenaline and noradrenaline, it appears that lower doses of adrenaline are more effective in promoting memory consolidation than noradrenaline.*

4.3 Methods: Part 2, Administration of β_2- and β_3-adrenoceptor agonists to prenatally hypoxic chicks.

The aim of Part 2 of this chapter was to determine whether memory consolidation can be promoted in prenatally hypoxic chicks using β_2- and β_3-AR agonists. I proposed that AR function may have been altered due to long-term exposure to circulating noradrenaline and adrenaline levels. If this is the case, β_2-ARs may become desensitized in response to excessive stimulation (Bouvier *et al.*, 1989; Carpene *et al.*, 1993; Rang *et al.*, 1995), whereas β_3-ARs may show very little, if any, desensitization (Carpene *et al.*, 1993; Nantel *et al.*, 1993). Therefore, it is hypothesised that stimulation of β_2-ARs by exogenous administration with β_2-AR agonists will be ineffective in promoting memory consolidation in prenatally hypoxic chicks, whereas administration of β_3-AR agonists will be able to consolidate labile memory seen in these compromised chicks.

4.3.1 Incubation and hatching

Fertile eggs were half-wrapped lengthways from either days 14-18, 10-18, or 10-14, and incubated with control eggs (uncovered) under the same conditions reported in Section 2.1.1. Chicks were allowed to hatch in the incubator, after which they were housed in groups of approximately 10 in communal brooders for 1-2 days until postnatal testing commenced.

4.3.2 Discriminated bead task

Procedures for the discriminated bead task have been described in Section 2.1.3. In the present study, three groups of chicks were examined, consisting of W14-18, W10-18, and W10-14 chicks. All of these chicks were trained on 100% anthranilate. As reported in Chapter 2, the memory in chicks from wrapped eggs trained on 100% anthranilate resembles what is seen with weakly reinforced training (e.g. 20% anthranilate) in normal chicks; that is long-term memory consolidation is not achieved. Each group consisted of 3 sub-groups, which received either (a) the β_2-AR agonist zinterol (3.0 pmol/ hemisphere), (b) the β_3-AR agonist CL316243 (0.3 pmol/hemisphere), or (c) saline, 20 minutes after training. These agents were injected bilaterally into the IMHV region of the chick brain (see Section 4.1.2) with a 27-gauge needle (volume: 5 µl/hemisphere). Memory retention was tested 120 minutes following training.

CL316243 (disodium (R,R)-5-[2-[[2-3-Chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate) was kindly donated by Dr. Tim Nash (Wyeth Pharmaceuticals, Sydney, Australia). Zinterol hydrochloride was obtained from Bristol-Myers Squibb (Noble Park, Australia).

4.3.3 Statistical Analysis

All data are presented as mean ± SEM. Data from the discriminated bead task were analysed using a one-way ANOVA with unweighted means. Dunnett's test was used for post hoc analysis. If data were found to be heterogeneous, square root or log transformations were performed and the most homogenous transformation was used for further analysis. Statistical significance was accepted at the level of $P<0.05$. Note usually 14-20 chicks per group are required in order that simple statistical tests of differences between means are sufficiently powerful to detect a difference of about one standard deviation (K. Ng, *personal communication*). In the experiments outlined below, 12 or less chicks per group were studied, which may have limited the power of subsequent statistical analyses.

4.4 Results: Part 2, Administration of β_2- and β_3-AR agonists to prenatally hypoxic chicks.

4.4.1 Discriminated bead task

4.4.1.1 Effectiveness of β-AR agonists in promoting memory consolidation

As mentioned in Section 4.1.1, if 20% anthranilate is used rather than 100% on the training trial, chicks form a labile memory, which lasts for only 30 minutes. By 120 min after weakly reinforced training, the memory disappears, and chicks peck at the red bead nearly as much as the blue bead (Crowe *et al.*, 1989b) (see Fig. 4.8 for DR score at 120 min after weakly reinforced training). Memory consolidation following weakly reinforced training can be promoted by injecting β_2- or β_3-AR agonists 20 min after weakly reinforced training into the IMHV (Gibbs & Summers, 2000). As indicated in Fig. 4.8, administration of β_2- and β_3-AR agonists clearly enhances memory consolidation, to levels indistinguishable from that obtained following strongly reinforced training (100% anthranilate).

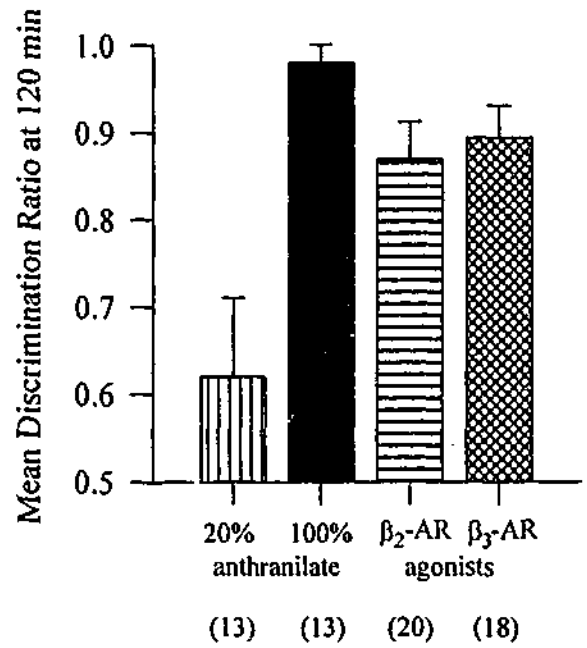


Fig. 4.8. *Effects on memory retention following training on 20% and 100% anthranilate, and the effects of administering β_2- and β_3-AR agonists 20 min after weakly reinforced training (20% anthranilate). When chicks are trained on 20% anthranilate rather than 100%, the memory is gradually lost, and by 120 min after training, chicks peck at the red bead nearly as much as the blue bead. Administration of either β_2- or β_3-AR agonists 20 min after weakly reinforced training is effective in consolidating weakly reinforced memory. Animal numbers are in parentheses. (Results for the β_2- and β_3-AR agonists from Gibbs, M. E. & Summers, R. J. (2000). Neuroscience, 95: 913-922).*

4.4.1.2 Administration of β_2- and β_3-AR agonists to W14-18 chicks

Analysis of variance (one-way) revealed a significant effect of β-AR agonist administration into the IMHV 20 min after weakly reinforced training in the W14-18 chicks ($F_{2,29}$=4.367, P=0.022; Fig. 4.9). Post hoc analysis revealed that injection of the β_3-AR agonist, CL316243, promoted memory consolidation in the W14-18 chicks (DR 0.87±0.05 vs. 0.60±0.07, P=0.021). Conversely, the β_2-AR agonist zinterol did not enhance memory consolidation. Retention levels were indistinguishable from that obtained from W14-18 chicks administered saline (DR 0.60±0.1 vs. DR 0.60±0.07, P=0.995).

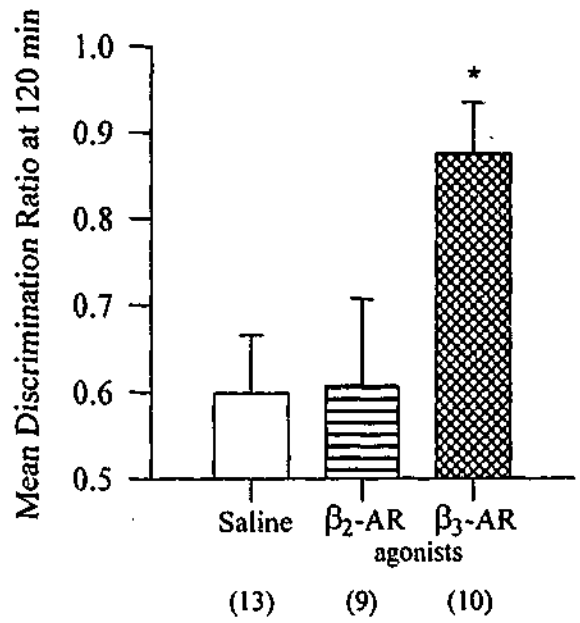


Fig. 4.9. The effects of administering saline, the β_2-AR agonist zinterol or the β_3-AR agonist CL316243, on memory retention in W14-18 chicks following training on 100% anthranilate. Administration of the β_3-AR agonist clearly enhanced memory consolidation. An asterisk (*) indicates value that is significantly different from saline value. Animal numbers are in parentheses.

4.4.1.3 Administration of β_2- and β_3-AR agonists to W10-18 chicks

Analysis of variance (one-way) revealed a significant effect of β-AR agonist administration in the W10-18 chicks ($F_{2,29}$=6.126, P=0.006; Fig. 4.10). In contrast to the results obtained from the W14-18 chicks, the β_2-AR agonist zinterol enhanced memory consolidation in the W10-18 chicks. Retention levels were significantly higher than that obtained from saline administered W10-18 chicks (DR 0.92±0.04 vs. 0.65±0.08, P=0.009). Consolidation was also promoted after the β_3-AR agonist CL316243 was administered (DR 0.92±0.06 vs. 0.65±0.08, P=0.013).

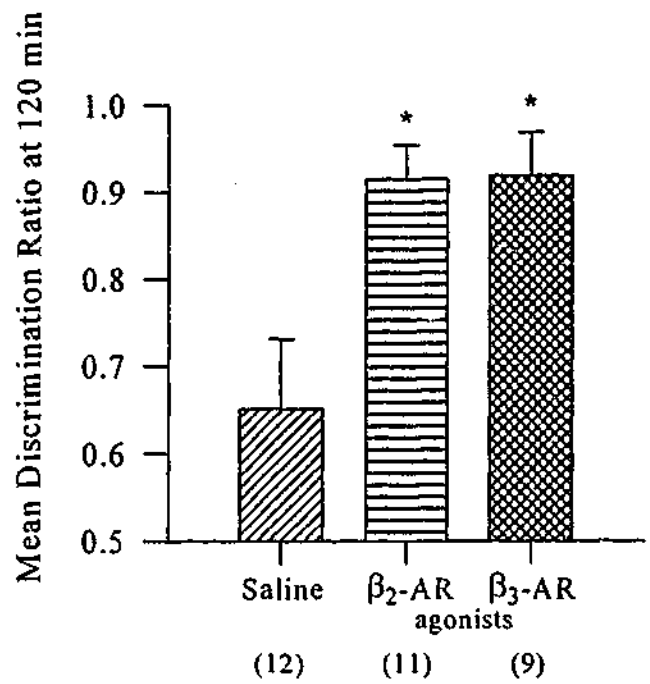


Fig. 4.10. *The effects of administering saline, the β_2-AR agonist zinterol or the β_3-AR agonist CL316243, on memory retention in W10-18 chicks following training on 100% anthranilate. Both the β_2- and β_3-AR agonists enhanced memory consolidation. Asterisks (*) indicate values that are significantly different from saline value. Animal numbers are in parentheses.*

4.4.1.4 Administration of β_2- and β_3-AR agonists to W10-14 chicks

Analysis of variance (one-way) revealed that neither the β_2- or β_3-AR agonists enhanced memory consolidation in the W10-14 chicks ($F_{2,32}$=2.721, P=0.081; Fig. 4.11). Of note, retention levels of the W10-14 chicks that were administered saline were slightly higher than observed previously.

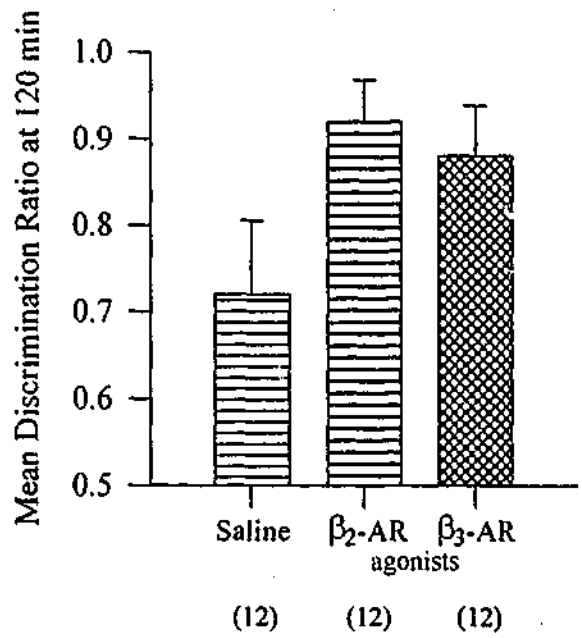


Fig. 4.11. *The effects of administering saline, the β_2-AR agonist zinterol or the β_3-AR agonist CL316243, on memory retention in W10-14 chicks following training on 100% anthranilate. Although the β_2- and β_3-AR agonists appeared to enhance memory consolidation, the increase in DR score was not significant. Animal numbers are in parentheses.*

4.5 Discussion: Part 1, The action of adrenaline in memory consolidation

The results from Part 1 of this chapter show that administration of adrenaline into the IMHV of the chick brain promotes the consolidation of a labile memory in a dose-dependant manner, similar to that seen with noradrenaline (Gibbs & Summers, 2000). The action of low doses of adrenaline was prevented by prior administration of the β_3-AR antagonist SR59230, whereas the action of intermediate doses of adrenaline was prevented by the β_{1+2}-AR antagonist propranolol. High doses of adrenaline inhibited memory consolidation, and this effect was prevented by the α_1-AR antagonist prazosin.

At present, it can not be determined which cells adrenaline acts on to influence memory storage. β-ARs are present on neurons and cerebral blood vessels (Cohen *et al.*, 1997), and may be localised on astrocytes (Aoki, 1992; Mantyh *et al.*, 1995). The action of adrenaline could potentially be at any one of these sites, and the effects of adrenaline on memory consolidation could be due to actions on neuronal communication, regional cerebral blood flow or cerebral metabolism (Gibbs & Summers, 2000).

Although the action of adrenaline on the different AR subtypes appears to be similar to that of noradrenaline (Gibbs & Summers, 2000), the potency of adrenaline in eliciting β-ARs mediated effects is higher than that of noradrenaline. This finding is in agreement with its pharmacology, where adrenaline has a higher affinity for β-ARs than noradrenaline (Rang *et al.*, 1995).

The results from Part 1 of this chapter demonstrated that stimulation of either β_2- or β_3-ARs in the IMHV of the chick brain with adrenaline consolidates memory. The ability of adrenaline to promote memory storage in dose-dependant manner, by acting at different AR subtypes, is similar to that of noradrenaline (Gibbs & Summers, 2000).

4.6 Discussion: Part 2, Administration of β_2- and β_3-AR agonists to prenatally hypoxic chicks.

We are not proposing the increased catecholamine levels post-hatch affects memory function. Instead, we are suggesting that AR function has been altered *in ovo*, due to an increase in catecholamine levels in response to the hypoxic insult. The results reported

in Part 2 of this chapter show that administration of β-AR agonists can promote memory consolidation in prenatally hypoxic chicks. Memory consolidation was promoted in the W10-18 chicks by administration of either the β_2-AR agonist zinterol, or the β_3-AR agonist CL316243. However, administration of the β_2-AR agonist to the W14-18 chicks did not enhance consolidation. Administration of the β-AR agonists to the W10-14 chicks did not enhance memory consolidation. Generally, retention levels of W10-14 chicks at 120 min after training are lower than that reported in this study, which may explain my administration of the β-AR agonists did not significantly enhance memory consolidation. These results suggest that the β_2-ARs may have been unresponsive or inactive in the W14-18 chicks. As mentioned in the introduction, the β_2-ARs may have become desensitized or down-regulated, possibly due to excessive stimulation from prolonged exposure to high levels of circulating plasma noradrenaline and adrenaline levels *in ovo*. The hypothesis that the β_2-AR agonist was unable to promote memory consolidation in the W14-18 chicks because the β_2-ARs were desensitized or down-regulated, is supported by the findings of Gibbs and Summers (*unpublished observation*), showing that the long acting β_2-AR agonist, formoterol, when given 24 hrs before weakly reinforced training, produced a similar result to those obtained with the W14-18 chicks in the present study; this is, memory was promoted by β_3-AR agonists but not by the β_2-AR agonists. These findings suggest that the cause of the cognitive impairment in the prenatally hypoxic chicks could be related to desensitization or down-regulation of β_2-ARs in the brain.

At present, we are unable to determine definitively from the results reported in this chapter whether the β_2-ARs were desensitized or down-regulated as a consequence of the elevated catecholamine concentrations. Receptor phosphorylation by cAMP-dependent protein kinases is one mechanism by which the phenomenon of desensitization develops during conditions of prolonged agonist exposure (Rang *et al.*, 1995; McGraw *et al.*, 1998). Future studies could determine whether AR desensitization has occurred in the areas of the chick brain that are involved in memory consolidation by measuring cyclic AMP activity (McGraw *et al.*, 1998). In addition, β-AR binding studies would provide valuable information about whether prolonged exposure to

catecholamines *in ovo* results in the reduction in the number of β-ARs (Manier *et al.*, 1987; Rang *et al.*, 1995).

4.6.1 Implications and Conclusions

The results from Part 1 of this chapter show that adrenaline acts via β_2- or β_3-ARs in the IMHV of the chick brain to consolidate memory. Results from Part 2 of this chapter demonstrate that memory consolidation was promoted in the W10-18 chicks by administration of either β_2- or β_3-AR agonists, however, administration of the β_2-AR agonist zinterol to the W14-18 chicks did not enhance consolidation. In support of this conclusion, administration of the β_2-AR agonist did not promote memory consolidation in the W14-18 chicks, which suggests a decrease in β_2-AR receptor numbers or a loss of β_2-AR responsiveness as a consequence of the prenatal hypoxic insult. It has previously been shown that postnatal hypoxia for 3 weeks in the rat decreases the number of β-ARs in the cerebral cortex (Kramer *et al.*, 1987), hence it is possible that receptor numbers may have been reduced in the W14-18 chicks.

The results from the present study do not shed light on why the W10-18 chicks have impaired memory. The activation of the β_2- and β_3-ARs with zinterol and CL316243, respectively, promoted memory consolidation in these animals, suggesting that β_2- and β_3-AR function was not altered in these animals. Since the W10-18 chicks also had impaired short-term memory, the type of prenatal insult in these animals could be different than in the W14-18 chicks. β_1-ARs, which are involved in short-term memory formation (Gibbs & Summers, 2002b), can become desensitized to some degree if exposed for prolonged periods to catecholamines (Carpene *et al.*, 1993). Future studies could assess whether β_1-AR function is altered following prenatal hypoxia. Whilst the W10-18 chicks did tend to have increased circulating catecholamine levels, this increase was not as great as in the W14-18 chicks, or controls. Therefore, the memory deficit in the W10-18 chicks may not be explained by alterations in the central catecholaminergic system, but could be due to subtle alterations in brain development.

In conclusion, the alterations observed in catecholamine concentrations in plasma and brain tissue reported Chapter 3, in conjunction with the inability of the β_2-AR agonist to

promote memory consolidation in the W14-18 chicks, supports the hypothesis that the functioning of the central catecholaminergic system may be altered following prenatal hypoxia, particularly if the insult is later in gestation.

Chapter 5

The effect of prenatal hypoxia on postnatal brain structure

IT IS NOW widely accepted that, in mammals, adverse prenatal conditions can impair brain development, with the severity and timing of the insult in relation to gestational age playing an important role in determining the extent and nature of the injury (Mallard *et al.*, 1995; Parer, 1998; Rees *et al.*, 1998). The overall aim of this chapter was to perform an examination of the brains of prenatally hypoxic chicks to determine whether the impaired ability of these animals to consolidate memories is associated with structural and/or neurochemical alterations in the brain, particularly in areas of the chick brain involved in memory formation. Since the nature of the memory impairment between the W14-18 and W10-18 chicks differed, with some of the W10-18 chicks having poor labile memory as well as impaired long-term memory consolidation, it may be expected that the impact of prenatal hypoxia on brain development will differ depending on the developmental stage at which the insult was induced.

Histologic and immunohistochemical techniques have been used to assess structural and neurochemical changes in the brain following prenatal hypoxia. The brains of both groups of wrapped eggs and controls were assessed postnatally (2 days after hatching), in order to determine whether there was an association between postnatal brain structure and brain function.

Previous studies have shown that chronic placental insufficiency, which leads to fetal hypoxemia in sheep (Robinson *et al.*, 1979; Cock & Harding, 1997) and guinea pigs (Jones & Parer, 1983), results in the delay of development of basic developmental processes such as neuronal migration and synaptogenesis in several regions of the brain including the cerebral cortex (Bisignano & Rees, 1988), hippocampus (Rees *et al.*, 1988), and cerebellum (Bisignano & Rees, 1988; Rees *et al.*, 1988; Nitsos & Rees, 1990; Mallard *et al.*, 1998). Injury to the brain following placental insufficiency *in utero* have also been reported, and include cortical white matter lesions (Mallard *et al.*, 1998), and proliferation of astroglia (Nitsos & Rees, 1990; Mallard *et al.*, 1998). Prenatal hypoxia in the chick embryo, induced by sealing half of the air cell with melted wax on day 2 of incubation until days 18-20, has been shown to result in a reduction in Purkinje cell size and density, as well as dendrite development, in the cerebellum (Lee *et al.*, 2001). Since experimentally induced prenatal hypoxia leads to alterations in brain development and structure, the first aim of the present study was to assess the brains of control and prenatally hypoxic chicks for any gross morphological changes that could be associated with impaired postnatal memory. As changes in the cerebral vasculature have been reported following prenatal hypoxia in the frontal cortex of fetal sheep (Mallard *et al.*, 1998; Rees *et al.*, 1998), and in the optic tectum of chick embryos (Ribatti *et al.*, 1989), the percentage of the brain parenchyma occupied by blood vessels was also assessed. This measure was used as an indicator of prenatal hypoxia and not directly as an indicator of altered memory function.

The second aim of this study was to assess the immunoreactivity of glutamine synthetase (GS), the astrocyte-specific enzyme, in the intermediate hyperstriatum ventrale (IMHV) of control and prenatally hypoxic chicks, to demonstrate effects on astrocyte numbers. Glial fibrillary acidic protein (GFAP) is the major structural protein in the intermediate filaments of astroglial cells (Eng *et al.*, 1971) and is frequently used as a marker for fibrous astrocytes, which are the predominant class of astrocytes in

white matter. Increases in GFAP immunoreactivity indicates that an astrocyte is in a reactive state. In the present study, however, the effects of prenatal hypoxia on astrocyte numbers, rather than the astrocyte response, was of primary interest. As mentioned in Section 1.12, there is compelling evidence for the involvement of astrocytes during the establishment and consolidation of memory in the chick (O'Dowd *et al.*, 1994a; Gibbs *et al.*, 1996; Gibbs & Summers, 2002a). Hence, it is possible that the impaired ability of the prenatally hypoxic chicks to consolidate memories may be due to a reduction in the number of astrocytes in the IMHV region of the chick brain, an area critically involved in memory consolidation (Kossut & Rose, 1984; Rose & Csillag, 1985; Sedman *et al.*, 1991; Gibbs & Summers, 2002b). Since the normal development of astrocytes in the chick embryo brain has not been fully investigated in either the IMHV or lobus parolfactorius (LPO), an additional aim was to study the ontogeny of astrocytes at the period over which hypoxia was induced, namely embryonic days 10, 14, 16, 18. Left-right asymmetries have been observed in synaptic parameters during development in the chick (Curtis *et al.*, 1989; Hunter & Stewart, 1989) and following passive avoidance learning (Stewart *et al.*, 1984; Gibbs *et al.*, 2002), hence, astrocyte and neuronal populations were examined in each hemisphere.

As reported in Chapter 3, there was an overall decrease in noradrenaline levels in the anterior forebrain of both groups of prenatally hypoxic chicks, as determined by high-performance liquid chromatography (HPLC). Whilst HPLC analysis indicated an overall decrease in noradrenaline, immunohistochemistry is required to localise specific regions within the anterior forebrain that may have been affected following the prenatal insult. Therefore, the third aim of the present study was to assess the immunoreactivity for tyrosine hydroxylase (TH)- the rate-limiting enzyme involved in the catecholamine synthesizing pathway, in the anterior forebrain, to elucidate the nature of the reduction in noradrenaline.

5.1 Materials and Methods

5.1.1 Incubation and hatching

Fertile eggs were half wrapped from days 14-18 and 10-18, respectively, and incubated with control eggs (uncovered) under the same conditions reported in Section 2.1.1. Chicks were allowed to hatch in the incubator, after which they were housed in groups

of approximately 10 in communal brooders until postnatal testing commenced (see Section 2.13).

5.1.2 Perfusion and tissue preparation

5.1.2.1 Embryo brain tissue

At embryonic days 8, 10, 14, 16, and 18, chick embryos were quickly removed from their shells and decapitated. These ages were chosen as they corresponded to the period over which hypoxia was induced in Chapter 2. Brains were carefully removed, then immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4. Once fixed, the brains were transferred to 70% alcohol and processed through to paraffin wax in an automated vacuum infiltration processor (VIP, Bayer Australia) overnight. Once complete, the tissue was embedded into paraffin blocks and 10 μm coronal sections were cut on a rotary microtome (Leitz Wetzlar, Germany). Sections were collected serially in sets of four, mounted on gelatin-coated slides and placed in a 37°C oven overnight. Every tenth slide was used for immunohistochemical staining with GS.

5.1.2.2 Two-day old brain tissue

At 2 days post-hatching, a group of chicks from both experimental groups and controls were deeply anaesthetised using Nembutal (pentobarbitone sodium, 60 mg/ml: 0.2 ml i.p.). Note: the animals used in this study were not the same animals used in Chapter 2. Chicks were perfused via the left ventricle of the heart with saline, then 4% paraformaldehyde in 0.1 M PB (pH 7.4), followed by 4% paraformaldehyde containing 10% sucrose. The right atrium of the heart was incised in order to allow the perfusate to escape. Once the brain was fixed, the liver and heart were removed and weighed. Brains remained in the skull of the chick for 1 hour, after which they were removed and weighed (see Tables 5.1 and 5.2 for brain weights) and post-fixed in 4% paraformaldehyde in 0.1 M PB (pH 7.4) for 4 hours. Brains were then transferred to 70% alcohol and processed through to paraffin wax using the automated VIP system (Bayer Australia). Once complete, the tissue was embedded into paraffin blocks and 10 μm coronal sections were cut on a rotary microtome (Leitz Wetzlar, Germany). Sections were collected serially in sets of three; the intervening 3 sections (30 μm in total) were discarded. The tissue was mounted on gelatin-coated slides and placed in a 37°C oven

overnight. Every fifth slide was stained with haematoxylin and eosin (H&E) for structural analysis.

Table 5.1. Brain and brain to body weight ratios of control and W14-18 chicks at 2 days post-hatching. Animal numbers are in parentheses.

	Control (n=7) mean±SEM	*Wrap days 14-18 (n=6)* mean±SEM	*P value*
Brain (g)	0.83±0.03	0.76±0.04	0.14
Brain/Body wt (g/g)	$0.022 \pm 8.1 \times 10^{-4}$	$0.023 \pm 4.0 \times 10^{-4}$	0.32

Table 5.2. Brain and brain to body weight ratios of control and W10-18 chicks at 2 days post-hatching. Brain weights of the W10-18 chicks were significantly reduced compared to controls. Asterisk (*) indicates value that is significantly different from control value. Animal numbers are in parentheses.

	Control (n=7) mean±SEM	*Wrap days 10-18 (n=7)* mean±SEM	*P value*
Brain (g)	0.89±0.02	0.82±0.02*	0.046
Brain/Body wt (g/g)	$0.022 \pm 4.6 \times 10^{-4}$	$0.022 \pm 4.5 \times 10^{-4}$	0.83

5.1.3 Haematoxylin and eosin staining for light microscopy

Sections were placed in Histolene (Grale Scientific, Australia) to remove the paraffin wax, dehydrated in descending concentrations of alcohol and stained with haematoxylin (6 min) and counter-stained with eosin (3 min). Sections were then rehydrated and mounted with DPX and coverslipped.

5.1.4 Immunohistochemistry

Immunoreactivity for GS and TH was localised on gelatin-coated slides using the avidin-biotin peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA). Sections from the region of interest were taken at matched levels and were stained simultaneously to ensure uniform conditions for subsequent quantitative analysis. Sections were firstly placed in Histolene (Grale Scientific, Australia) to remove the paraffin wax, then dehydrated in descending concentrations of alcohol to water. To

allow for better penetration of the antibody for GS, slides were pre-treated with Proteinase K (Boehringer Mannheim, Germany). In this instance, slides were placed in buffered formalin for 10 min after being cleared in water. Sections were then given 3 washes (5 min each) in 0.1 M PB (pH 7.4), after which, they were placed in a solution containing 0.001% Proteinase K in PB for 15 mins at 37°C. Following this, the treatment for GS and TH sections were the same. Sections were given 3 x 5 min washes with 0.1 M PB. To block the activity of endogenous peroxidases, 0.3% hydrogen peroxide in methanol was then placed on each section for 20 min, after which they were given 3, 5 min washes with 0.1 M PB. Bovine serum albumin (BSA, 4%; Sigma. St. Louis, MO) was then placed on each section for 30 min. BSA acts as a blocking serum and binds surface antigens to reduce non-specific and background staining. Sections were then incubated in the appropriate concentration of primary antibody (Table 5.3) diluted in primary diluent (consisting of 0.1 M PB, 2% BSA and 0.3% tritonX-100) either for 48 (GS) or 72 hours (TH) at 4°C. TritonX-100 perforates cell membranes, allowing the primary antibody to enter and bind to the specific antigen. Negative controls were prepared by omitting the primary antibody from the incubation solution. In this instance, immunoreactivity failed to occur.

Table 5.3. The dilutions of the 1 °and 2 °antibodies used for immunohistochemistry.

Antibody	*Source*	*1 °antibody*	*2 °antibody*	*ABC kit dilution*
anti-glutamine synthetase (GS)	Chemicon	1:1000; 48 hrs	1: 400; 45 min	1:1:200; 45 min
anti-tyrosine hydroxylase (TH)	Chemicon	1:100; 72 hrs	1: 100; 24 hrs	1:1:200; 90 min

Following the incubation period for the primary antibody, sections were given 3 x 5 min washes with 0.05 M PB. This removed any excess antibody thereby reducing background staining. Sections were then incubated in the appropriate concentration of secondary antibody (biotinylated anti-mouse IgG; Table 5.3) diluted in secondary diluent (consisting of 0.1 M PB, 2% BSA) either for 45 min (GS) or 24 hrs (TH). Thirty minutes prior to use, the ABC was made up to the appropriate dilution using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA, GS: elite; TH: standard, Table 5.3). Following the incubation period for the secondary antibody,

sections were given 3 x 5 min washes with 0.05 M PB. The prepared ABC solution was then added to each section for 45 (GS) or 90 min (TH), after which sections were given 3 x 5 min washes with 0.05 M PB to remove the excess ABC. The chromagen, 3,3' diaminobenzidine (DAB), containing hydrogen peroxide, was then added to each section and left for 20 min. The peroxidase of the ABC releases oxygen from the hydrogen peroxide, which oxidises the DAB forming a brown precipitate. Sections were given 3 x 5 min washes with 0.05 M PB, counterstained with 0.01% thionin for 3-5 min, then rehydrated and mounted with DPX and coverslipped.

5.1.5 Qualitative and quantitative analysis

5.1.5.1 Quantitative analysis of the astrocyte/neuron ratio in the embryo brain

The number of GS-immunoreactive cells in the IMHV and LPO of the chick embryo was assessed to determine when astrocytes are present during development. The number of neurons was also determined within these regions to obtain the astrocyte/neuron ratio. Neurons were defined on the basis of being GS-negative and having a clear nucleus and a well defined nucleolus. The ratio of astrocytes/neurons was not assessed in the day 8 or 10 embryo brains as qualitative analysis of this tissue revealed that the astrocytes were not present and these ages. Subsequently, two animals from each age group (E14, E16, and E18) were analysed using sections taken at matched levels. The ratio of astrocytes/neurons in the IMHV and LPO was determined using an Olympus microscope (BX50), to which an automated stage and colour video camera were connected. The ratio of astrocytes/neurons was analysed using Computer Assisted Stereological Toolbox (C.A.S.T Grid, Olympus, Denmark) software. The area of the IMHV and LPO were demarcated at a final magnification of x40. High image resolution was achieved by placing immersion oil over the sections at a final magnification of x400. Three sections of the IMHV and two sections of the LPO were analysed from each animal, with three fields from each hemisphere being randomly selected for sampling. The numbers of GS-immunoreactive cells and neurons counted within the IMHV and LPO, respectively, were pooled for each animal and a mean value was calculated. Mean values for the control and experimental groups were then determined and expressed as the ratio of GS- immunoreactive cells to neurons per $mm^2 \pm$ SEM.

5.1.5.2 Haematoxylin and eosin stained sections of the 2 day-old brain tissue

Sections from control and prenatally hypoxic chicks were qualitatively assessed for evidence of brain injury, including lesions, gliosis, dying cells, and infarction. As changes in the cerebral vasculature have been reported following prenatal hypoxia, the percentage of the brain parenchyma occupied by blood vessels was assessed. The proportion of brain parenchyma occupied by blood vessels was determined on H&E sections comprising the rostral forebrain containing both the IMHV and LPO. Five sections from each animal were projected at a final magnification of x100, and one field from each hemisphere was randomly selected for sampling. A grid (10 x 10 cm) of evenly spaced points (1 cm intervals) was placed over the section. The number of points falling on blood vessels was counted and this number was expressed as a percentage of the total number of points on the grid. Results from each animal were pocled and a mean value was calculated. A mean of means ± SEM was calculated for each group.

5.1.5.3 Quantitative analysis of the astrocyte/neuron ratio in 2 day-old brain tissue

The number of GS- immunoreactive cells in the IMHV was assessed to determine whether prenatal hypoxia altered the number of astrocytes. The number of neurons was also determined within this region to obtain the astrocyte/neuron ratio. The procedure used to calculate this ratio was the same as for the embryo brains (see Section 5.1.5.1), except that five sections from each animal were analysed. Results from each animal were pooled and a mean value was calculated. A mean of means ± SEM was calculated for each group.

5.1.6 Statistical analysis

Not all measurements were made on all animals; the numbers used for each experiment are indicated. All measurements were made on coded slides, with the code not being broken until all measurements were made. Data are presented as mean of means ± SEM for each group. The ratio of astrocytes/neurons in the embryo brains was analysed using a one-way analysis of variance (ANOVA) for repeated measures. Post hoc analysis was performed using a test of least significant difference (LSD). All other data were analysed using an unpaired t-test. Statistical significance was accepted at the level of $P<0.05$.

5.2 Results

5.2.1 Brain development in the chick embryo

It was not my intention to construct an ontogenetic atlas of the chick embryo brain. Instead, the primary interest was to determine what structures were present at each embryonic age, and to see when astrocytes developed. Since there currently appears to be no ontogenetic study of the chick embryo brain, only structures that could be confidently detected at older ages were labelled. Note that the tissue was extremely fragile at embryonic days 8 and 10, which made it difficult to collect perfect sections, and thus label specific regions of the brain.

5.2.1.1 Ontogeny of astrocytes

Qualitative examination of the E8 and E10 brain tissue revealed that astrocytes were not present at either of these ages (E8: Fig. 5.1A,B; E10; Fig. 5.1C,D). Examination of the regions adjacent to the lateral ventricles at E8 and E10 revealed the presence of precursor cells and immature neurons and/or astrocytes (E8: Fig. 5.1B; E10: Fig. 5.1D). At E10, there was some background staining but no clear cellular staining. By E14 (Fig. 5.1E,F), astrocytes were clearly present and had short processes. At this age, it also appeared that the parenchyma was less densely packed with neurons. By E16 (Fig. 5.1G,H), the size of astrocytes and neurons both appeared to increase. The appearance of astrocytes and neurons at E18 (Fig. 5.1I,J) is similar to that at E16, although the meshwork of positively stained processes had increased.

5.2.1.2 Structural development

At E8 (Fig. 5.2), the lateral ventricle and optic tectum are clearly present. The ectostriatum, hippocampus, lamina hyperstriatica, and lamina frontalis superior are also evident. By E10 (Fig. 5.3), the hyperstriatum accessorium is present, and the optic tectum has developed significantly. By E10, the cerebellum has not developed. By E14 (Fig. 5.4A), the hyperstriatum ventrale, lamina medullaris dorsalis, LPO and neostriatum are clearly present. Between E10 and E14, the cerebellum has developed (Fig. 5.4B), and the size of the ventricle within the optic tectum has decreased. By E16 (Fig. 5.5), the olfactory bulb is present, and the folia of the cerebellum are evident. The appearance of the brain at E18 is similar to that at E16 (Fig. 5.6).

Fig. 5.1. Photomicrographs of coronal sections of embryo brains at days 8, 10, 14, 16 and 18 of gestation (A, C, E, G, I, respectively) stained with glutamine synthetase and thionin. Panels B, D, F, H, and J show at high power, neurons (black arrow), immature neurons and/or astrocytes (small black arrow) and GS-immunoreactive cells (clear arrow). At E10, there was some background staining but no clear cellular staining. By embryonic day 14, astrocytes are present in the chick brain. Abbreviations: E; ectostriatum; HA: hyperstriatum accessorium; Hp: hippocampus; HV: hyperstriatum ventrale; LH: lamina hyperstriatica; LMD: lamina medullaris dorsalis; LPO: lobus parolfactorius; N: neostriatum; VL: ventriculus lateralis. Scale bar: A: 1.3 mm; B, D, F, H, J: 23 μm; C: 1.0 mm; E: 1.4 mm; G, I: 1.7 mm.

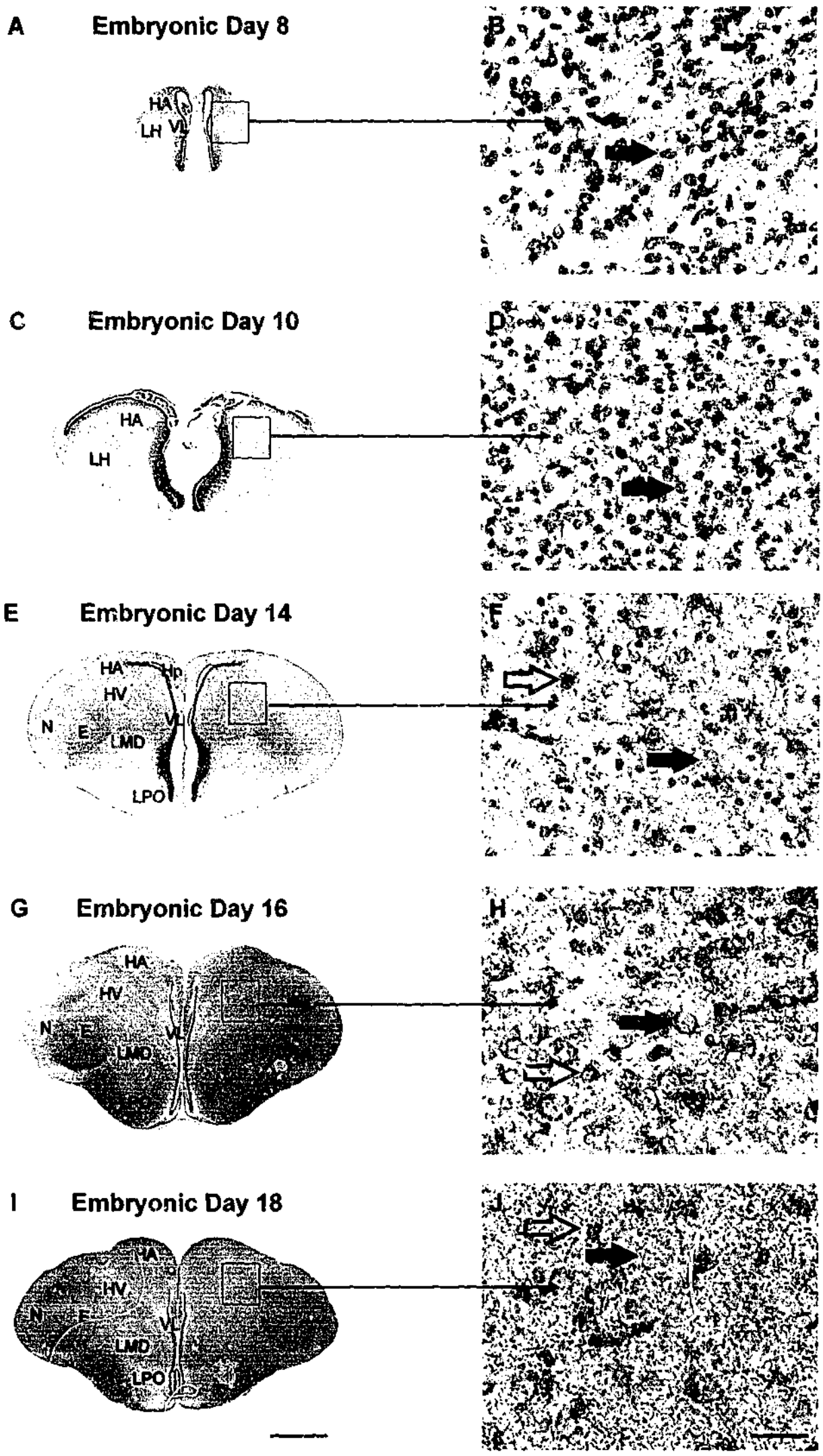

A
Embryonic Day 8
HA
LH
VL
B
C
Embryonic Day 10
HA
LH
D
E
Embryonic Day 14
HA
Hp
HV
N
E
LMD
VL
LPO
F
G
Embryonic Day 16
HA
HV
N
E
LMD
VL
H
I
Embryonic Day 18
HA
HV
N
E
LMD
VL
LPO
J

Fig. 5.2. Photomicrographs of coronal sections of embryo brains at day 8 of gestation stained with glutamine synthetase and thionin. Sections are 100 μm apart. It is evident that glia are not present at E8. Abbreviations: HA: hyperstriatum accessorium; LH: lamina hyperstriatica; OT: optic tectum; VL: ventriculus lateralis; VT: ventriculus teci mesencephali. Note that the tissue was extremely fragile at this age and it was difficult to collect perfect sections. Scale bar: 1-6: 1.35 mm; 7-12: 1.5 mm.

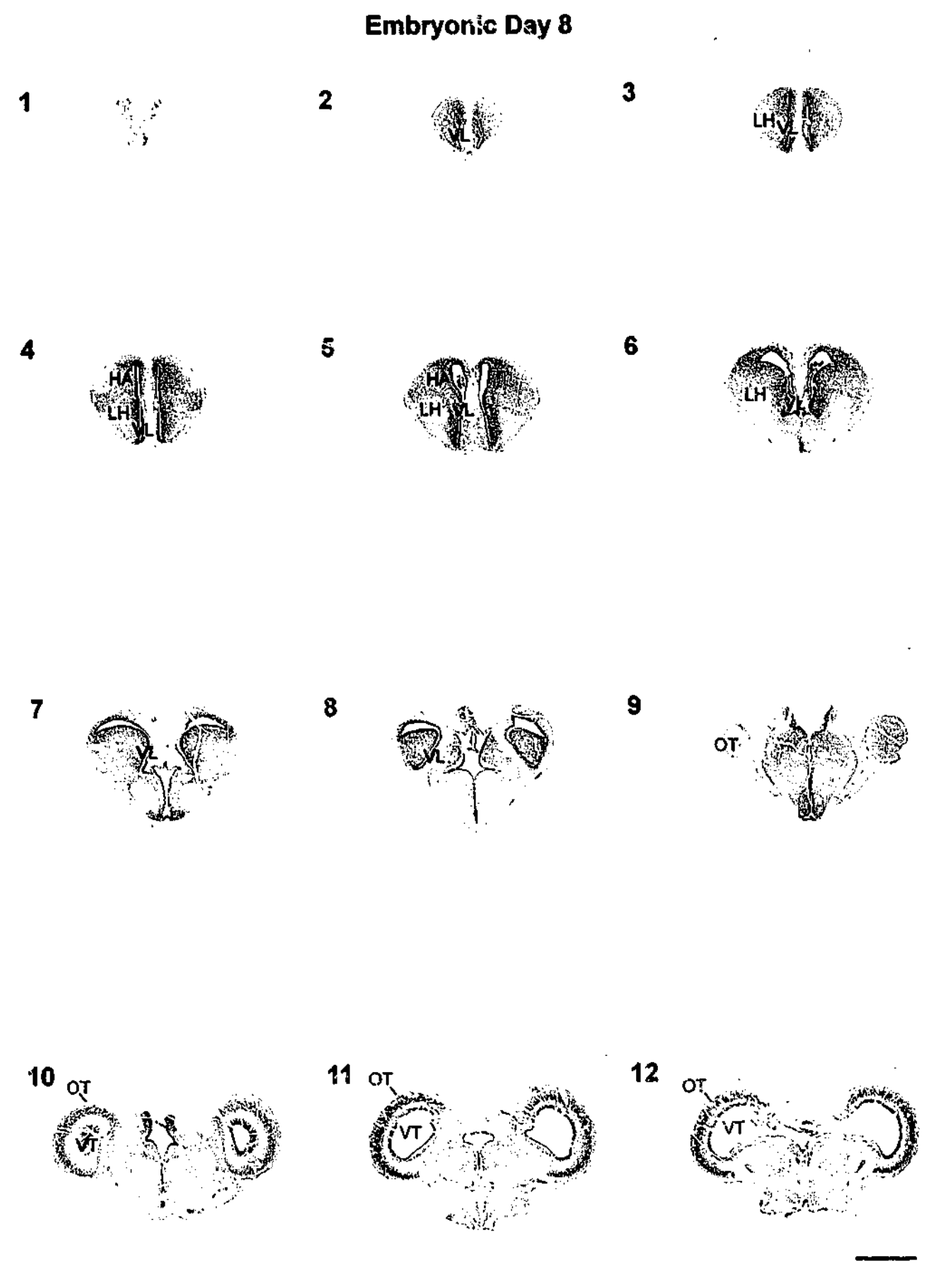


Fig. 5.3. Photomicrographs of coronal sections of embryo brains at day 10 of gestation stained with glutamine synthetase and thionin. Sections are 100 μm apart. It is evident that glia are not present at E10. Note that the tissue was extremely fragile at this age and it was difficult to collect perfect sections. Sections 7 and 8 are not labelled due to poor tissue quality. Abbreviations: HA: hyperstriatum accessorium; LH: lamina hyperstriatica; OT: optic tectum; VL: ventriculus lateralis; VT: ventriculus teci mesencephali. Scale bar: 1-6: 1.0 mm; 7-13: 1.2 mm.

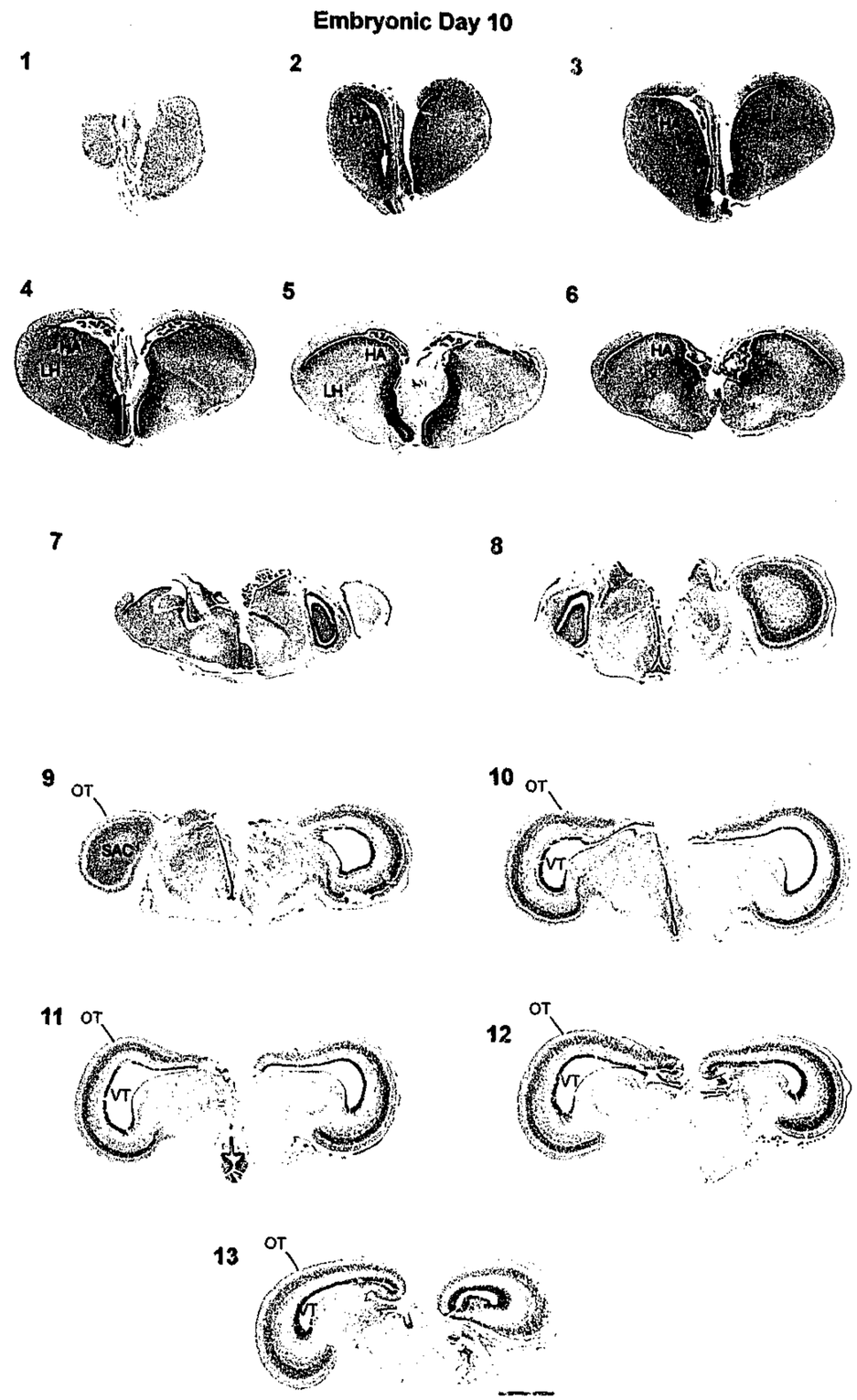


Fig. 5.4A. Photomicrographs of coronal sections of embryo brains at day 14 of gestation stained with glutamine synthetase and thionin. Sections are 100 μm apart. Abbreviations: AId: archistriatum intermedium, pars dorsalis; E; ectostriatum; HA: hyperstriatum accessorium; Hp: hippocampus; HV: hyperstriatum ventrale; LMD: lamina medullaris dorsalis; LPO: lobus parolfactorius; N: neostriatum; NC: neostriatum caudale; OV: nucleus ovoidalis; PVCL: plexus choroideus ventriculi lateralis (choroid plexus within lateral ventricle); ROT: nucleus rotundus; VL: ventriculus lateralis; VMN: nucleus ventromedialis hypothalami. Scale bar: 1-10: 1.1 mm.

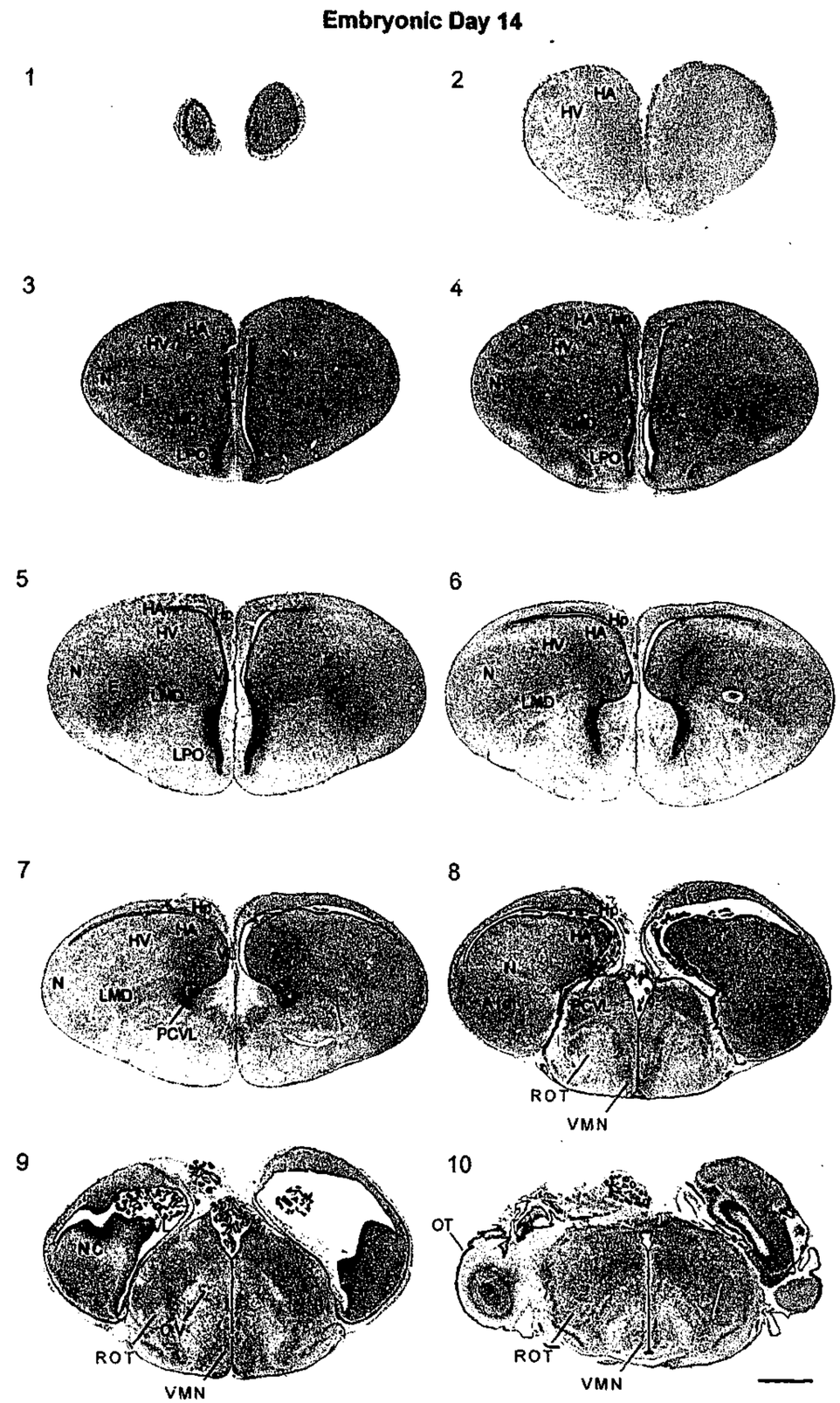


Fig. 5.4B. Photomicrographs of coronal sections of embryo brains at day 14 of gestation stained with glutamine synthetase and thionin. Sections are 100 μm apart. Abbreviations: Cb: cerebellum; EW: nucleus nervi oculomotorii, pars accessoria; Ipc: nucleus isthmi, pars parvocellularis; MLd: nucleus mesencephalicus lateralis, pars ventralis; PL: nucleus pontis lateralis; SAC: striatum album centrale; SCE: stratum cellulare externum; SPL: nucleus spiriformis lateralis; OT: optic tectum; VT: ventriculus teci mesencephali. Scale bar: 11-15: 1.2 mm.

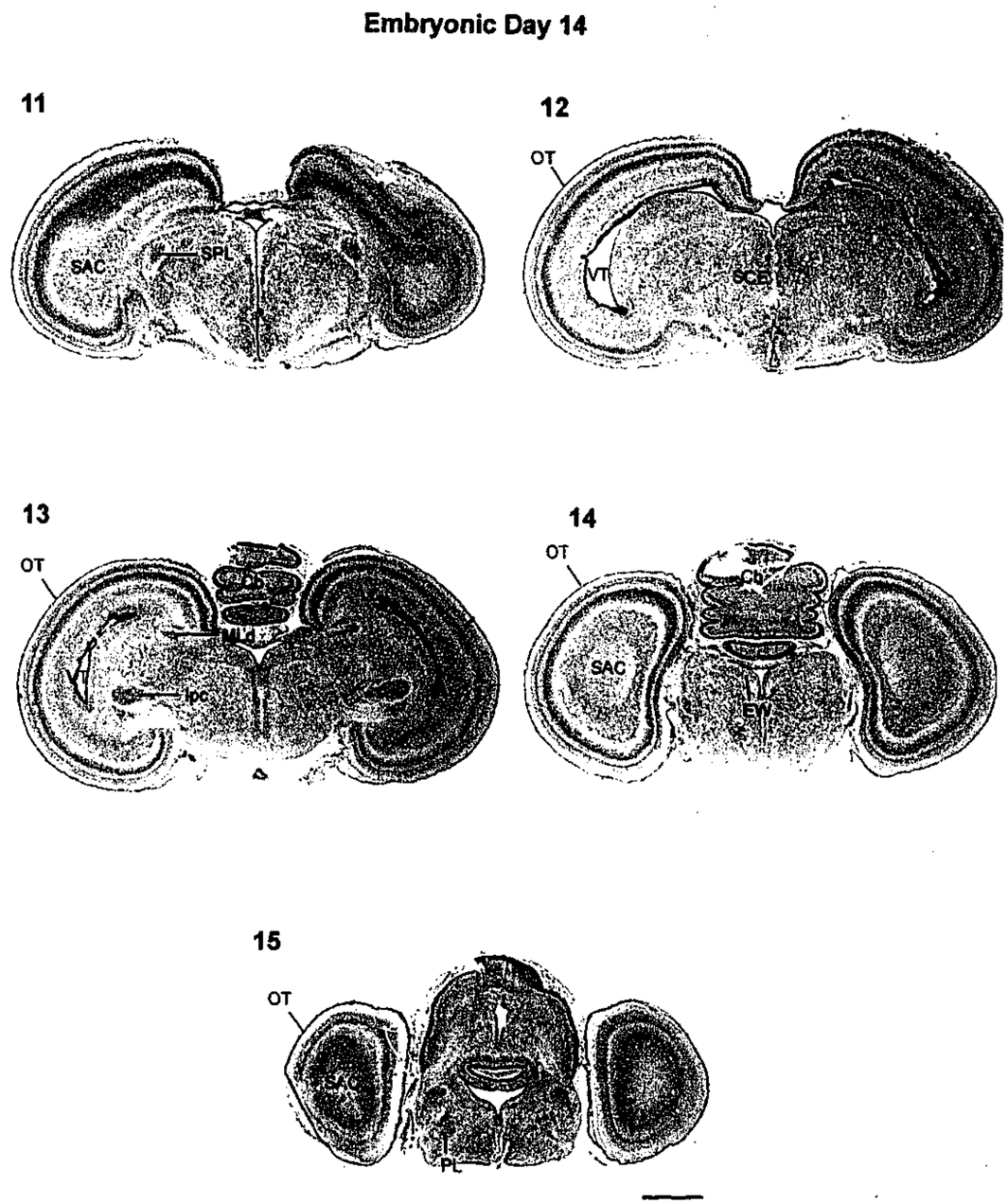


Fig. 5.5. Photomicrographs of coronal sections of embryo brains at day 16 of gestation stained with glutamine synthetase and thionin. Sections are 200 μm apart. Abbreviations: Cb: cerebellum; DA: tractus dorso-archistriaticus; E: ectostriatum; EW: nucleus nervi oculomotorii, pars accessoria; HA: hyperstriatum accessorium; Hp: hippocampus; HV: hyperstriatum ventrale; ICo: nucleus intercollicularis; Ipc: nucleus isthmi, pars parvocellularis; LMD: lamina medullaris dorsalis; LPO: lobus parolfactorius; MLd: nucleus mesencephalicus lateralis, pars ventralis; N: neostriatum; nBOR: nucleus opticus basalis; OB: olfactory bulb; OT: optic tectum; OV: nucleus ovoidalis; PVCL: plexus choroideus ventriculi lateralis (choroid plexus within lateral ventricle); SAC: striatum album centrale; Tn: nucleus taeniae; VL: ventriculus lateralis; VMN: nucleus ventromedialis hypothalami; VT: ventriculus teci mesencephali. Scale bar: 1-6: 1.8 mm; 7: 1.7 mm: 8,9: 1.9 mm; 10: 1.6 mm.

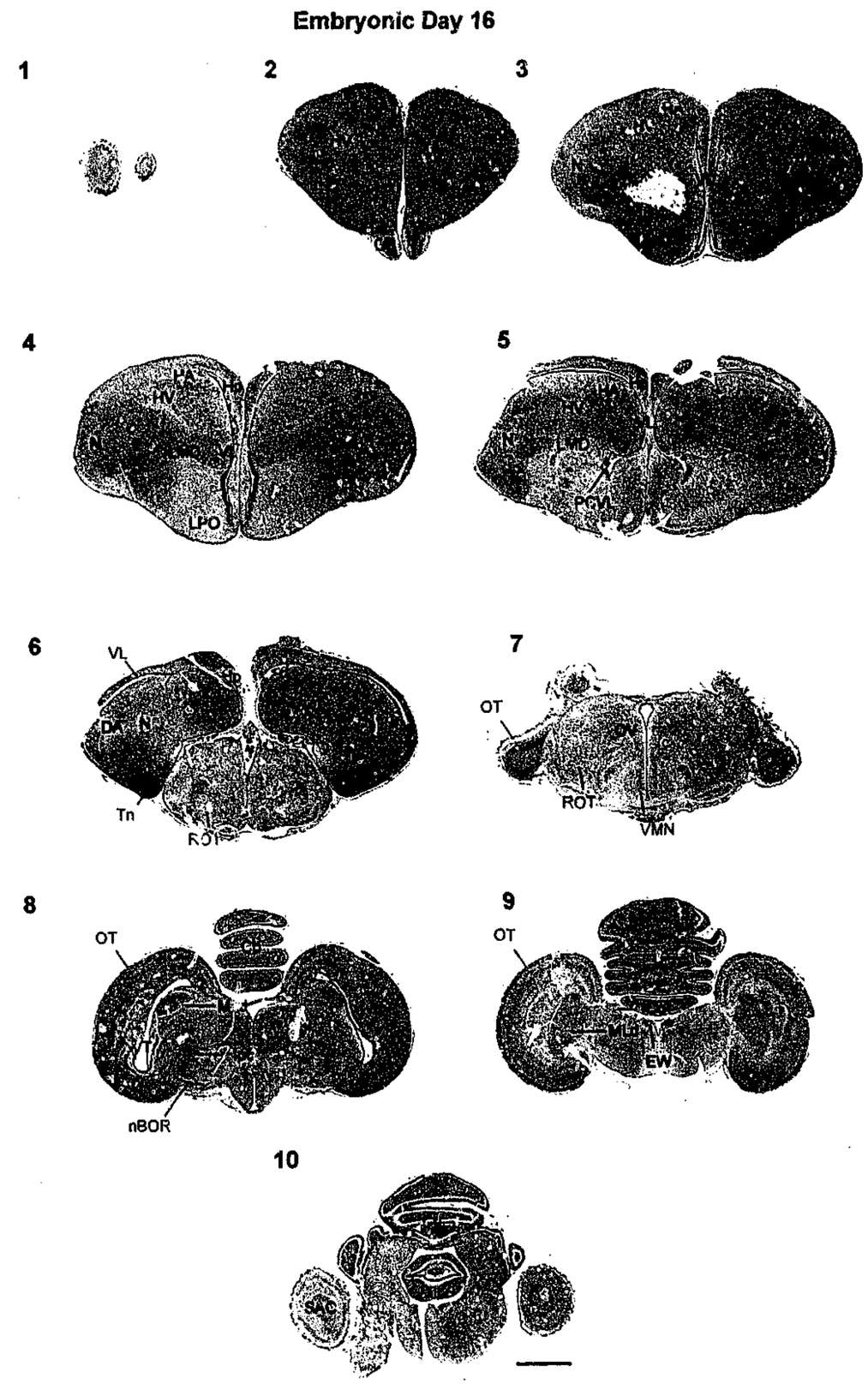
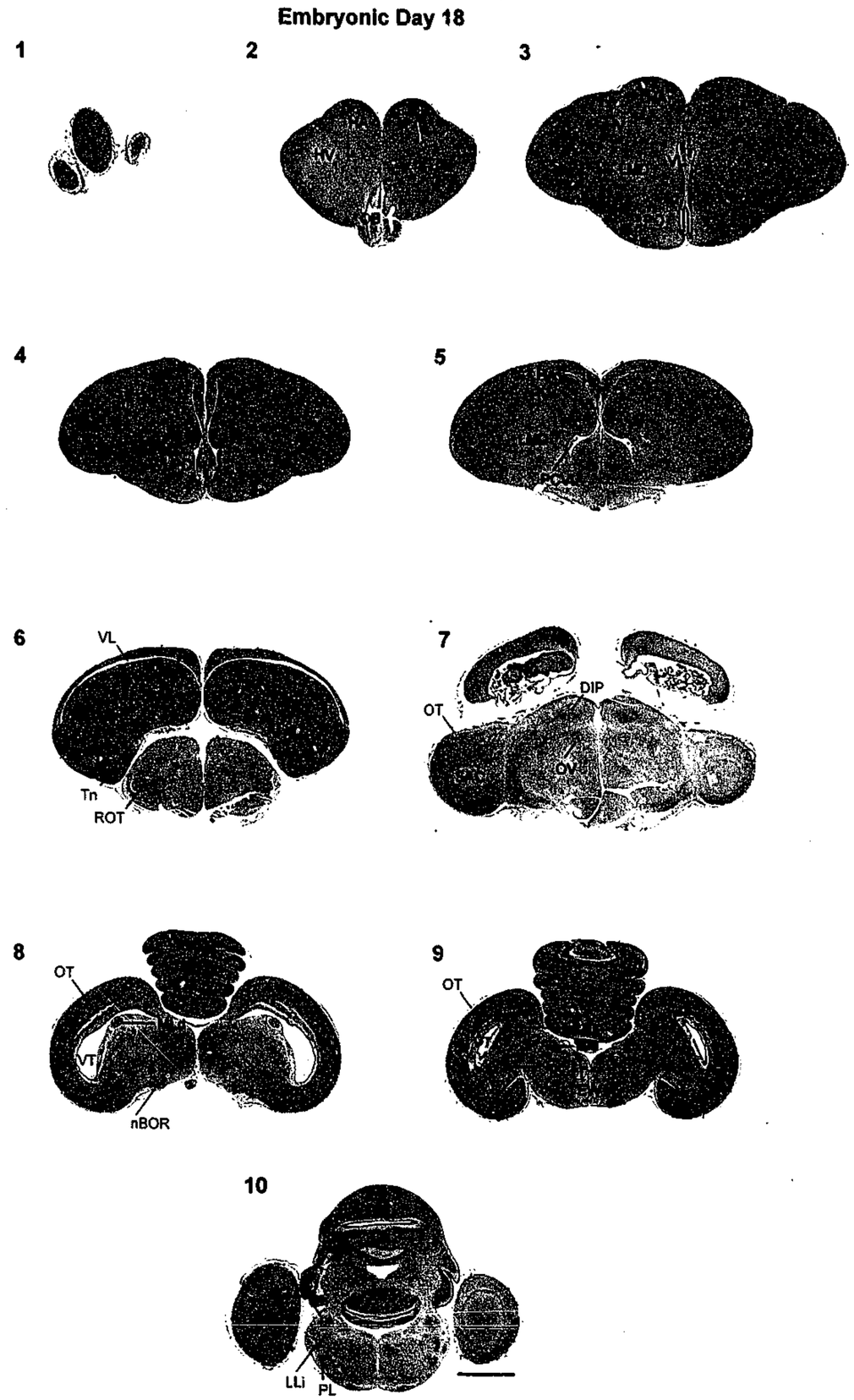


Fig. 5.6. Photomicrographs of coronal sections of embryo brains at day 18 of gestation stained with glutamine synthetase and thionin. Sections are 200 μm apart. The appearance of the brain at E18 is similar to that at E16. Cb: cerebellum; DA: tractus dorso-archistriaticus; DIP: nucleus dorsointermedius posterior thalami; E; ectostriatum; EW: nucleus nervi oculomotorii, pars accessoria; HA: hyperstriatum accessorium; Hp: hippocampus; HV: hyperstriatum ventrale; ICo: nucleus intercollicularis; Ipc: nucleus isthmi, pars parvocellularis; LLd: nucleus lemnisci lateralis, pars dorsalis; LLi: nucleus lemnisci lateralis, pars intermedia; LMD: lamina medullaris dorsalis; LPO: lobus parolfactorius; MLd: nucleus mesencephalicus lateralis, pars ventralis; N: neostriatum; nBOR: nucleus opticus basalis; NC: neostriatum caudale; OB: olfactory bulb; OT: optic tectum; OV: nucleus ovoidalis; PL: nucleus pontis lateralis; PVCL: plexus choroideus ventriculi lateralis (choroid plexus within lateral ventricle); SAC: striatum album centrale; Tn: nucleus taeniae; VL: ventriculus lateralis; VT: ventriculus teci mesencephali. Scale bar: 1-10: 2 mm.



5.2.1.3 Ontogeny of astrocytes in the IMHV

Although there was a tendency for the density of astrocytes to increase from E14 to E16, analysis of variance (one-way) revealed that there was no effect of age (F=4.146, P=0.137; Fig. 5.7) on the density of astrocytes in the IMHV. There was no difference in the density of astrocytes between the left and right hemispheres (F=3.315, P=0.166). The density of neurons remained relatively constant from E14 to E18, hence, there was no effect of age (F=2.238, P=0.254) on the density of neurons in the IMHV, and there were no hemispheric differences (F=0.761, P=0.447). This trend was also evident for the ratio of astrocytes/neurons (age: F=1.624, P=0.333; hemisphere: F=1.007, P=0.390).

5.2.1.4 Ontogeny of astrocytes in the LPO

The density of astrocytes and neurons in the LPO followed a similar trend to that in IMHV. Analysis of variance (one-way) revealed that there was no difference between the three different embryonic ages in the density of astrocytes in the LPO (F=4.712, P=0.119; Fig. 5.8). In addition, there was no difference in the density of astrocytes between the left and right hemispheres (F=0.079, P=0.797). There was no effect of age (F=1.488, P=0.356) or hemisphere (F=0.670, P=0.473) on the density of neurons in the LPO. This trend was the same for the ratio of astrocytes/neurons in the LPO (age: F=1.303, P=0.391; hemisphere: F=0.001, P=0.980).

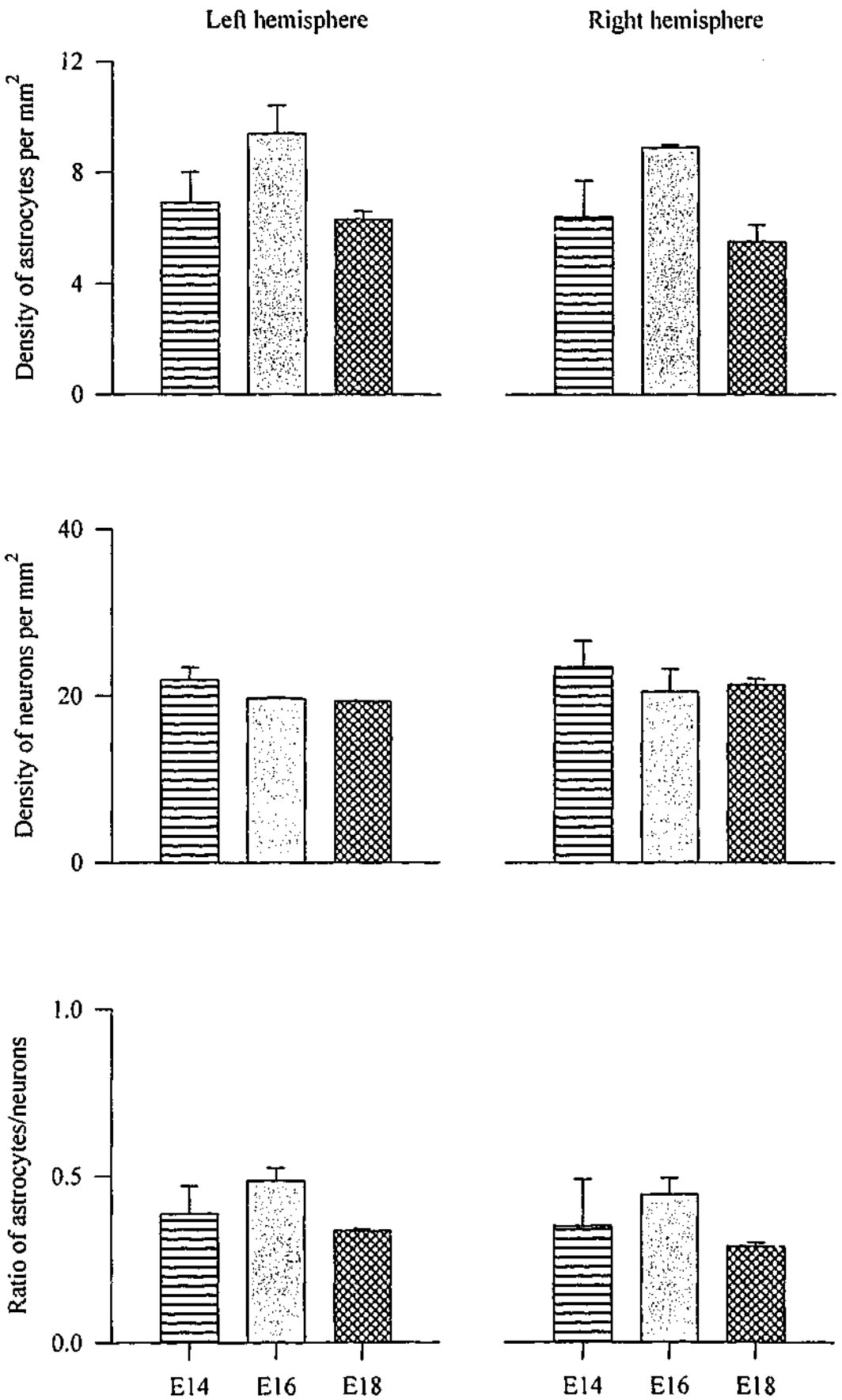


Fig. 5.7. The density of astrocytes and neurons in the IMHV of the chick embryo brain at embryonic days 14 (E14), *16* (E16) *and 18* (E18). *Although there was a tendency for the density of astrocytes to increase from E14-E16, this increase was not statistically significant. The density of neurons remained relatively constant throughout the later stages of development.*

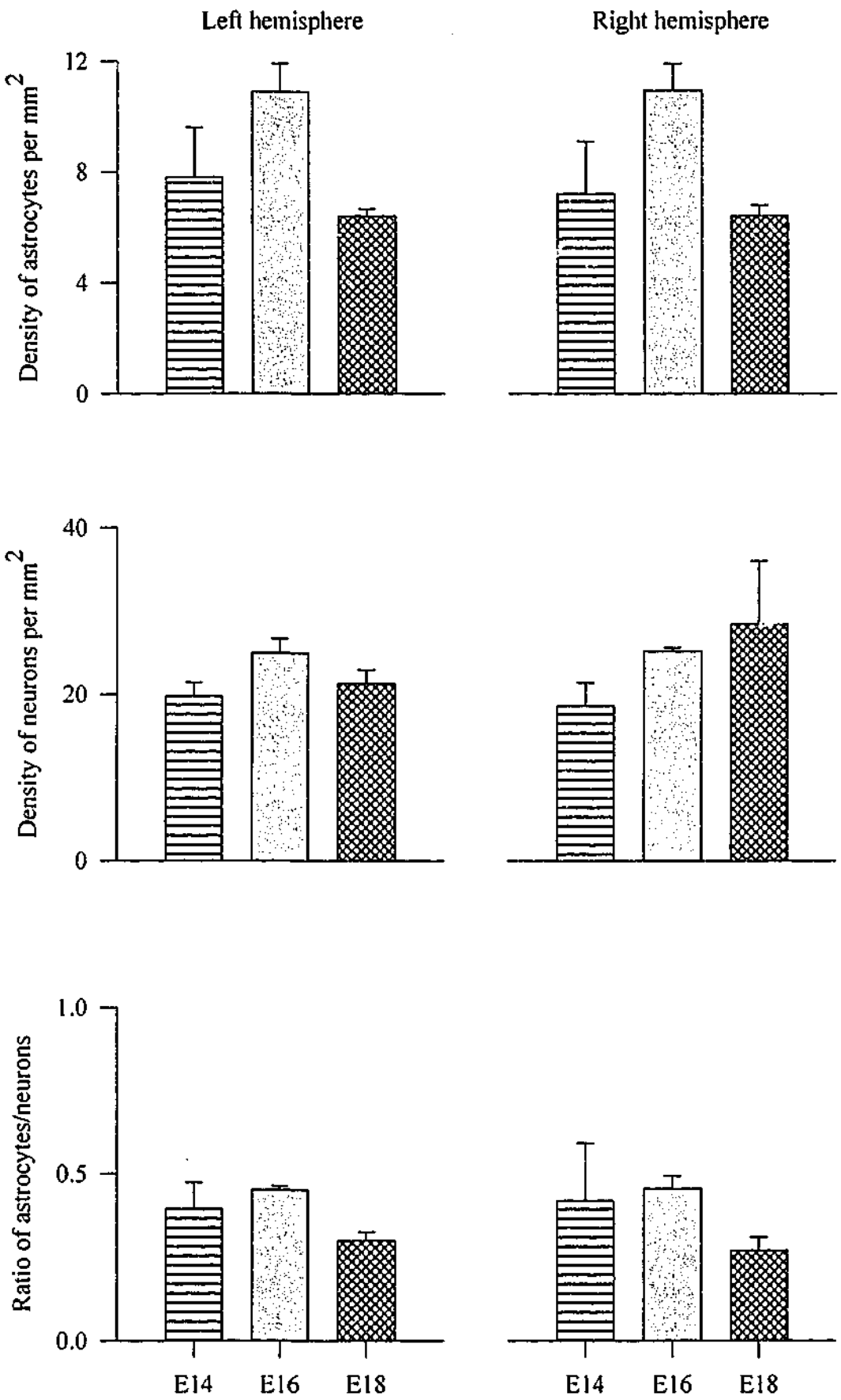


Fig. 5.8. The density of astrocytes and neurons in the LPO of the chick embryo brain at embryonic days 14 (E14), *16* (E16) *and 18* (E18). *Although there was a tendency for the density of astrocytes to increase from E14-E16, this increase was not statistically significant.*

5.2.2 Qualitative and quantitative analysis of the 2 day-old brain tissue

5.2.2.1 Structural analysis

Examination of the H&E stained sections revealed no overt structural abnormalities in the brains of control (Fig. 5.9A,B) or W14-18 chicks (Fig. 5.9C,D). Dying cells or gliosis were not noted in any areas of the brain. This was also the case for the W10-18 chicks (micrographs from W10-18 chicks are not shown).

5.2.2.2 Percentage of the brain parenchyma occupied by blood vessels

Quantitative analysis revealed no difference in the percentage of the brain parenchyma occupied by blood vessels between control (Fig. 5.10A and Fig. 5.9B) and W14-18 chicks at 2 days (F=0.087, P=0.773; Fig. 5.10A and Fig. 5.9D). This was also the case for the W10-18 chicks (F=0.329, P=0.578; Fig. 5.10B).

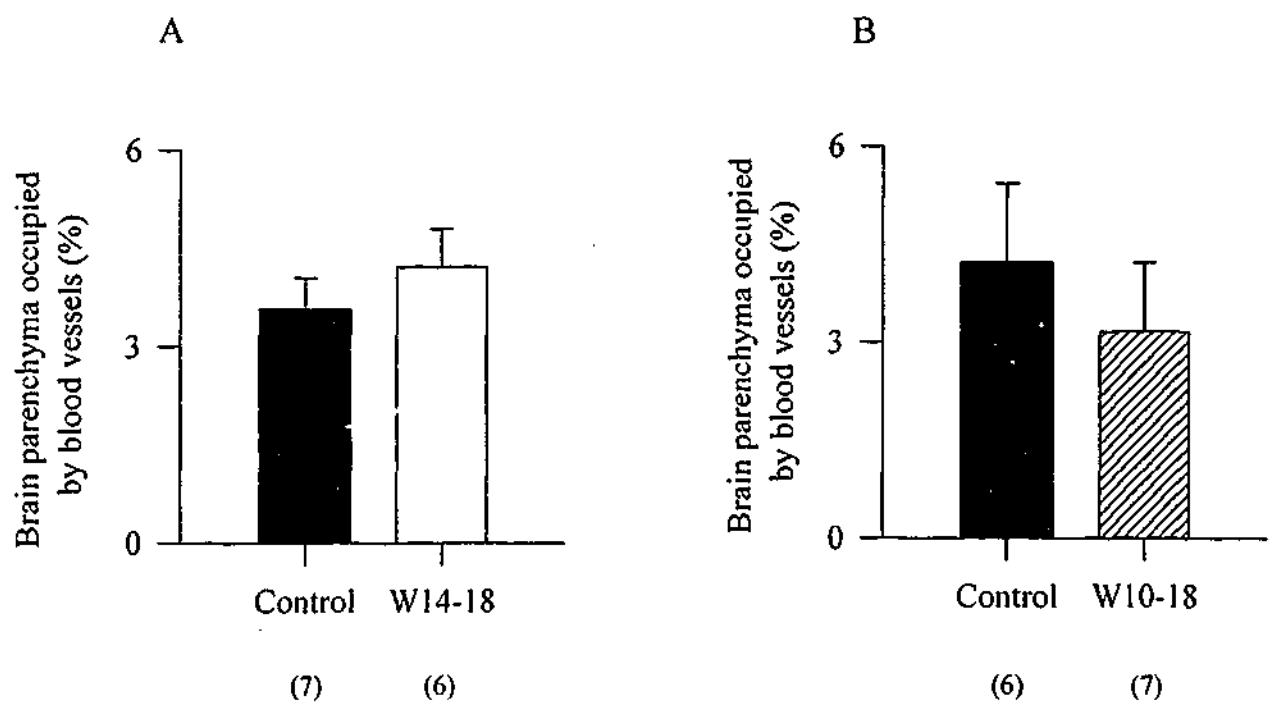
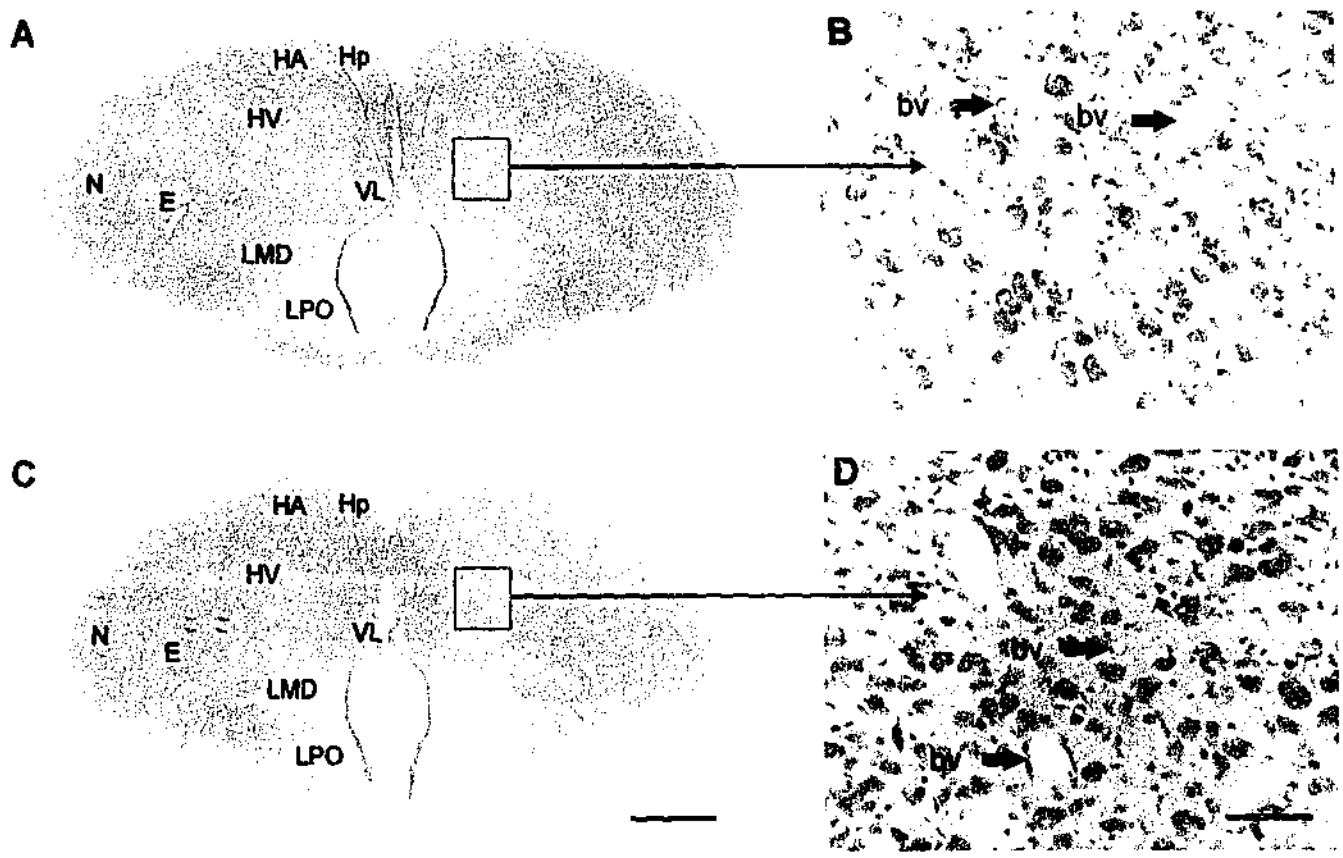


Fig. 5.10. The percentage of brain parenchyma occupied by blood vessels in control chicks, W14-18 chicks (A) *and W10-18* (B) *chicks 2 days after hatching. There was no difference in the percentage of the brain parenchyma occupied by blood vessels between controls and prenatally hypoxic chicks. Animal numbers are in parentheses.*

Fig. 5.9. Photomicrographs of coronal sections of 2 day-old chick brains stained with haematoxylin and eosin. Panels A and B are from a control animal. Panels C and D are from a W14-18 chick. There were no overt structural abnormalities in the brains of control or W14-18 or W10-18 chicks (not shown). Dying cells or gliosis were not noted in any areas of the brain. Quantitative analysis revealed no difference in the percentage of the brain parenchyma occupied by blood vessels between control and W14-18 chicks at 2 days (B, D). This was also the case for the controls and the W10-18 chicks (not shown). Abbreviations: bv: blood vessel; E; ectostriatum; HA: hyperstriatum accessorium; Hp: hippocampus; HV: hyperstriatum ventrale; LMD: lamina medullaris dorsalis; LPO: lobus parolfactorius; N: neostriatum; VL: ventriculus lateralis. Scale bar: A, C: 1.8 mm; B, C: 58 μm.


A
HA
Hp
HV
N
E
VL
LMD
LPO
B
bv
bv
C
HA
Hp
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N
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VL
LMD
LPO
D
bv
bv

5.2.3 Quantitative immunohistochemical analysis

5.2.3.1 Astrocyte/neuron ratio in the W14-18 chicks

Analysis of variance (one-way) revealed that there was no difference in the density of astrocytes in the IMHV between control and W14-18 chicks (F=0.758, P=0.403; Fig. 5.11), or the left and right hemispheres (F=0.966, P=0.347). Although there was no difference between the two groups in the density of neurons in the IMHV (F=1.310, P=0.277), post hoc analysis revealed that the density of neurons in the left hemisphere was greater than that in the right in the W14-18 (F=15.071, P=0.003). The ratio of astrocytes/neurons was not different between the controls and the W14-18 chicks (F=2.542, P=0.139), or between the left and right hemispheres (F=2.628, P=0.133).

5.2.3.2 Astrocyte/neuron ratio in the W10-18 chicks

Analysis of variance (one-way) revealed that there was no difference in the density of astrocytes in the IMHV between control and W10-18 chicks (F=0.327, P=0.579; Fig. 5.12), or the left and right hemispheres (F=0.010, P=0.922). Although there was no difference between the two groups in the density of neurons in the IMHV (F=1.172, P=0.302), post hoc analysis revealed that the density of neurons in the left hemisphere was greater than that in the right in the W10-18 chicks (F=5.249, P=0.043). The ratio of astrocytes/neurons was not different between the controls and the W10-18 chicks (F=1.205, P=0.296), or between the left and right hemispheres (F=0.031, P=0.862). Note that the density of astrocytes and neurons tended to be higher in the W10-18 chicks and their controls compared to the W14-18 and their controls. This may be due to the difference in brain weights between the two cohorts of prenatally hypoxic chicks and controls. Given this disparity, each cohort was examined individually.

5.2.3.3 Tyrosine hydroxylase immunoreactivity

As reported in Chapter 3, the level of noradrenaline, as determined by HPLC, was found to be reduced in the anterior forebrain of the two groups of prenatally hypoxic chicks. Whilst HPLC analysis indicated an overall decrease in noradrenaline, immunohistochemistry was required to localise specific regions within the anterior forebrain that may have been affected following the prenatal insult. The immunoreactivity of TH- the rate-limiting enzyme involved in the catecholamine synthesizing pathway, was assessed in the anterior forebrain to elucidate the nature of

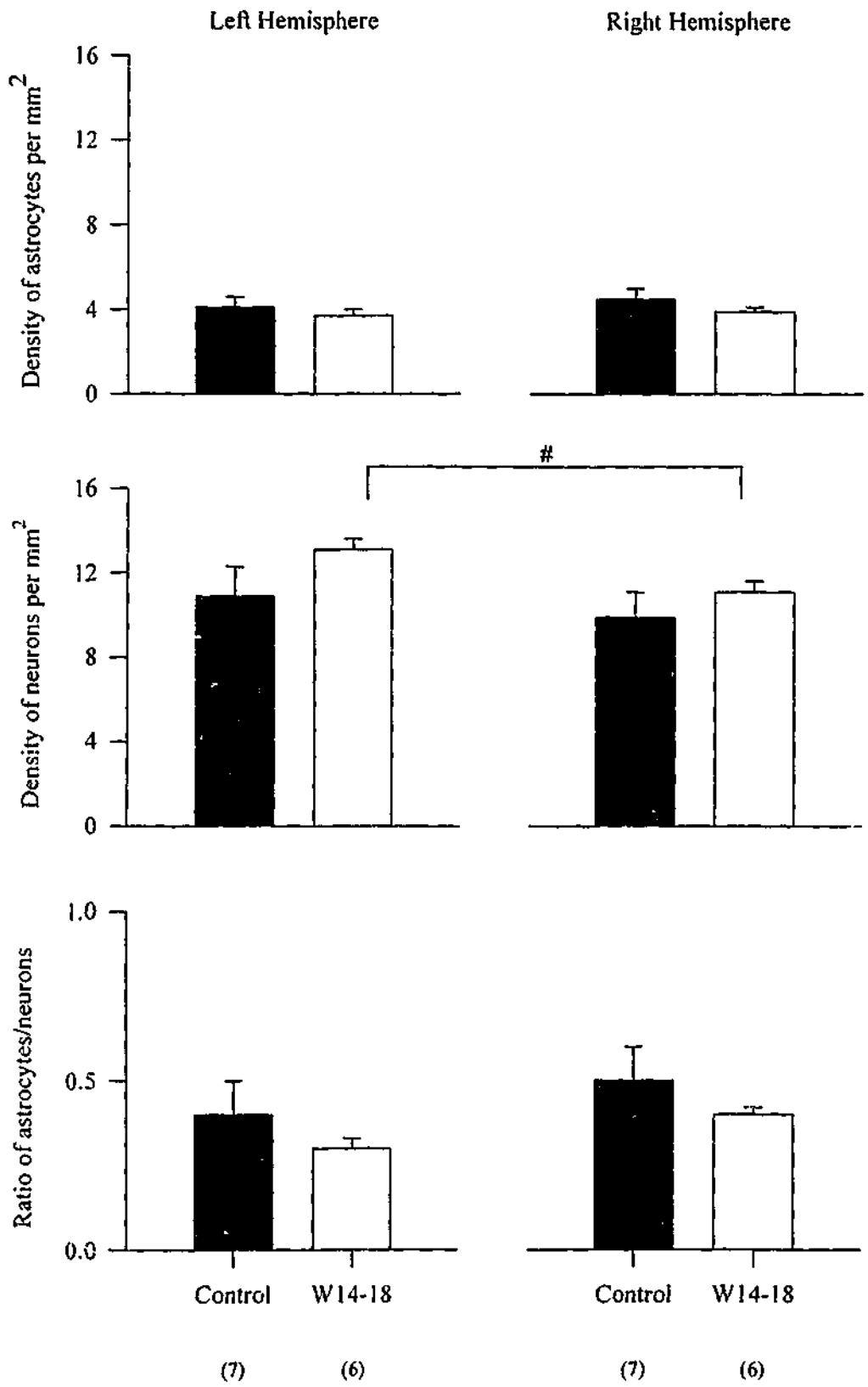


Fig. 5.11. The density of astrocytes and neurons in the IMHV region of the chick brain for control and W14-18 chicks 2 days post-hatching. There was no difference in the density of astrocytes or neurons in the IMHV between control and W14-18 chicks, however, the W14-18 chicks had more neurons in the left hemisphere than the right. # indicates a significant difference between the left and right hemispheres. Animal numbers are in parentheses.

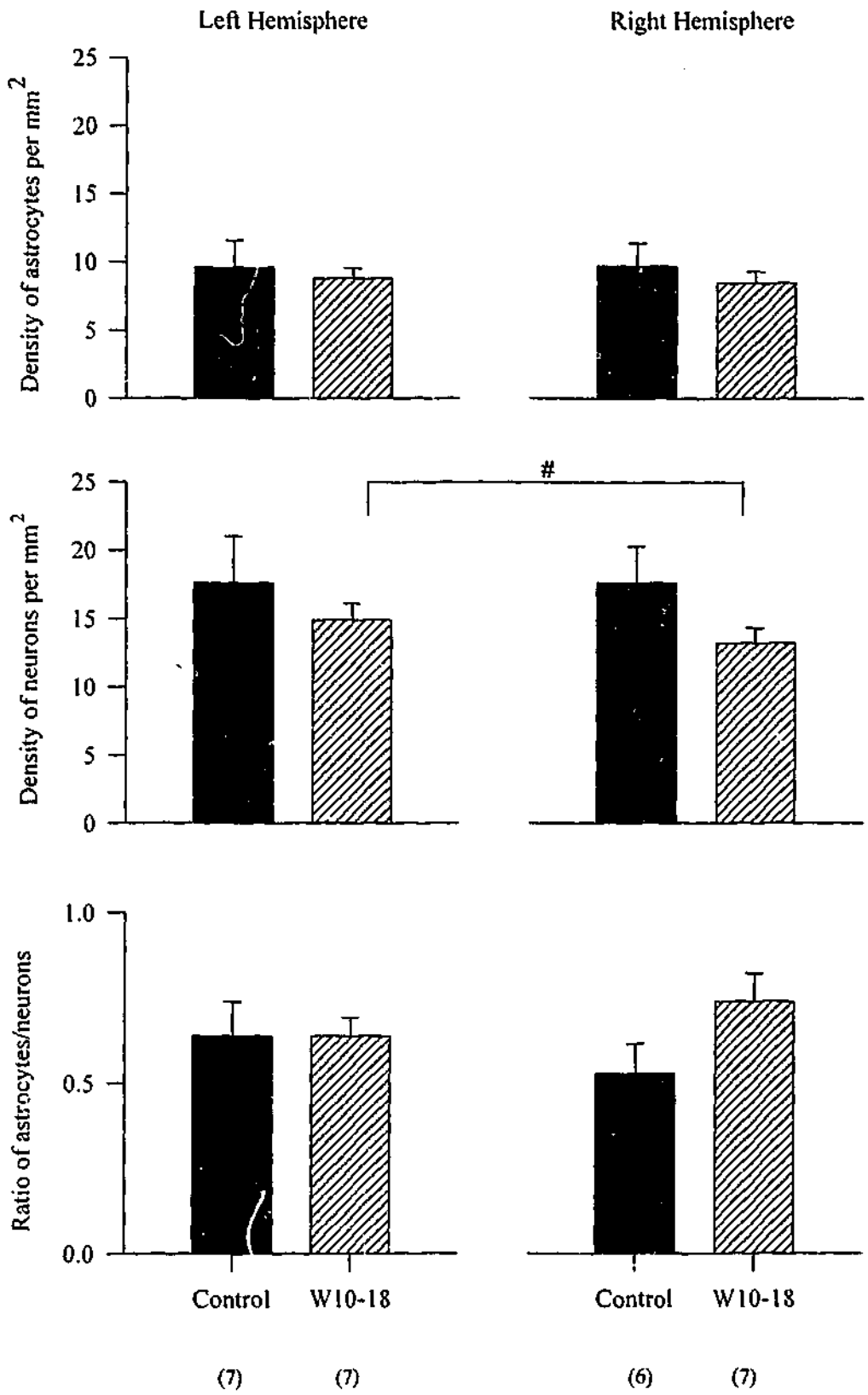


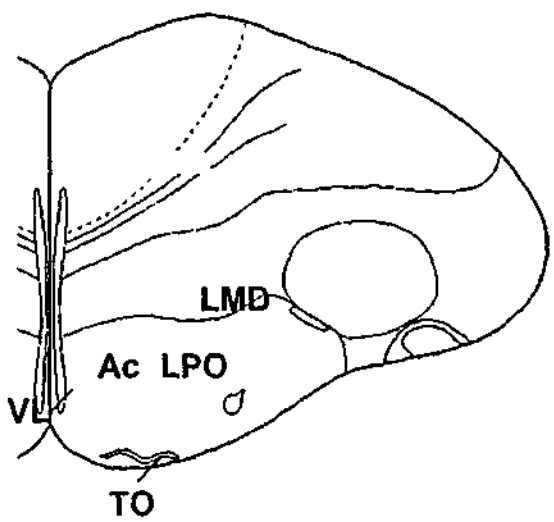
Fig. 5.12. The density of astrocytes and neurons in the IMHV region of the chick brain for control and W10-18 chicks 2 days post-hatching. There was no difference in the density of astrocytes or neurons in the IMHV between control and W10-18 chicks, however, the W10-18 chicks had more neurons in the left hemisphere than the right. # indicates a significant difference between the left and right hemispheres. Animal numbers are in parentheses.

the reduction in noradrenaline. The anterior forebrain contains the LPO, an area of the chick brain involved in memory formation and consolidation (Kossut & Rose, 1984; Rose & Csillag, 1985; Gibbs & Summers, 2002b), hence, this area was the focus for analysis.

TH immunoreactivity was quite varied between groups. In the control tissue for the W14-18 chicks, 2 out of 7 animals had staining in the LPO in the rostral region (not shown). The other animals had no staining in this region (Fig. 5.13A). More caudally, there was intense staining in the tuberculum olfactorium in 5 out of 7 animals, with little staining in the LPO (Fig. 5.13B). In the W14-18 tissue, 5 out of 6 had no staining in the LPO or tuberculum olfactorium in the rostral region (Fig. 5.13C). In the caudal regions (Fig. 5.13D), TH immunoreactivity was most intense in the tuberculum olfactorium in 5 out of 6 animals. There appeared to be a reduction in TH immunoreactivity in the brains of these animals compared to their controls, which is consistent with the results obtained from the HPLC analysis.

In the control tissue for the W10-18 chicks, 3 out of 7 animals had intense staining in the nucleus accumbens, LPO and tuberculum olfactorium in the rostral region (Fig. 5.14A). One animal had staining localised to the nucleus accumbens and tuberculum olfactorium (Fig. 5.14C), and 3 animals did not have staining in any of these regions. In the caudal region, TH immunoreactivity was intense in the LPO and tuberculum olfactorium regions in 5 out of 6 animals (Fig. 5.14B,D). In the W10-18 tissue (Fig. 5.14E,G), 1 out of 6 animals had intense staining in the nucleus accumbens, LPO and tuberculum olfactorium in the rostral region (Fig. 5.14E). In the other 5 animals, staining was localised to the nucleus accumbens (Fig. 5.14G). Caudally, there was intense staining in the LPO and tuberculum olfactorium in all 7 animals (Fig. 5.14F,H). It is apparent from these findings that the TH immunoreactivity in W10-18 tissue did not reflect the reduction in noradrenaline levels as detected by HPLC.

Fig. 5.13. Dark-field photomicrographs of coronal sections of 2 day-old chick brains stained with tyrosine hydroxylase (TH) in a control (A, B) and W14-18 chick (C, D). In the control tissue for the W14-18 chicks, 2 animals out of 7 had staining in the lobus paraolfactorius in the rostral region (not shown). The other animals had no staining in this region (A). More caudally, there was intense staining in the tuberculum olfactorium in 5 out of 7 animals, with little staining in the lobus paraolfactorius (B). In the W14-18 tissue, 5 out of 6 had no staining in the lobus paraolfactorius in the rostral region (C). In the caudal regions (D), TH immunoreactivity was most intense in the tuberculum olfactorium in 5 out of 6 animals. Abbreviations: Ac: nucleus accumbens; LMD: lamina medullaris dorsalis; LPO: lobus paraolfactorius; TO: tuberculum olfactorium; VL: ventriculus lateralis. Scale bars: A-D: 400 μm.



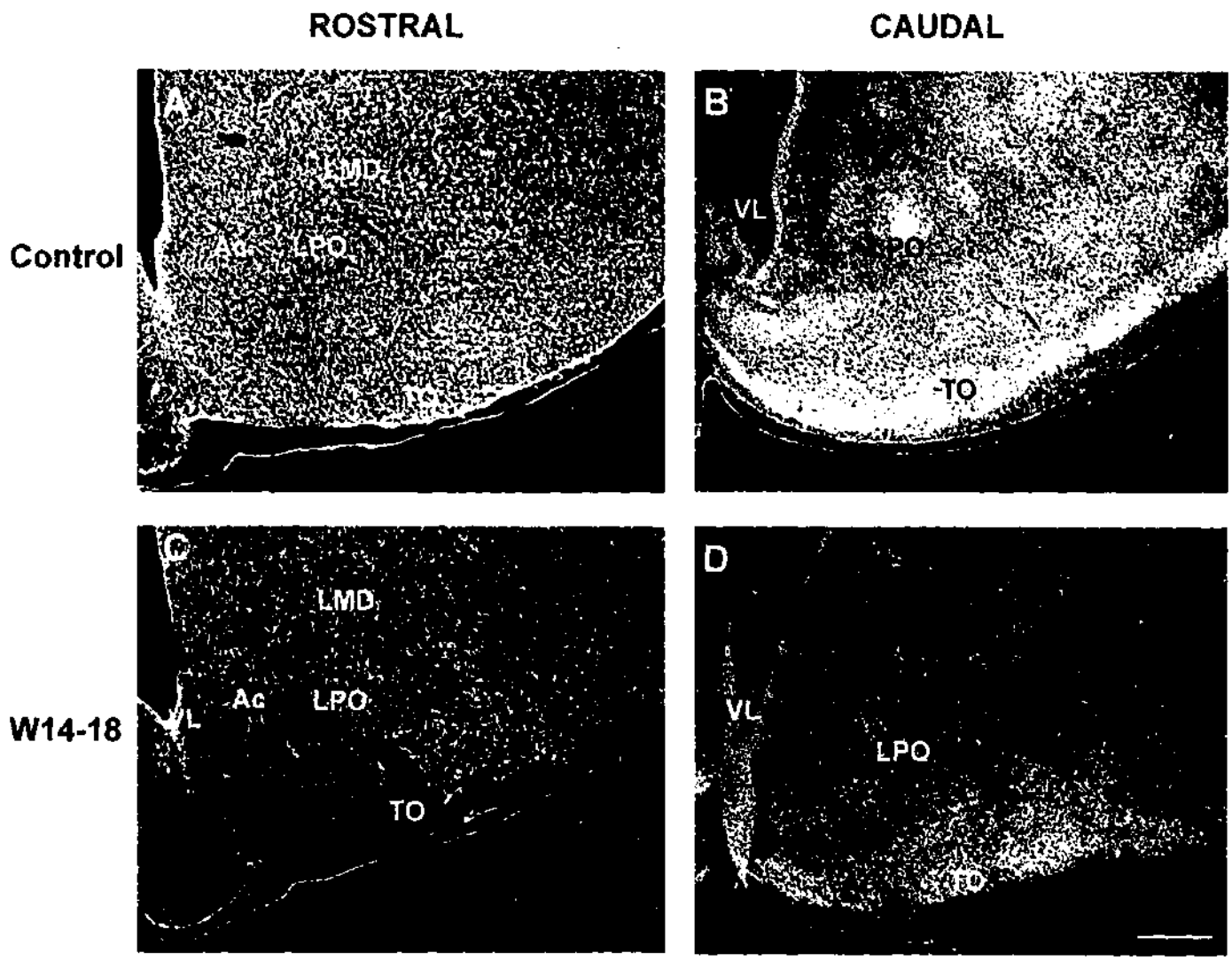
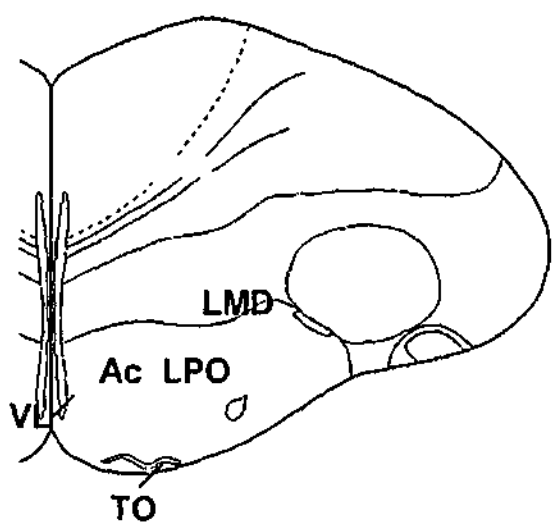
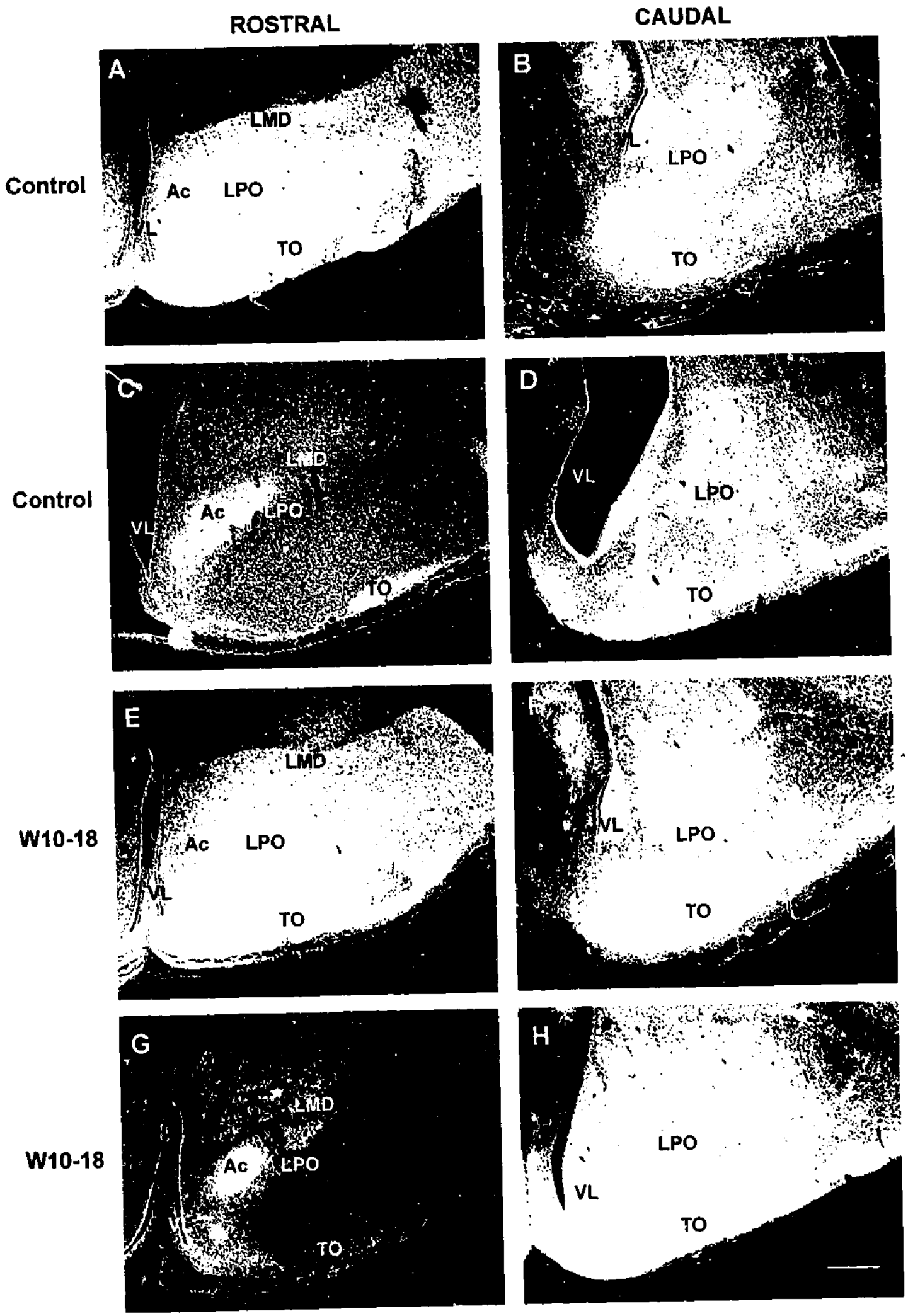

ROSTRAL
CAUDAL
Control
W14-18
A
B
C
D
LMD
Ac
LPO
VL
PO
TO

Fig. 5.14. Dark-field photomicrographs of coronal sections of 2 day-old chick brains stained with tyrosine hydroxylase (TH) in control (A-D) and W10-18 chicks (E-H). In the control tissue for the W10-18 chicks, 3 out of 7 animals had intense staining in the nucleus accumbens, lobus paraolfactorius and tuberculum olfactorium in the rostral region (A). One animal had staining localised to the nucleus accumbens and tuberculum olfactorium (C), and 3 animals did not have staining in any of these regions. In the caudal region, TH immunoreactivity was intense in the lobus paraolfactorius and tuberculum olfactorium regions in 5 out of 6 animals (D). In the W10-18 tissue, 1 out of 6 animals had intense staining in the nucleus accumbens, lobus paraolfactorius and tuberculum olfactorium in the rostral region (E). In the other 5 animals, staining was localised to the nucleus accumbens (G). Abbreviations: Ac: nucleus accumbens; LMD: lamina medullaris dorsalis; LPO: lobus paraolfactorius; TO: tuberculum olfactorium; VL: ventriculus lateralis. Scale bars: A, B: 400 μm; C-H: 390 μm.




ROSTRAL
CAUDAL
Control
Control
W10-18
W10-18
A
B
C
D
E
G
H
LMD
Ac
LPO
VL
TO

5.3 Discussion

The aim of this chapter was to perform a structural and neurochemical examination of the brains of control and prenatally hypoxic chicks. The data presented show that prenatal hypoxia from either day 10 or 14 of incubation until day 18 does not result in gross structural damage in the brain after hatching. Whilst it was proposed that prenatal hypoxia may have altered cerebral vasculature, the percentage of the brain parenchyma occupied by blood vessels was not different between controls and prenatally hypoxic chicks. The density of astrocytes and neurons in the IMHV was not altered following prenatal hypoxia. Data from this chapter also demonstrated that astrocytes are present in the embryo brain from E14.

5.3.1 Structural analysis

Analysis of the brains of the prenatally hypoxic chicks 2 days after hatching revealed that there were no gross structural alterations such as necrosis, infarction or gliosis. The absence of any gross pathologies in the brains of chicks from wrapped eggs, in spite of a significant impairment in postnatal memory, is of interest. It has been suggested that some prenatal insults may not result in obvious focal pathologies or lesions, but rather altered development of synaptic connectivity or neural transmission (see Altman, 1986; Morgane *et al.*, 1993; Fiala *et al.*, 2002). Mental retardation has been consistently associated with abnormalities in dendrite structure, and in the shape and density of dendritic spines (Huttenlocher, 1970, 1991; Kaufmann & Moser, 2000); schizophrenia (Garey *et al.*, 1987; Roberts *et al.*, 1996) has also been associated with pathologies of spine distribution and shape. The relationship between spine pathology and cognitive deficits is of particular importance. As principal sites of synaptic input, spines play a key role in connectivity in the brain. Thus alterations in spine morphology resulting from hypoxia or malnutrition, for example, are expected to have a significant impact on mental function. Subtle changes in spine structure or connectivity may also underlie less extreme conditions, such as minimal brain dysfunction and learning disabilities (see Altman, 1986; Morgane *et al.*, 1993). Thus, in the present study, subtle changes in synaptic connectivity, or dendritic spine morphology, may have contributed to the impairments in learning and memory. Future quantitative ultrastructural studies and/or Golgi staining of dendritic spines would be required to assess this possibility.

Alternatively, the lack of any gross structural abnormalities in the brains of the hypoxic chicks in the present study could be due to the hypoxic chicks that did sustain overt brain damage not surviving, or being unable to endure the process of hatching. Unlike in compromised mammalian pregnancies where births can be assisted in order to improve the chances of survival, this is not the case in the chick embryo. The successful hatching of the chick embryo requires the precise timing of specific events, such as the internalisation of the yolk sac, and internal pipping. Unfortunately, if these events do not occur, human intervention will not improve the chances of survival. It would therefore be of interest in future studies to examine the brains of the hypoxic embryos that do not hatch, to see if overt brain damage is present in these animals.

5.3.2 Immunohistochemistry

5.3.2.1 Brain development in the chick embryo

To date, there has been no complete study into the ontogeny of astrocytes in the IMHV and LPO in the chick embryo. Curtis *et al.* (1989) examined the number of astrocytes in the IMHV at days 16 and 19 of incubation. However, complete quantitative data was not presented due to methodological issues with the point count technique used to estimate astrocyte numbers (Curtis *et al.*, 1989).

The results of the current analysis of brain development in the chick embryo indicate that the proportion of neurons in the chick embryo brain is nearly twice that of astrocytes. This difference is similar at 2 days after hatching. Astrocytes were found to be present at E14 but not at the earlier ages examined (e.g. E8 and E10). It was expected that the density of neurons in the IMHV and LPO would have decreased with embryonic age, due to neuronal process development or programmed cell death (Oppenheim, 1991). However, in the present study, neuronal density remained relatively static, particularly in the IMHV. Although greater than 50% of motor and sensory neurons die between embryonic days 6-10 in the chick embryo (Hamburger, 1975; Hamburger *et al.*, 1981; Oppenheim *et al.*, 1982), the specific timing of cell death in the IMHV and LPO is not known. It should be noted that density counts do not establish whether total neuronal numbers are altered, as the volume of the nucleus of interest is not taken into consideration. To unequivocally establish the number of neurons in a particular nucleus at each age, appropriate stereological techniques that estimate the

volume of the area of interest and neuronal numbers per unit volume would need to be employed. However, in the case of the IMHV, monitoring volume changes during development is hampered by the lack of obvious lateral boundaries of this region (Curtis *et al.*, 1989).

5.3.2.2 Astrocyte/neuron ratio at 2 days post-hatching

Distortions in the ratio of astrocytes/neurons as a consequence of a prenatal insult may produce various abnormalities, including the failure of neurons to properly migrate and assemble into functional units, particularly if there is an effect on radial glial cells. In addition, disproportionate cell loss resulting in altered astrocyte/neuron ratios may produce alterations in the brain, in particular, between interacting neuronal systems that are transmitter specific. Given that astrocytes are present in the chick brain at E14 and not at E10, it could be expected that the period of hypoxia from days 14-18 of incubation might impact on astrocyte development. Whilst the results from the present study suggest that the density of astrocytes and neurons in the IMHV is not altered following prenatal hypoxia, it cannot be determined whether astrocyte function is altered. As mentioned in Section 1.12, inhibiting the conversion of astrocytically accumulated glutamate to glutamine (Gibbs *et al.*, 1996), or the *de novo* formation of glutamate in astrocytes from glucose, impairs memory consolidation (O'Dowd *et al.*, 1994a). Furthermore, the energy demands for the formation of long-term memory are met by glycogenolysis, a process which is virtually restricted to astrocytes (O'Dowd *et al.*, 1994b). Hence, if astrocyte function is altered, memory function could be impaired. Future studies could assess whether energy metabolism within the brain is impaired by measuring cytochrome oxidase activity. It has previously been demonstrated that prenatal hypoxia (Royer *et al.*, 2000) and neonatal hypoxia-ischemia (Vannucci *et al.*, 1996) in the rat decreases glucose transporter (GLUT) proteins gene expression. These results suggest that glucose transport and utilisation are reduced following a hypoxic insult. mRNA levels of GLUT transporter proteins could be investigated to assess whether glucose transport is altered the brains of the prenatally hypoxic chicks.

Although left-right asymmetries have been observed in synaptic parameters during development in the chick (Curtis *et al.*, 1989; Hunter & Stewart, 1989) and following passive avoidance learning (Stewart *et al.*, 1984; Gibbs *et al.*, 2002), the significance of the increase in density of neurons in the left hemisphere in both groups of prenatally

hypoxic chicks is yet to be determined. As mentioned above, density counts do not to establish whether total neuronal numbers are altered.

5.3.2.3 Tyrosine hydroxylase immunoreactivity

As reported in Chapter 3, HPLC analysis indicated an overall decrease in noradrenaline in the anterior forebrain of the prenatally hypoxic chicks. Immunohistochemistry was used to determine whether the immunoreactivity of TH was altered in specific regions in the anterior forebrain following the prenatal insult. At present, we can not definitely determine whether TH immunoreactivity was altered in the prenatally hypoxic chicks due to the variability and intensity of the staining.

The anterior forebrain was collected for HPLC analysis as it contained the LPO, an area of the chick brain critically involved in memory formation and consolidation (Kossut & Rose, 1984; Rose & Csillag, 1985; Gibbs & Summers, 2002b). TH immunoreactivity within this region was therefore of particular interest. Examination of the control brain tissue for the W14-18 chicks revealed that there was no TH immunoreactivity in the LPO, instead, there was intense staining in tuberculum olfactorium. In the W14-18 tissue, TH immunoreactivity was also limited to the tuberculum olfactorium, and appeared to be reduced compared to controls, which is consistent with the results obtained from HPLC analysis. Conversely, in the control tissue for the W10-18 chicks, intense TH immunoreactivity was evident in some animals in the LPO, nucleus accumbens, and tuberculum olfactorium. This trend was also apparent in the W10-18 chicks; this finding does not reflect the reduction in noradrenaline levels as detected by HPLC.

The nucleus accumbens and tuberculum olfactorium have not been identified as playing a role in memory processing in the chick, furthermore, the interaction between these two regions and the LPO is not known. The TH immunoreactivity, which was evident in the LPO, nucleus accumbens and tuberculum olfactorium, suggests that these three regions could be the major source of catecholamines in this area of the anterior forebrain. Thus, the reduction in noradrenaline levels in the anterior forebrain as detected by HPLC, could possibly be in the LPO, nucleus accumbens or tuberculum olfactorium. In order to obtain a better understanding of the normal distribution of catecholaminergic fibres and terminals in the areas of the chick brain involved in

memory, other enzymes involved in the catecholamine synthesizing pathway, such as dopamine-β-hydroxylase (noradrenaline and adrenaline) and phenylethanolamine-*N*-methyl transferase (adrenaline) could be assessed in the future, however, this was beyond the scope of the present study.

5.3.3 Cerebral vasculature

The percentage of brain parenchyma occupied by blood vessels was used as an indicator of prenatal hypoxia. Under chronically hypoxic conditions in mammals, cerebral blood vessel number and diameter have been shown to increase (Ribatti *et al.*, 1989; Rees *et al.*, 1997; Mallard *et al.*, 1998). This alteration in cerebral vasculature is thought to be due to the stimulating effect of low O_2 concentration on angiogenesis (LaManna *et al.*, 1998). It has been suggested that angiogenesis may be partly mediated by the potent angiogenic growth factor VEGF, which contains a hypoxia-sensitive motif (Makino *et al.*, 2001; Oosthuyse *et al.*, 2001). The dilatation of pre-existing blood vessels, or the formation of new vessels under hypoxic conditions, would result in an increase in cerebral blood flow, thereby maintaining oxygenation of the brain tissue. This may be a basis for brain sparing and increased brain weights, expressed in relation to body weight, in the presence of chronic hypoxemia (Rees *et al.*, 1997; Mallard *et al.*, 1998). However, in the present study, the percentage of the brain parenchyma occupied by blood vessels was not altered at 2 days after hatching. Since the relative brain weights of the W14-18 and W10-18 chicks were not increased at 2 days after hatching, this result is perhaps not surprising. Future studies could confirm whether cerebral oxygenation was maintained during the hypoxic insult by using markers of oxidative or nitrosative stress.

Preliminary results in a parallel study being performed in our laboratory of 8 day-old brain tissue from control and prenatally hypoxic chicks suggested that the percentage of brain parenchyma occupied by blood vessels was increased in the W10-18 chicks compared to their respective controls. There appeared to be no difference in percentage of brain parenchyma occupied by blood vessel between W14-18 chicks and their controls. At this age, the brain weights relative to body weight of the W10-18 were increased. The significance of this late increase in the percentage of the brain parenchyma occupied by blood vessels is yet to be determined.

5.3.4 Cellular events that may lead to memory impairment

Several studies on the developing nervous system have shown that *N*-methyl-D-aspartate (NMDA) receptor activation by glutamate, regulates neuronal survival, axonal growth and connectivity (Balazas *et al.*, 1988; see McDonald & Johnston, 1991; Simon *et al.*, 1992; Burgoyne *et al.*, 1993; see Danbolt, 2001). Under normal conditions, glutamate released from nerve terminals in taken up from the extracellular space by surrounding neurons and astrocytes. However, under hypoxic conditions, glutamate levels can become neurotoxic (Penning *et al.*, 1994; Henderson *et al.*, 1998). The major mode of glutamate neurotoxicity is believed to be via NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate (KA) receptors, causing a toxic influx of Ca^+ into the cell, resulting in the eventual lysis of cell membranes (Puka-Sundvall *et al.*, 2000; Zipfel *et al.*, 2000). In the present study, excessive activation of NMDA receptors via elevated glutamate release during the hypoxic insult could have resulted in reduced growth of neuronal processes or connectivity; the later having implications for cognitive function.

The NMDA receptor is also thought to be involved in learning and memory (Collingridge & Bliss, 1987; Rickard *et al.*, 1994). In the chick, intracranial administration of the NMDA receptor antagonist 2-amino-5-phosphopentanoate immediately after or prior to learning, results in amnesia for the passive avoidance task at 80-90 min post-training (Rickard *et al.*, 1994). This data suggest that processes dependent on NMDA receptor activation are necessary for memory consolidation in the chick. NMDA receptor numbers have been shown to decrease following hypoxia (Mishra & Delivoria-Papadopoulos, 1992); a decrease in which could have implications for memory consolidation. Alterations in receptor numbers or binding could also be involved in the alteration in memory function observed in the prenatally hypoxic chicks.

Studies in the chick have shown that morphological changes occur at the dendritic and synaptic level in the IMHV and LPO following learning (Stewart *et al.*, 1984; Patel *et al.*, 1988). Morphological changes in dendritic spines have been hypothesised to underlie long-term potentiation (LTP), a long lasting form of synaptic plasticity which is thought to be related to learning and memory (Yuste & Bonhoeffer, 2001; Muller *et al.*, 2002). Thus, subtle changes in dendritic spine morphology or synaptic connectivity

as a consequence of prenatal hypoxia may have contributed to the impairments in learning and memory observed in the prenatally hypoxic chicks.

5.3.5 Conclusions

The results from this chapter show that restricting gas exchange across the eggshell from either day 10 or 14 of incubation until day 18 does not result in overt structural alterations in the brain, or alterations in the ratio of astrocytes/neurons in the IMHV. Furthermore, the percentage of parenchyma occupied by blood vessels was not altered in the brains of the prenatally hypoxic chicks at 2 days. However, at 8 days post-hatch, there was a suggestion that the percentage of parenchyma occupied by blood vessels was increase in the W10-18 chicks. The lack of any gross structural abnormalities in the brains of the hypoxic chicks in the present study may be due to the elimination of hypoxic chicks that sustained overt brain damage, owing to their inability to hatch successfully. Taken together, these results suggest that the impaired ability of chicks from wrapped eggs to consolidate memories is not due to overt structural alterations in the brain. This is not to say that the period of prenatal hypoxia did not result in subtle perturbations in synaptogenesis or neuronal connectivity, for example, or a disruption in the functional and metabolic interactions between neurons and astrocytes. Since memory is complex, the cognitive impairment observed in both groups of prenatally hypoxic chicks could be due to a disturbance in a number of different biological processes that are required for the formation and consolidation of memory.

Chapter 6

General Discussion

ADVERSE PRENATAL CONDITIONS that reduce oxygen (O_2) and nutrient supply to the human fetus have been implicated in a number of neurological disorders such as cerebral palsy (Nelson & Ellenberg, 1986; Blair & Stanley, 1988), minimal brain dysfunction disorders (see Altman, 1986), learning disabilities (Marlow *et al.*, 1993), and Attention Deficit Hyperactivity Disorder (ADHD) (Accardo & Whitman, 1992). This thesis has examined the effects of prenatal hypoxia, in the presence of normal nutrient supply, on several parameters, including; postnatal growth, memory function, postnatal brain structure, and the central catecholaminergic system. The results from each study reported in this thesis have been discussed in detail in the respective experimental chapters. In this chapter, the significance of the major findings arising from this thesis will be discussed, and how these results further our understanding of the postnatal consequences of prenatal compromises. The directions for future research in field will also be considered.

6.1 The chick as a model of prenatal compromise

The majority of experimental studies that have examined the effects of prenatal hypoxia on fetal brain development and somatic growth have used mammalian animal models. Techniques that aim to induce fetal hypoxemia often cause fetal hypoglycemia, as well as metabolic, cardiovascular and endocrine perturbations. This ultimately makes it difficult to determine whether subsequent impairments in brain development and fetal growth are due to O_2 deprivation or altered nutrient supply. Furthermore, when inducing fetal hypoxemia in mammals, there are often confounding effects of maternal and placental physiology, and materno-fetal nutrient transfer. The chick embryo is now emerging as a valuable animal model to assess the responses and adaptations to prenatal insults, such as hypoxia. As the chick develops independently of its mother (the mother is only needed to maintain an adequate physical environment), maternal and placental factors that might normally influence fetal responses in mammals, are not of concern as confounding factors. In the chick egg, nutrient supply from the yolk sac and albumin is separated from the area of gas exchange, thereby enabling the direct effects of hypoxia to be studied. The supply of nutrients can also be manipulated independently by the removal of albumin (Miller *et al.*, 2002). The effect of prenatal hypoxia on postnatal neurological function can be readily assessed in the chick, using established behavioural tasks that have been used for 30 years to assess memory formation and consolidation (Gibbs & Summers, 2002b). This is of particular advantage since behavioural testing in the early postnatal period in precocial mammals, particularly sheep, is often difficult to perform as most tasks that assess learning rely on the offspring's desire to be with its mother as reinforcement (Camm *et al.*, 2000).

Through the pioneering work of Blanco and co-workers, it appears that the physiological responses to hypoxia on the cardiovascular system in the chick embryo parallel those seen in humans and other mammalian species. Furthermore, alterations in somatic (Metcalfe *et al.*, 1981; McCutcheon *et al.*, 1982; Stock & Metcalfe, 1987; Asson-Batres *et al.*, 1989) and organ growth (McCutcheon *et al.*, 1982), as well as blood gas status (Mulder *et al.*, 2000; Tazawa *et al.*, 1971b), parallel the changes observed in hypoxic mammalian fetuses. Thus, the chick embryo appears to be a suitable animal model for fetal physiology research.

In spite of the advantages of using the chick embryo over mammalian models, there are some limitations. Unlike in the sheep fetus for example, chick eggs/embryos cannot be catheterised for long periods of time due to risk of infection. Furthermore, the chick has a small blood volume, which limits the volume of blood that can be collected as well as sampling frequency.

6.2 Half-wrapping eggs as a means of inducing hypoxia

Half-wrapping eggs as a means of inducing hypoxia does not readily allow for the determination of embryonic blood gas status. However, I have shown that half-wrapping eggs from either day 10 or day 14 of incubation leads to a decrease in PO_2, and an increase in hematocrit. PCO_2 was increased in the eggs wrapped from day 14, but not from the eggs wrapped from day 10.

I have shown in several studies that chicks from wrapped eggs have impaired memory function, therefore, the cognitive impairment induced by half-wrapping eggs is reproducible. Recent studies have shown that prenatal hypoxia, induced from days 14-18, 10-18 and 10-14 of incubation by reducing incubator O_2 concentration to 14%, results in the same impairment in memory observed in the studies presented in this thesis when the chick eggs were half-wrapped (C. Rodricks and I. Rose, *personal communication*). This finding is significant, and it confirms that the deficit due to half-wrapping eggs with an impermeable membrane is due to hypoxia.

6.3 Mechanisms involved in the postnatal memory impairment observed in the prenatally hypoxic chicks

Several factors could have contributed to the memory impairment observed in the prenatally hypoxic chicks, including hypoxia-induced increase in sympathetic activity, hypoxia-induced corticosterone release, or increased partial pressure of CO_2. Alterations in blood pH, or increases in temperature as a consequence of wrapping, may have also contributed to the impairment in memory. In this thesis, the possible role of the central catecholaminergic system in the postnatal memory impairment was examined, since noradrenaline plays a significant role in memory consolidation (Gibbs & Summers, 2000). The brains of the prenatally hypoxic chicks were also examined to determine whether brain development and/or overt injury had occurred following the prenatal

insult, since several studies in sheep (Bisignano & Rees, 1988; Rees *et al.*, 1988; Mallard *et al.*, 1998) and guinea pigs (Nitsos & Rees, 1990; Mallard *et al.*, 1999; Mallard *et al.*, 2000) have shown that fetal hypoxemia causes alterations in brain development.

6.3.1 Impairments in memory function and the central catecholamine system

It is not proposed that alterated catecholamine levels post-hatch are affecting memory function in these experiments. Instead, I suggest that adrenoceptor function may be altered *in ovo*, if catecholamine levels are increased in response to a hypoxic insult. In normal postnatal chicks, increasing noradrenaline (Gibbs & Summers, 2002b) and adrenaline levels (Chapter 4) in the brain by exogenous administration promotes memory consolidation. Both transmitters/hormones function in a similar fashion via an action at β_2-, β_3-, and α_1-adrenoceptors (Gibbs & Summers, 2002b). Thus with acute administration of noradrenaline and adrenaline, activation of β_2- and β_3- adrenoceptors will promote the consolidation of memory. It is not expected that a chronic exposure will have the same effect as an acute administration.

Plasma catecholamine levels have been shown to increase following prenatal hypoxia *in ovo* (Mulder *et al.*, 2000), and as reported in Chapter 3, noradrenaline levels were high postnatally (adrenaline also tended to be significantly increased). High plasma catecholamine levels during development *in ovo* could influence both peripheral and central neural processes, as the blood brain barrier of the chick is not fully developed until after hatching (Stewart & Wiley, 1981; Liebner *et al.*, 1997). The chick embryo is able to increase plasma noradrenaline and adrenaline concentrations in response to 5 min of acute hypoxemia at day 13 and 16, respectively (Mulder *et al.*, 2000). The timing of the increased noradrenaline and adrenaline levels coincide approximately with the times that the hypoxic insult was induced in the studies outlined in this thesis. Prolonged exposure to noradrenaline and adrenaline will lead to the down-regulation or desensitisation of β_2-adrenoceptors in the brain. Hence, there will be reduced numbers of β-adrenoceptors for subsequent postnatal memory processing. Reduced β-adrenoceptor density in the cerebral cortex (Kramer *et al.*, 1987) has been reported following postnatal hypoxia for 3 weeks in the rat, although the β-subtype which was affected was not specified. This finding further supports the evidence that adrenoceptor

density is altered following an insult during a critical period of development. Future studies could determine whether prolonged exposure to catecholamines *in ovo* results in the reduction in the number of β-adrenoceptors by performing β-adrenoceptor binding studies. In addition, measuring Camp activity would establish whether adrenoceptor desensitization has occurred in the chick brain following prenatal hypoxia.

It should be noted that plasma catecholamine levels were variable, particularly in the W14-18 and W10-18 chicks, with some animals showing extremely high levels. This could be due to differences in the degree of hypoxia induced by wrapping, or variability between animals in their responses to the prenatal hypoxic insult. Increasing the sample size in future studies could reduce this variability.

Noradrenaline has been shown to play a neurotrophic role during brain development (Kasamatsu & Pettigrew, 1976; Berger-Sweeney & Hohmann, 1997; Levitt *et al.*, 1997; Herlenius & Langercrantz, 2001). Noradrenaline levels were shown to be decreased in the anterior forebrains of the prenatally hypoxic chicks, which could have affected neuronal activity or neuronal plasticity in these animals. There is now considerable evidence suggesting that the level of monoamines are reduced in the brains of patients suffering neurodevelopmental disorders such as Rett Syndrome (Okado *et al.*, 2001) and Alzheimer's disease (Hertz, 1989; Dringenberg, 2000). Neurotransmitter disturbances, including noradrenaline imbalance, have also been reported in suffers of schizophrenia, depression, and ADHD (Russell *et al.*, 2000; Bradshaw, 2001).

The proposition that disturbances in the functioning of the central catecholaminergic system and/or alterations in neuronal plasticity during critical periods of development can cause cognitive impairments, is an important consideration that requires further investigation.

6.3.2 Impairments in memory function and brain structure

There is compelling evidence suggesting that adverse prenatal conditions result in altered brain development and neurological impairments in the postnatal period. It is believed that prenatal compromises may disrupt the normal development of the brain, or cause structural alterations, that could potentially affect postnatal brain function. In light

of previous findings in mammals that showed impaired brain development following prenatal hypoxia, the brains of the prenatally hypoxic chicks were examined to determine whether alterations in brain function could be explained by alterations in brain development. There appeared to be no overt structural damage in the brains of the hypoxic chicks, or alterations in the proportion of glia/neurons in the areas of the brain involved in memory consolidation in the chick. However this is not to say that subtle alterations in synaptic connectivity or dendritic spine morphology or number, for example, may have occurred in these animals as a consequence of the prenatal insult. Future quantitative ultrastructural studies and/or Golgi staining of dendritic spines would be required to assess this possibility. The functional and metabolic interactions between neurons and astrocytes (e.g. GLUT transporters) may have been disrupted in the prenatally hypoxic chicks. These parameters should be addressed in future studies.

It is important to note that the brains that were examined were from the chicks that survived the prenatal insult and successfully hatched. It is possible that chicks that did sustain gross structural abnormalities did not survive, or were unable to hatch. Unlike in compromised mammalian pregnancies where births can be assisted in order to improve the chances of survival, this is not the case in the chick embryo, where the precise timing of specific behavioural events is required for the successful hatching of the chick embryo.

Preliminary results suggested that the percentage of brain parenchyma occupied by blood vessels was increased in the W10-18 chicks compared to their respective controls. This finding suggests that alterations in cerebral vasculature may take time to develop and may not be evident at 2 days after hatching, Future studies could investigate the long-term changes in cerebral vasculature following a prenatal insult.

The finding that no overt tissue damage or distortions in the ratios of cell types were evident in the brains of the prenatally hypoxic chicks is significant. This suggests that the cognitive impairment is likely to be due to subtle rather than overt perturbations in brain development. Subtle damage as a consequence of a prenatal insult could considerably occur in human populations.

6.4 Summary

This thesis has shown that prenatal hypoxia from mid-to late gestation can impair memory function after birth. Using the chick as a model, the timing of the prenatal insult, rather than its duration, appears to play a critical role in determining the nature of the memory impairment. In spite of the possible role of the central catecholaminergic system in the memory impairment observed in the prenatally hypoxic chicks, there could be other possible contributing factors. It must be remembered that memory is complex, and that subtle alterations in neural development could impact of any of the cellular processes thought to be involved in memory formation in the chick, as well as other possible memory mechanisms involving synpatic connectivity, second-messenger protein kinases, NMDA receptor activation and long-term potentiation.

6.5 Overall significance of study

The chick embryo was used as an animal model in the studies outlined in this thesis as it offered the unique opportunity to study the effects of prenatal hypoxia on postnatal memory function. The effects of hypoxia alone, in the absence of undernutrition, cannot be readily determined in mammalian models. Furthermore, assessing memory in developing postnatal mammals is often difficult. Drawing parallels to mammalian species, in particular the human situation, results from this thesis suggest that fetal hypoxia alone, in the absence of fetal undernutrition, can impair memory function after birth. The finding of an association between prenatal hypoxia alone and postnatal memory impairment could have implications for human pregnancies complicated with reduced oxygen delivery to the fetus, such as during placental insufficiency or umbilical cord occlusion.

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