Neural and synaptic plasticity in the chick brain after passive avoidance learning

Thesis

NEURAL AND SYNAPTIC PLASTICITY IN THE CHICK BRAIN AFTER PASSIVE AVOIDANCE LEARNING

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ABSTRACT

The avian hippocampus (Hp) is believed to be homologous to mammalian hippocampus both from a developmental and an anatomical view-point. In one-day old domestic chicks (*Gallus domesticus*), studies have demonstrated that it may play a key role in the acquisition of one-trial passive avoidance learning (PAL) where the aversive experience is exposure to a bitter tasting substance, methyl anthranilate.

In the present study, following PAL the numerical density of asymmetric axospinous synapses decreased by 36% in the dorsal Hp of the right hemisphere of MeA trained compared to control birds, 6 hours after training. In contrast, 24h post PAL there is a 33% decrease in numerical density of asymmetric axodendritic synapses in the MeA trained group in relation to the same area of control chicks.

Cell proliferation studies using the thymidine analogue, bromodeoxyuridine, (BrdU) demonstrated a 47% reduction in cell proliferation in the dorsal Hp of the MeA trained group 24h later in comparison with controls, which disappears after 9 days. In nucleus taeniae of amygdala and the arcopallium dorsale and intermediale, there are no differences between birds 24h or 9 days post BrdU injection but in olfactory bulb of MeA trained chicks cell labelling increases by 95% and 71.4% respectively, compared to control and water-trained birds, 24 h after PAL. The increase is more dramatic 9 days post PAL, when the MeA-trained group shows a 259% and 314% increase respectively in relation to control and water-trained animals. Following PAL apoptotic studies in the Hp 24h, 5 and 9 days post BrdU injection demonstrated that there were no differences in cell death between the different groups.

Radioimmunoassay measurements of cortisol in chick forebrain tissue demonstrated longer term increase in levels of steroid in the chick Hp compared to arcopallium and striatum mediale 20 minutes after training, indicating that PAL is a stressful experience which may explain synaptic density and cell proliferation reduction observed after PAL.

Declaration

I declare that the research studies presented in this thesis are original and that the thesis does not exceed 100000 words. Part of this work has been published as an abstract

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List of Abbreviations

A	arcopallium
AA	anterior arcopallium
AChE	acetylcholinesterase
ACHT	Adrenocorticotropic hormone
AD	arcopallium dorsale
ADX	adrenalectomy
AI	arcopallium intermediale
AL	ansa lenticularis (AL)
AM	arcopallium mediale
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
APH	area parahippocampalis
APP	amyloid beta/A4 protein precursor
AR	androgen receptors
at	axon terminal
AVT	area ventralis tegmenti
B	basal amygdala
BDA	biotinylated dextran amine
BDNF	Brain derived neurotrophic factor
bFGF	basic fibroblast growth factor
BLA	basolateral amygdala
BLP	basolateral amygdaloid nucleus, posterior part
BMA	basomedial amygdaloid nucleus, anterior part
BMP	basomedial amygdaloid nucleus, posterior part
BO	bulbus olfactorius (olfactory bulb)
BrdU	5-bromo-2-deoxyuridine
BST	bed nucleus of stria terminalis
CA	cornus ammonis
CaMKII	Ca^{2+} calmodulin kinase type II
CAMs	cell adhesion molecules
CDL	area corticoidea dorsolateralis
CeA	central amygdaloid nucleus
CIO	capsula interna occipitalis

CORT	corticosterone
CPa	commissurae pallii
CPi	cortex piriformis
CPP	cortex prepiriformis
CPu	caudate putamen
CRF	corticotrophin-releasing-factor
CTB	choline toxin B subunit
DA	tractus dorso-arcopallialis
DAB	diaminobenzidine
den	dendrite
DG	dentate gyrus
DGCL	dentate gyrus cellular layer
DHEA	dehydroepiandrosterone
DHp	dorsal hippocampus
DL	dorsolateral Hp
DLd	dorsal dorsolateral Hp
DLv	ventral dorsolateral Hp
DM	dorsomedial Hp
DMA	nucleus dorsomedialis anterior thalami
DMAl	nucleus dorsomedialis anterior thalami, pars lateralis
DMAm	nucleus dorsomedialis anterior thalami, pars medialis
EAA	excitatory amino acid
EFPs	evoked field potentials
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERα	estrogen type α receptors
ERβ	estrogen type β receptors
FL	field L
FPL	fascicullus prosencephali lateralis
GA	Golgi apparatus
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein

GKAPs	guanylate kinase associated proteins
GP	globus pallidus
GRs	glucocorticoids receptors
H or Hp	hippocampus
HA	hyperpallium apicale
HD	hyperpallium densocellulare
HF	hippocampal formation
HI	lateral limb of V-shaped area
HI	hyperpallium intercalatum
HL	nucleus habenularis lateralis
HM	nucleus habenularis medialis
HPA	hypothalamic-pituitary-adrenal
Ht	triangular part of the hippocampus
HVC	High Vocal Center
ICE	interleukin-1β converting enzyme
IGF-1	insulin-like growth factor 1
IL	interleukin
IMHV	intermediate medial hyperstriatum ventrale
IMM	intermediate medial mesopallium
i.p.	intraperitoneal
ITM	intermediate term memory
La	lateral amygdala
LaDL	lateral amygdala, dorsolateral part
LaM	lamina mesopallialis
LaVL	lateral amygdala, vetrolateral part
LaVM	lateral amygdala, vetromedial part
LFS	lamina frontalis superior
LFSM	lamina frontalis suprema
LHy	region lateralis hypothalami
LoC	locus coeruleus
LPO	lobus parolfactorius
LPS	lamina pallio-subpallialis
LPS	Lipopolysaccharide

LSO	organum septi laterale
LTM	long term memory
LTP	long term potentiation
M	mesopallium
MARCKS	myristoylated alanine rich C kinase substrate
Me	medial amygdala
MeA	methylanthranilate
MD	mesopallium dorsale
mGluRs	metabotropic glutamate receptors
mit	mitochondrion
MNM	mediorostral nidopallium/mesopallium
mo	molecular layer
MRs	mineralcorticoid receptors
MV	mesopallium ventrale
N	nidopallium
Na^+/K^+ ATPase	sodium-potassium adenosine trisphosphatase
NBM	nucleus basalis magnocellularis
NCAM	neural cell adhesion molecule
nCPa	nucleus commissurae pallii
NDC	dorsocaudal nidopallium
NDB (FDB)	nucleus diagonalis Brocae
NeuN	nuclear neuronal marker
NGF	nerve growth factor
NL	nidopallium laterale
NMDA	N-methyl-D-aspartate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
NSE	neuron-specific enolase
NSTL	nucleus striae terminalis lateralis
NT	Neurotrophins
NT-3	Neurotrophin-3

NT-4/5	Neurotrophin-4/5
nTSM	nucleus tractus septopallio-mesencephalicus
OA	nucleus olfactorius anterior
OM	tractus occipito-mesencephalicus
OMPFC	orbital and medial prefrontal cortex
OV	nucleus ovoidalis
PAL	passive avoidance learning
PCVL	plexus choroideus ventriculi lateralis
PHA-L	Phaseolus vulgaris leucoagglutin
PHN	nucleus periventricularis hypothalami
PKA	Protein kinase A
PLR	prolactin releasing hormone
PoA	posterior pallial amygdala
PSA-NCAM	polysialylated neural cell adhesion molecule
PSD	postsynaptic density
PSD-95	postsynaptic density protein of 95 kD MW
PVN	nucleus parventricularis magnocellularis
PVt (PV)	pallidum ventrale
RET	relative electron transmission
ROT	nucleus rotundus
RSd	nucleus reticularis, superior, pars dorsalis
RSP	posterior retrosplenial cortex
SA	spine apparatus
SAP-102	synapse-associated protein of 102 kD MW
SCN	suprachiasmatic nucleus
SER	smooth endoplasmic reticulum
SL	lateral septum
SM	medial septum
SMe	stria medullaris
SNc	substantia nigra pars compacta
SPC	segmented, completely partitioned synapses
SPf	substance P field
SPm	medial substance P field

StL	striatum laterale
STM	short term memory
StM	striatum mediale
SVZ	subventricular zone
T	thyroxine
TCA	tricarboxylic acid
TdT	terminal deoxynucleotidyl transferase
TeO	tectum opticum
thal	thalamus
TnA	nucleus taeniae of the amygdala
TrkB	tyrosine kinase B
TrO	tractus opticus
TSM	tractus septopallio-mesencephalicus
TuO	tuberculum olfactorium
Va	vallecula telencephali
VC	ventral core of Hp
VHp	ventral hippocampus
VIP	vasoactive intestinal polypeptide
VL	ventrolateral Hp
VL	ventriculus lateralis
VM	ventromedial Hp
VMN	nucleus ventromedialis hypothalami
VO	ventriculus olfactorius
VS	ventral striatum

PREFACE

The mammalian hippocampus has been intensively investigated in relation to its role in the processes of learning and memory (Olds et al., 1990; Gould et al., 1999b; Ramirez-Amaya et al., 2001) and in particular its involvement in long term potentiation. It has been demonstrated that the mammalian hippocampus shows synaptic remodelling (Ramirez-Amaya et al., 1999; Popov et al., 2004) which appears to be a correlate of long term potentiation (LTP) (Toni et al., 1999; Toni et al., 2001), which may provide a model of learning (Bliss and Lomo, 1973).

The avian hippocampus has been suggested to be homologous to the mammalian hippocampus (Kallen, 1962; Casini et al., 1986; Erichsen et al., 1991; Krebs et al., 1991). However, the avian hippocampus has not been extensively examined and knowledge about its function and its participation in learning models is limited. Studies have shown that it is affected by ischaemia (Horner et al., 1996) and spatial learning (Barnea and Nottebohm, 1994; Regolin and Rose, 1999; Shiflett et al., 2004). In the domestic chicks *Gallus domesticus* it is involved in imprinting (McCabe and Horn, 1994; Sadananda and Bischof, 2004) and one-trial passive avoidance learning (PAL) (Sandi et al., 1992; Unal et al., 2002). It is still unclear, though, how the avian hippocampus is affected by PAL, which is unlikely to be exclusively related to the learning of a spatial task. Studies in the rat have shown that the hippocampus can be affected by other types of learning such as fear conditioning (Winocur, 1997; Shors et al., 2002) whilst stress has been also demonstrated to alter synaptic plasticity (Magarinos and McEwen, 1995; Gould et al., 1997; Gould and Tanapat, 1999; Sandi et al., 2003).

The aim of this study is to examine the effects of passive avoidance learning in the chick hippocampus from two different aspects; synaptogenesis and neurogenesis. It has been shown that the avian hippocampus, in a similar way to the mammalian hippocampus (Schlessinger et al., 1975; van Praag et al., 1999; van Praag et al., 2002), exhibits neurogenesis

in adulthood (Barnea and Nottebohm, 1994). Studies in rats (Kempermann et al., 1997a; Gould et al., 1999b; Shors et al., 2002) and avians (Alvarez-Buylla et al., 1988b; Alvarez-Buylla et al., 1992; Dermon et al., 2002) have implied that learning paradigms enhance cell proliferation or survival of neurones (Kempermann et al., 1998; Gould et al., 1999b). There is also the possibility that PAL may ease cell death as cell proliferation. In this connection, apoptotic studies will demonstrate any differences caused by PAL, whilst at the same time revealling basal levels of cell death in the control group at different time points after learning.

The rat hippocampus is considered part of the 'limbic system' (Sapolsky et al., 1985a) – an interconnected network of brain regions participating in emotional processing and learning (Papez, 1995). It has been demonstrated that the hippocampus is affected by stress in rats (Sapolsky et al., 1985b) and primates (Ohl et al., 2000; Fuchs et al., 2001) and by learning tasks in mammals (Moser et al., 1995; van Praag et al., 2002) and chicks (Good, 1987; Unal et al., 2002). The chick hippocampus shows connectivity with the limbic arcopallium (Szekely, 1999), which is homologous to the mammalian amygdala (Davies et al., 1997), the nucleus taeniae of amygdala (Szekely and Krebs, 1996; Cheng et al., 1999); with the latter being connected to the bulbus olfactorius (Reiner and Karten, 1985), an area strongly affected by strong odours (McKeegan, 2002) and therefore associated with PAL (Richard and Davies, 2000). Therefore, cell proliferation studies were conducted in these areas in order to investigate the effects of passive avoidance learning in cell birth.

Because the mammalian hippocampus can be influenced by stress and show impairments in memory formation (Bodnoff et al., 1995; McEwen and Sapolsky, 1995; Conrad et al., 1996) as well as structural alterations (Gould and Tanapat, 1999; Fuchs et al., 2001; Vyas et al., 2002), a cortisol study was undertaken in this thesis (cortisol has already been identified in chick plasma, Idler et al., 1976; Kalliecharan and Hall, 1976) to measure the

levels of this adrenal steroid in brain tissue, in particular in the arcopallium, striatum mediale (an area significantly affected by PAL, Dermon et al., 2002) and in the hippocampus.

Chapter 1

INTRODUCTION

The introduction of this thesis is aimed to present to the reader some of the topics that will be discussed below and help them understand the connection between learning and memory and their structural effects on certain areas of the chick limbic system. Relevant comparisons are made to mammalian literature where pertinent.

1.1 LEARNING AND MEMORY IN THE CHICK BRAIN

The chick is a good model to test learning paradigms, as well as the formation of memory, because the young domestic chick is precocial and learns a great deal of information during its first few days of life. Memory formation in the day old chick can be divided into three stages (Gibbs and Ng, 1979a; b, Fig. 1.1). A short term stage (STM) is available for 10 minutes after training and its activation is attributed to neuronal hyperpolarization caused by increases in potassium conductance, then an intermediate stage memory (ITM) follows from 15 minutes until 50 minutes after training caused by sodium-potassium adenosine trisphosphatase (Na^+/K^+ ATPase) and finally the long term stage (LTM) which starts 55 minutes post training and is characterised by post-translational glucosylation of pre-existing proteins, followed by a second wave of glycoprotein synthesis de novo (Crowe et al., 1994; Freeman et al., 1995, fig. 1.2.)

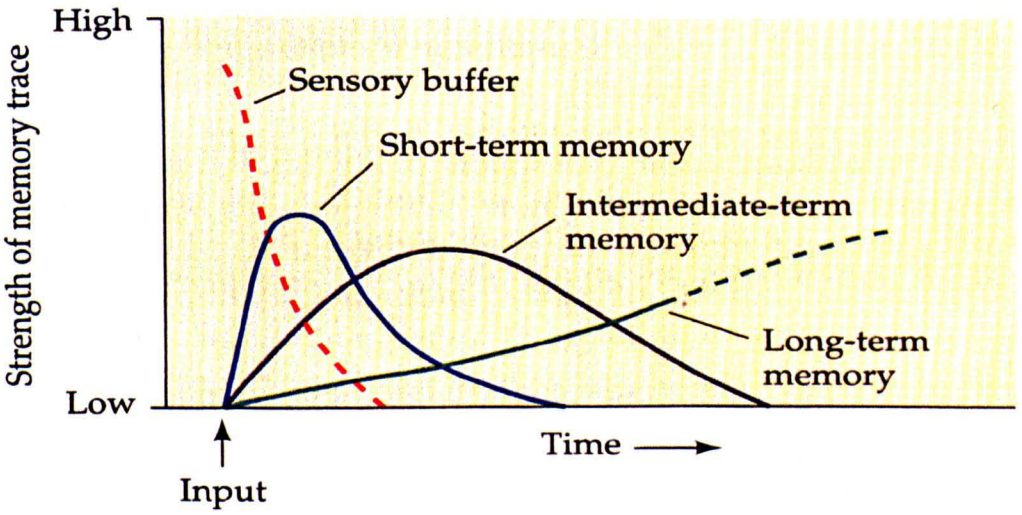

Fig 1.1. Stages of memory formation in relation to time (adapted from McGaugh, 1968)

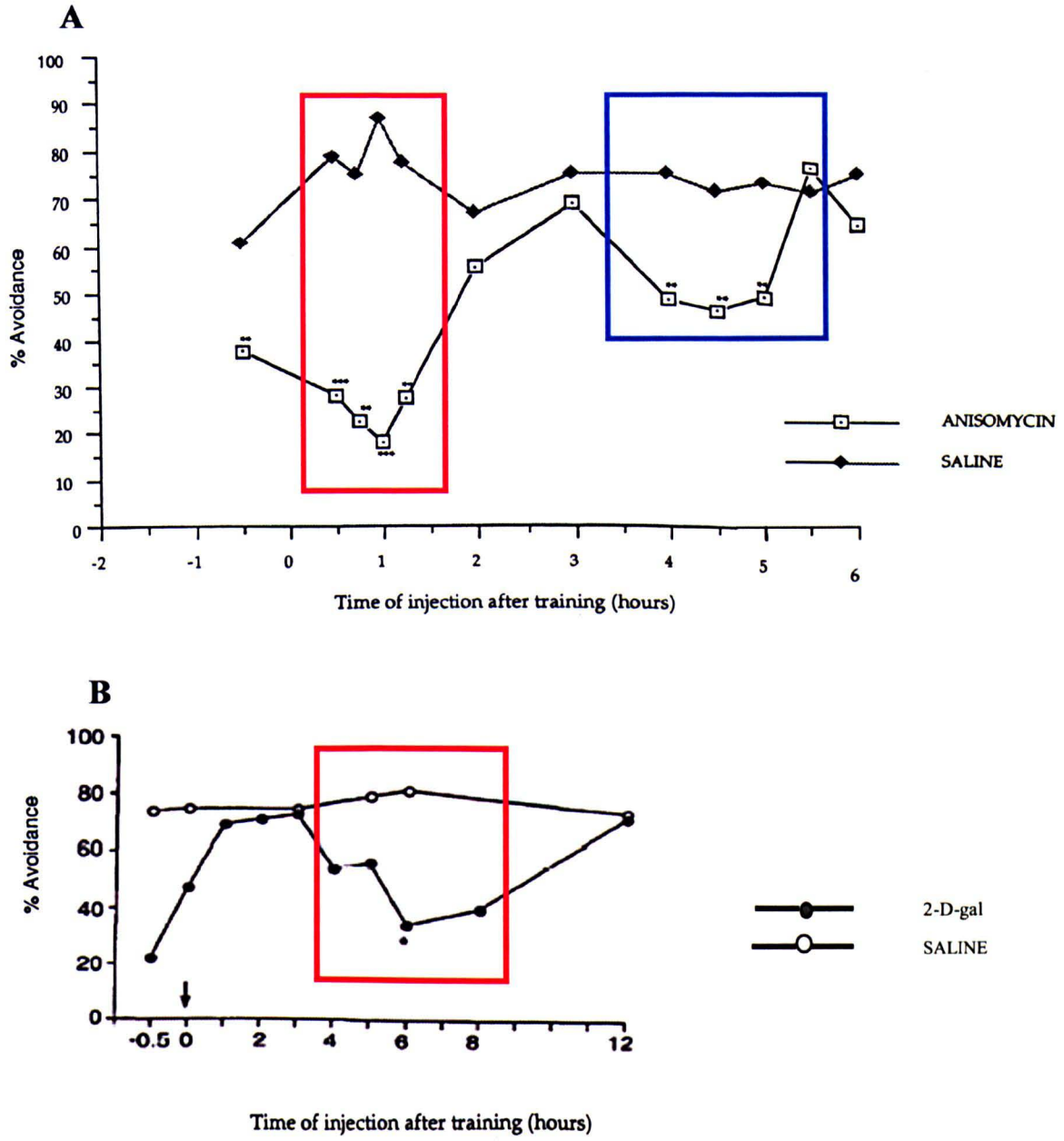

Fig 1.2. Timecourse of double wave of glucoprotein formation and the effect of anisomycin (A) and 2-deoxygalactose (2-D-gal) (B). In diagram A, animals injected at 30 min before or up to 1.5 h after training and 4-5 hours after training showed amnesia when tested 24 hours after training, **P<0.01, ***P<0.001 (graph adapted from Freeman et al., 1995). In diagram B, injections of 2-deoxygalactose 5.5-8 h after training resulted in amnesia at 24h, *P<0.05 (graph adapted from Rose and Jork, 1987)

On the other hand, memory retrieval follows a series of biochemical steps starting with the flux of extracellular calcium (Gibbs et al., 1979), then glutamate and finally N-methyl-D-

aspartate (NMDA) receptor activation (Rickard et al., 1994). In chicks, the immediate early genes *c-jun* and *c-fos* are also activated (Anokhin et al., 1991; Anokhin and Rose, 1991; Mileusnic et al., 1996). A very important discovery to enlighten memory formation processes was that long term memory is protein synthesis dependent (Davis and Squire, 1984). Although anisomycin (protein synthesis inhibitor), if injected before training causes amnesia within one hour leading psychologists to the wrong conclusion that beyond that time point memory formation is protein synthesis independent (Gibbs and Ng, 1977). However, Freeman and colleagues (1995) have proven that there are two time windows for protein synthesis, since anisomycin injections made 4-5 hours after training were amnestic when animals were tested 24h after the training. Although memory formation events involve the upregulation of NMDA receptors 30 min after training, 5,5 hours post-training, AMPA receptors are activated but not NMDA (Steele and Stewart, 1995). Finally, it has been demonstrated that two waves of glycoprotein synthesis occur (Scholey et al., 1993; Crowe et al., 1994), including cell adhesion molecules (CAMs), which have been suggested to participate in learning and synaptic remodelling procedures (Luthl et al., 1994; Rusakov et al., 1994; Skibo et al., 1998).

Long-term memory can be defined as declarative (memory for facts and events-that is) and non-declarative (procedural-memory for performance such as motor skills and learning of rules and procedures-how to) memory (Fig. 1.3). A further subdivision of declarative is into episodic and semantic.

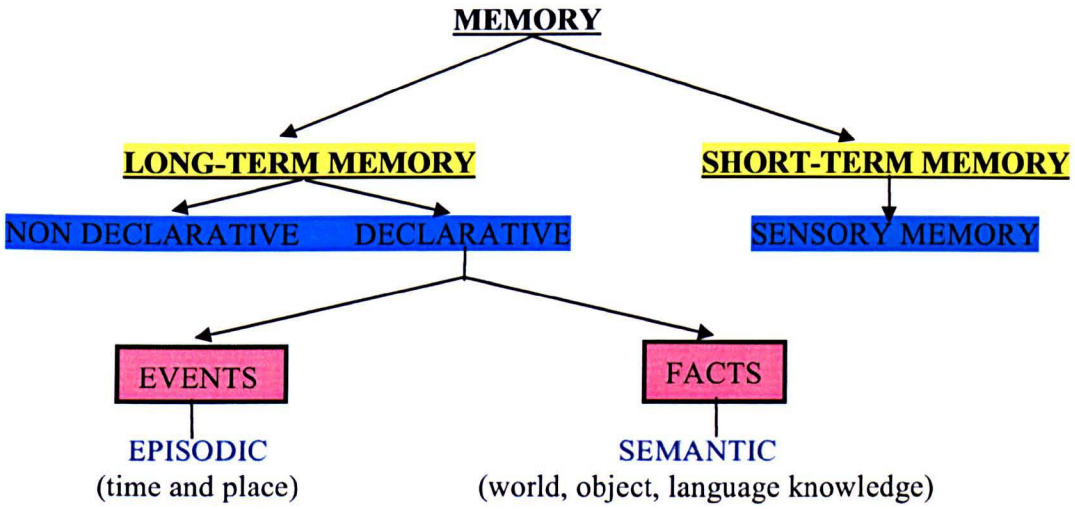

Fig.1.3 Drawing representing the structure of human memory demonstrating the relationship between different forms of declarative memory (adapted from Squire and Liss, 1968).

Behavioural studies in domestic chicks have been conducted mainly by using two well established learning tasks, the passive avoidance learning (PAL) (Cherkin, 1969) and imprinting (Horn et al., 1979; McCabe and Horn, 1988). During PAL the chicks are presented with a chrome bead dipped in a bitter tasty substance, methylanthranilate (MeA), which they peck and then demonstrate disgust responses, including head shaking, emission of distress sounds, bill wiping and backward movements. After pecking the bead, they will avoid a similar but dry bead for several days. In contrast, animals that are trained with a bead dipped in water will continue to peck when re-tested (Mileusnic et al., 1980; Dermon et al., 2002). The two main structures that participate in PAL are the intermediate medial mesopallium (IMM) and striatum mediale (StM) (Rose and Csillag, 1985; Curtis et al., 1989; Lowndes and Stewart, 1994), although studies have proven the importance of other structures in memory formation or retention after PAL (Sandi et al., 1992; Lowndes and Davies, 1994). Imprinting is based on the preference of young chicks to follow an object after having been exposed to it (Bateson, 1966; Bolhuis, 1991).

Imprinting has been demonstrated to activate the IMM by causing synaptic and molecular changes (McCabe et al., 1981; Horn, 1998). Bilateral lesions to the IMM before or

3 h after imprinting have been demonstrated to prevent the chick from learning the imprinting paradigm (McCabe et al., 1981), indicating that this area is essential for memory acquisition and retention. Ablations to the IMM 6 h after training had no effects, indicating that the IMM is not required for memory recall (McCabe et al., 1982). Some of the changes in the IMM after imprinting include an increase in NMDA receptors (McCabe and Horn, 1991), increase in phosphorylation (Sheu et al., 1993), mRNA expression (Meberg et al., 1996) and in the amount of myristoylated alanine rich C kinase substrate (MARCKS) (Solomonia et al., 2003). There is also release of γ-aminobutyric acid (GABA) and taurine (McCabe et al., 2001), activation of early gene *c-fos* and product Fos (Suge and McCabe, 2004), increase in neural cell adhesion molecule (NCAM) (Solomonia et al., 1998) and clathrin heavy chain protein (Solomonia et al., 1997), which is responsible for vesicle recycling during synaptic plasticity processes (Maycox et al., 1992). Synaptic changes demonstrated at electron microscope level also occur during imprinting (Bradley et al., 1981; Horn et al., 1985) and increased neuronal activity has been recorded (Bradford and McCabe, 1994). Studies in the chick hippocampus have not, however, shown any gene or neuronal activity after imprinting (McCabe and Horn, 1994; Suge and McCabe, 2004).

Passive avoidance learning activates a number of biochemical and molecular procedures such as uptake of 2-deoxyglucose (Kossut and Rose, 1984; Rose and Csillag, 1985), amyloid beta/A4 protein precursor (APP) upregulation (Mileusnic et al., 2000) muscarinic (Rose et al., 1980) and δ-opioid receptor binding (Csillag et al., 1993), *in vivo* and *in vitro* L-leucine incorporation (Mileusnic et al., 1980; Schliebs et al., 1985) and activation of the early genes *c-fos* and *c-jun* around 40 minutes after training (Anokhin and Rose, 1991) and of their products 1-2 hours later (Freeman and Rose, 1995). At the same time as Fos production, an increase in calcium flux in synaptoneurosomes has been observed (Salinska et al., 1999). A training paradigm similar to PAL has been carried out in hens and has shown an

increase in the levels of glucose available for utilisation from the brain (Gibbs and Summers, 2002) via activation of β_2 and β_3 adrenoreceptors. Memory reinforcement may be taking place due to neurotransmitter release caused by glucose increase, an example being the case of acetylcholine (Kopf et al., 2001). Additionally, changes in synaptic plasticity have been observed after PAL, such as an increase in synaptic density in the IMM (Doubell and Stewart, 1993; Stewart and Rusakov, 1995) and StM (Stewart et al., 1984; Hunter and Stewart, 1993), redistribution of neural cell adhesion molecule (NCAM) in the synaptic junction (Rusakov et al., 1994) and interestingly a different pattern of morphological changes has been demonstrated after learning for symmetrical and asymmetrical synapses (Stewart et al., 1987).

Many studies have focused on the activation of NMDA receptors after PAL in chicks (Burchuladze and Rose, 1992), since it has been demonstrated that they participate in LTP (Bliss and Collingridge, 1993). Another glutamate receptor is that for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), which along with NMDA plays a key role in LTP (Bliss and Collingridge, 1993). AMPA has been suggested to help the activation of NMDA receptors by providing the postsynaptic depolarization in order for NMDA receptors to reduce their blockage caused by Mg^{2+} (Bliss and Collingridge, 1993). Although studies are controversial as far as the participation of AMPA in memory formation is concerned (Burchuladze and Rose, 1992; Stewart et al., 1992; Rickard et al., 1994), indications point to the assumption that AMPA may be taking part in memory retention or recall (Steele and Stewart, 1995).

There have been increases in NMDA receptor binding in the left IMM and StM 30 minutes after PAL (Stewart et al., 1992), which presumably result from an increased release in neurotransmitter glutamate which causes an upregulation of NMDA receptors (Steele et al., 1995) and as a result memory enhancement. Furthermore, 3 h after PAL, NMDA receptor

binding has been identified also in other areas such as the hippocampal formation (Stewart et al., 1993).

Neurotrophins (NT) have been suggested to take part in synaptic plasticity and memory formation. Brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) participate in the induction of LTP (Castren et al., 1993), whilst NGF, neurotrophin 3 (NT-3) and neurotrophin-4/5 (NT-4 /5) are involved in spatial memory (Fischer et al., 1987) and NGF is involved in memory consolidation of passive avoidance training in rats (Ricceri et al., 1994). In chicks, however, only BDNF is implicated in memory consolidation (Johnston and Rose, 2001) and transition from short to long term memory. Injection of BDNF antibodies influences the levels of SNAP-25 and syntaxin (Johnston et al., 1999) and therefore changes synaptic efficiency (Johnston and Rose, 2001). It has been suggested that an intracellular signal is transmitted via tyrosine kinase B (TrkB) receptors affecting GABAergic systems (McKay et al., 1999).

The chick brain shows asymmetry after learning (Stewart et al., 1984) as well as in perception of stimuli. The left avian hemisphere is concerned with discrimination and categorisation of object related cues (Prior et al., 2004) and individual stimuli like inhibiting pecks at distracting objects (Mench and Andrew, 1986), whilst the right hemisphere is essential for spatial tasks (Andrew, 1991). Studies have shown that lesions of the right IMM distract memory recall at the early stages of memory, whilst the same does not happen after ablations of the left IMM (Gilbert et al., 1991; Andrew, 1999). As Andrew (1999) has implied, the human and the chick brain use the right hemisphere to preserve information available for use, whereas the left hemisphere is responsible for operations that belong or are part of the information maintained from the right hemisphere.

Nitric oxide (NO) has been suggested to take part in learning and memory as well as synaptic plasticity procedures that trigger memory formation (Haley et al., 1992; Hawkins et

al., 1998; Prast and Philippu, 2001) and modulation of aggression (Nelson et al., 1995). NO has been demonstrated to be a potential retrograde synaptic messenger in LTP (Gally et al., 1990; Haley et al., 1992), whilst nitric oxide synthase has been located in hippocampal spines (Burette et al., 2002). Studies for the colour discrimination in cockerels have shown that lesions on the left hemisphere around the time of training impair memory formation (Rickard and Gibbs, 2003) as it has been also observed in chicks (Patterson et al., 1990). Therefore it has been suggested that the left hemisphere is activated immediately after learning, with neuronal (nNOS) and endothelial (eNOS) nitric oxide synthase isoforms being necessary for memory formation (Rickard and Gibbs, 2003). In rats, however, the production of LTP requires activation of either isoform (eNOS or nNOS) (Son et al., 1996). Inhibition of eNOS in the right hemisphere occurs only if the inhibitors are administered during the intermediate stage of memory formation (15-55 minutes post training). In contrast, inhibitors of nNOS have no effects (Rickard and Gibbs, 2003). Therefore, these authors suggested that nNOS is activated around the time of training, whilst eNOS is activated 10-25 minutes after training in the right hemisphere. Gibbs et al. (2003) concluded that in chicks the left hemisphere plays a crucial role in memory formation whilst the right hemisphere participates in ITM procedures. Other studies have shown the presence of NOS in the chick brain, demonstrating that the striatum mediale contains an abundance of NOS labelled neurones and extensive NOS labelled neurite arborisation (Panzica et al., 1994; von Bartheld and Schober, 1997), unlike the IMM and the arcopallium. The striatum mediate receives strong dopaminergic input from the area ventralis tegmenti (AVT) and substantia nigra pars compacta (SNc) (Moons et al., 1994); NO can affect the release of dopamine (Lonart et al., 1993), the latter modulating glutamatergic neurotransmission, which is involved in LTP and learning (Madison et al., 1991; Rogers, 1993).

Lipopolysaccharide (LPS) is part of the membrane of Gram negative bacteria and infection causes the induction of cytokines interleukin IL-1β and IL-6 and the activation of hypothalamic-pituitary-adrenal (HPA) axis resulting in increased levels of circulating corticosteroids (Zuckerman et al., 1989). It has been shown to impair the performance of rats for the water maze training (Shaw et al., 2001), the induction of LTP (Bellinger et al., 1993) and passive avoidance learning in one day old chicks (Sell et al., 2001). It has been also demonstrated to disrupt the ITM and as a result consolidation of LTM, but leaving striatum mediale intact by disrupting the Na^+/K^+ ATPase activity and rising corticosterone levels (Sell et al., 2003).

PAL has been shown to be a stressful experience elevating plasma corticosterone levels (Sandi and Rose, 1997). Interestingly, injections of corticosterone in the IMM just before or after weak training (10% MeA), enhance memory retention (Sandi and Rose, 1994a) whilst administration of glucocorticoid receptors antagonists cause amnesia for the strong training (100% MeA) (Sandi and Rose, 1994b). Furthermore, injections of corticosterone in the IMM enhance protein synthesis for 6 hours (Sandi et al., 1995).

1.2 WHY STUDY THE CHICK HIPPOCAMPUS?

The hippocampus is strongly involved in learning of spatial tasks in rats (Moser et al., 1993; Olsen et al., 1994; Ramirez-Amaya et al., 2001) and birds (Biegler et al., 2001; Watanabe, 2002). The chick hippocampus has been demonstrated to participate also in other forms of learning such as the passive avoidance learning (Unal et al., 2002). It has been suggested that the chick hippocampus has bilateral connectivity with the intermediate medial mesopallium (IMM) (Bradley et al., 1985) and ipsilateral connections with the striatum mediale (StM) (Atoji et al., 2002)-two regions that are closely associated with PAL (Rose and

Csillag, 1985; Stewart and Rusakov, 1995; Csillag, 1999). Therefore, it could also be affected by PAL.

1.3 THE HIPPOCAMPUS

1.3.1 The mammalian hippocampus

The hippocampal formation is in the mammalian brain and consists of 6 anatomically discrete sections (Fig. 1.4): entorhinal cortex, para- and presubiculum, the subiculum proper, Ammon's horn and dentate gyrus (Amaral and Witter, 1995). The hippocampus is responsible for episodic, declarative, contextual and spatial learning and memory (McEwen, 2001).

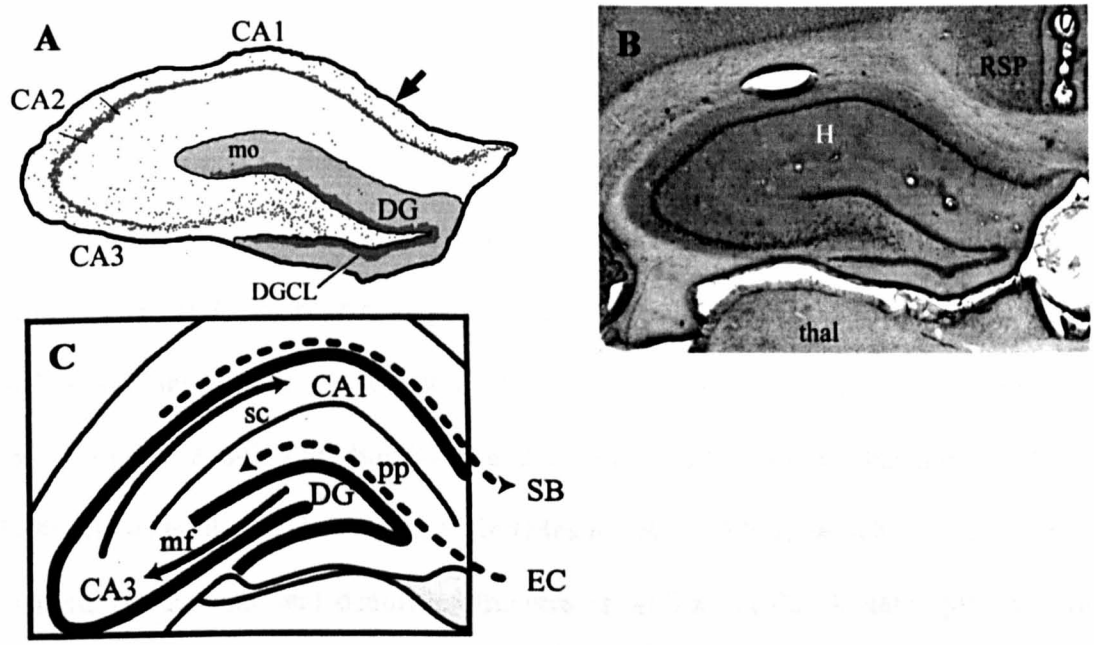

Fig 1.4. A. Representation of the mammalian hippocampus. Arrow indicates input from the subiculum. CA: cornus ammonis (Ammon's horn), DG: dentate gyrus, DGCL: dentate gyrus cellular layer, mo: molecular layer. B. Coronal section of hippocampus. H: hippocampus, thal: thalamus, RSP: posterior retrosplenial cortex (Popov et al., 2004, fig.1, page 253). C. Connectivity representation. Mf: mossy fibre pathway, SB: subiculum, sc: Schaffer pathway, pp: perforant pathway (Hough et al., 2002, fig.1, page 298).

LTP is an extensively studied model to explore the synaptic changes that underlie learning and memory in the hippocampus (Bliss and Lomo, 1973; Bliss and Collingridge, 1993). LTP is an increase in synaptic activity that results from brief, high frequency

stimulation of afferent fibres (Barnes, 1979) and depends on coincident depolarization of both pre- and postsynaptic regions (Malinow and Miller, 1986). It produces mossy fibre synaptogenesis in CA3 (Adams et al., 1997) whilst other studies have demonstrated that LTP increases the number of axospinous synapses in CA1 (Chang and Greenough, 1984) and causes new synapse formation (Toni et al., 1999; Toni et al., 2001). Although the induction of LTP in CA3 is NMDA-receptor independent (Escobar et al., 1997), in CA1 in order for LTP to be induced, NMDA receptors must be activated via glutamate and simultaneously they should produce sufficient depolarization of the postsynaptic membrane to relieve a Mg^{2+} block in the ion channel and permit the entrance of calcium in the postsynaptic terminal (Lynch and Baudry, 1984; Malenka et al., 1988)

The hippocampus participates in various types of learning and most notably spatial water-maze training (Morris, 1984), as well as aversive types of learning, such as the passive avoidance response where animals receive electric shocks when entering a dark compartment (Black et al., 1977; Lorenzini et al., 1996). It responds physiologically to several hormones such as adrenal steroids (Lupien et al., 1998; Joels, 1999; Lathe, 2001), adrenaline (Miyashita and Williams, 2004), estradiol (Lam and Leranth, 2003), thyroid hormones (Meaney et al., 2000; Matos et al., 2002) and serotonin (Meaney et al., 2000), which can provoke changes in synaptic connections and dendritic structure as well as in the dentate gyrus volume during development and in adult life (Lupien et al., 1998).

In mammals, the hippocampus can be damaged by stroke (Strong et al., 1990; Sieber et al., 1997; Frerichs, 1999; Letechipia-Vallejo et al., 2001) and head trauma (Fowler et al., 2002) and is sensitive to ageing and repeated stress (Sapolsky, 1992; McEwen and Magarinos, 1997). The dentate gyrus exhibits experience-dependent structural changes due to the continuation of neurogenesis (Altman and Das, 1965; Schlessinger et al., 1975; Gould et al., 1997; Gould et al., 1999b; Gould et al., 1999c) and synapse formation (Ramirez-Amaya et al.,

1999; Ramirez-Amaya et al., 2001) into adulthood, whilst it is also affected by environmental stimuli (Kempermann et al., 1997a; Winocur, 1997; Wood et al., 1999). Especially in adult life the reduction of neurogenesis as well as other pathological conditions can alter dramatically the volume of hippocampus resulting in a loss of memory and the inability of learning. Aged rats show impaired performances for tasks that involve spatial recognition (e.g. radial arm maze, water maze training, Morris, 1984; Gallagher and Burwell, 1989; Ward et al., 1999) possibly because of alterations in visual ability (O'Steen et al., 1995; Lindner et al., 1997) or stress responses (Bodnoff et al., 1995; Mabry et al., 1996). Dysfunction of hippocampus can cause vegetative and endocrine abnormalities as well as memory and cognitive deficits (Zola-Morgan et al., 1986; Rempel-Clower et al., 1996) with episodic memory (information about 'where-when-what', (Clayton et al., 2003) being disrupted more than semantic memory (Vargha-Khadem et al., 1997).

Previous studies have exhibited that the dorsal and ventral hippocampus participate in different memory processes and lesions caused in the abovementioned areas affect behavioural learning in diverse ways (Hughes, 1965; Sinnamon et al., 1978). Dorsal hippocampus lesions prevented rats from learning the water maze training unlike ventral lesions (Moser et al., 1993; Moser et al., 1995). In addition, the former caused impairments in the preservation of memory after passive avoidance response (Winocur and Bindra, 1976; Black et al., 1977; Cogan and Reeves, 1979; Walsh et al., 1984). The ventral hippocampus in rats has efferent connections with several subcortical regions like the basolateral amygdala (Ebert et al., 1995) and the medial prefrontal cortex (Ishikawa and Nakamura, 2003) the latter participating in the regulation of fear emotions and fear related behaviour (Morgan et al., 1993; Quirk et al., 2000), which may indicate that the ventral hippocampus may play a key role in autonomic, emotional, social and reproductive procedures (Moser and Moser, 1998). On the other hand lesions to the dentate gyrus by the microtubule disrupter colchicine (Sutherland et al., 1983),

or adrenalectomy (Conrad and Roy, 1993) impair spatial learning in rats similarly to ischaemic lesions of the CA1 region (Volpe et al., 1992; Olsen et al., 1994). These data imply that the dorsal and the ventral hippocampus control different functions and that there is a combination of diverse connections and tasks in the hippocampus (Moser and Moser, 1998).

1.3.2 The chick hippocampus

The avian hippocampal formation, which includes the hippocampus (Hp) and the area parahippocampalis (APH) (Karten and Hodos, 1967), occupies the dorsomedial part of the telencephalon and originates from the medial pallium (Atoji et al., 2002). It is considered to be homologous to the mammalian hippocampus (see Table 1.1 for comparison of regions), because from an embryological aspect it emerges from the same part of the developing neural tube and is likewise positioned relative to the lateral ventricle as does the mammalian hippocampus (Kallen, 1962).

Mammalian Brain	Avian Brain
Hippocampus (Hp)	Hippocampus (Hp)
Lateral (La) and accessory basal amygdala (parts of the basolateral amygdala-BLA)	Intermediate arcopallium (AI)
Basal amygdala (part of the basolateral amygdala-BLA)	Arcopallium dorsale (AD)
Medial amygdala (Me)	Nucleus taeniae of amygdala (TnA)
Olfactory bulb (BO)	Olfactory bulb (BO)
Neocortex	Avian Wulst, nidopallium, mesopallium

Table 1.1. Table illustrating homologue structures between the mammalian and avian brain.

Studies have shown that there are parallels in gene expression during development (Puelles et al., 2000). Furthermore, it shows resemblance in connectivity (Casini et al., 1986; Szekely, 1999; Atoji et al., 2002; Hough et al., 2002), neurotransmitter content (Erichsen et

al., 1991; Krebs et al., 1991) and electrophysiological properties similar to the theta rhythm (Siegel et al., 2000).

The input and output connections of the avian hippocampus (see Fig 1.5) are similar to those of the mammalian hippocampus since both regions receive projections from the contralateral hippocampus, the thalamus, the hypothalamus, the raphe nuclei and the locus coeruleus (Benowitz and Karten, 1976; Krayniak and Siegel, 1978; Casini et al., 1986; Trottier et al., 1995), project to the hypothalamus and the septal nuclei, whilst demonstrate reciprocal connectivity with the diagonal band and arcopallium (amygdala in mammals) (Bons et al., 1976; Bouille et al., 1977; Casini et al., 1986; Lorenzini et al., 1996; Szekely and Krebs, 1996).

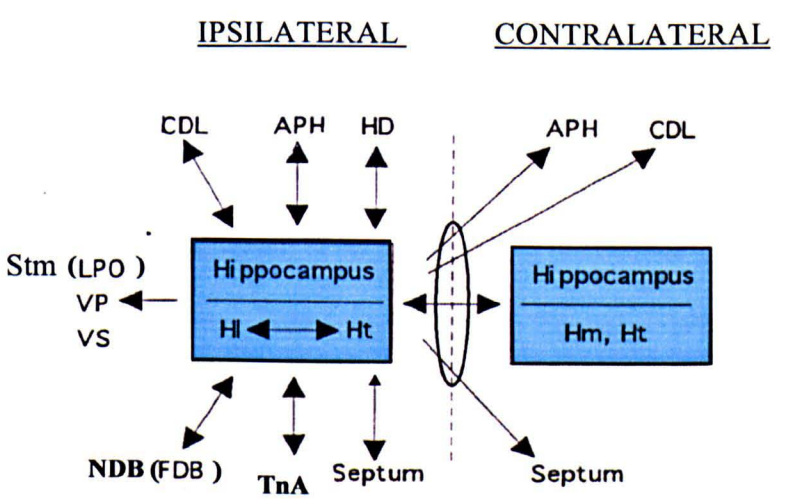

Fig 1.5. Drawing illustrating the afferent and efferent connections of the dorsal hippocampus. Double arrowheads show reciprocal connections. The septum receives more afferents in relation to efferents and therefore the arrowhead is smaller. The dashed line divides the brain into right and left side. The oval shape indicates the oval commisure (drawing from Atoji et al., 2002). APH: area parahippocampalis, TnA: nucleus taeniae of the amygdala, Stm (LPO): striatum mediale, VS: ventral striatum, HD: hyperpallium densocellulare, CDL: area corticoidea dorsolateralis, HI: lateral limb of V-shaped area, Ht: triangular part of the hippocampus, PVt (PV): pallidum ventrale, NDB (FDB): nucleus of the diagonal band (from Atoji et al., 2002, fig 14, page 194).

Additionally, there appear to be morphological similarities of neuronal types in the hippocampi of these different species (Molla et al., 1986; Montagnese et al., 1996; Tombol et al., 2000). However, one of the differences (Bingman et al., 2003) centres on the pallial

sensory inputs into the HF, which exhibit anatomical differences between pigeons and rats. Through evolution, the cerebral hemispheres have enlarged (Bingman et al., 2003) and have formed the neocortex in mammals and the anterior forebrain Wulst in birds. Regions within the neocortex and avian Wulst are the sources of visual input into HF that give the visual information to the hippocampus to include space perception as part of memory (Bingman et al., 2003).

The avian hippocampus appears to be a much simpler structure than the mammalian hippocampus due to its lack of lamination, in other words the absence of cytoarchitectonically distinct layers. This cytoarchitectural difference makes it difficult to characterize the avian hippocampal formation into regional subdivisions. Based on immunocytochemical (Erichsen et al., 1991), cell morphology (Molla et al., 1986), connectivity (Casini et al., 1986; Szekely, 1999; Atoji et al., 2002) and electrophysiological data (Siegel et al., 2002) the ventral hippocampal formation resembles the mammalian Ammon's horn. The dorsal avian hippocampus in contrast is considered to be equivalent to the dentate gyrus and the APH to the subiculum (Casini et al., 1986; Krebs et al., 1991; Szekely, 1999; Atoji et al., 2002). Specifically, Erichsen et al. (1991) divided the avian hippocampus into 7 subregions (Fig 1.5.), from area 1 to 7. Area 2 corresponds to mammalian Ammon's horn (CA1-CA3), area 3 to the dentate gyrus, area 4 to the hilus, area 6 to the subiculum and area 7 to the entorhinal cortex.

In the zebra finch, 4 subregions have been suggested, the V formation that corresponds to Ammon's horn, the DM that relates to the dentate gyrus, the DL that matches up to the subiculum (although the division of the DL areas is not unmistakably characterized) and SP field that relates to the entorhinal cortex (Szekely and Krebs, 1996, Fig. 1.6; Szekely, 1999).

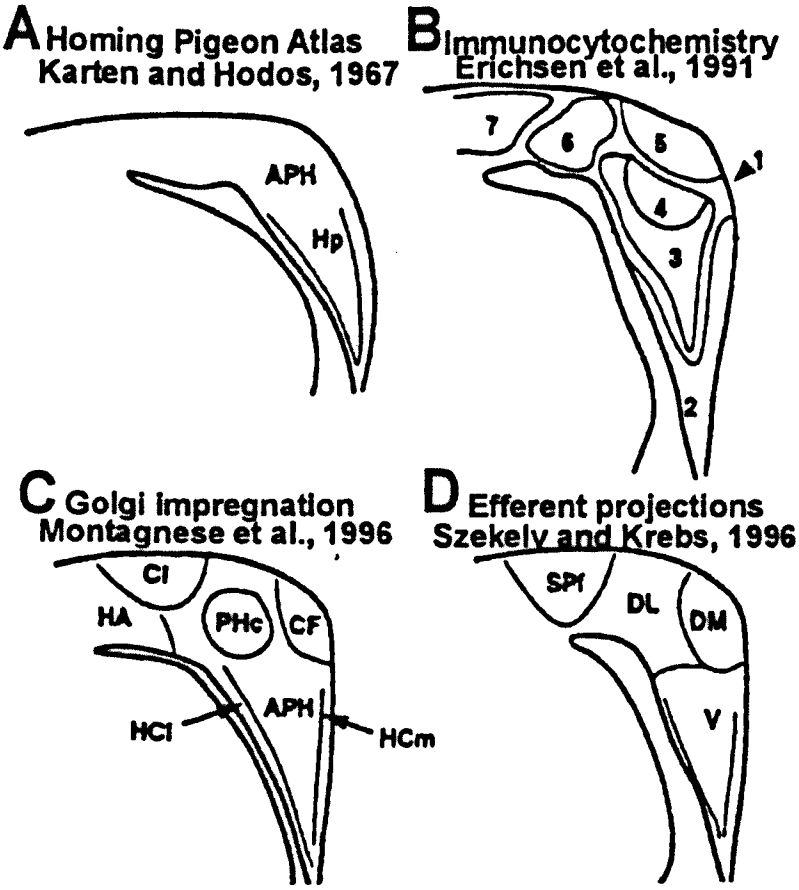

Fig. 1.6. A.The avian HP and APH according to the pigeon atlas of Karten and Hodos, 1967. B Avian subdivisions of the HF based on immunocytochemical markers. 1.alveus, 2.Ammon's horn, 3. hilus, 4, 5.dentate gyrus, 6. subiculum, 7. entorhinal cortex. C. Avian HF based on Golgi techniques. CI: entorhinal cortex, HCm : Ammon's horn, PHc: subiculum. D. Subdivisions based on anterograde and retrograde pathway tracing techniques. DM: dentate gyrus, V: Ammon's horn, DL: subiculum, SPf: entorhinal cortex (from Siegel et al., 2002, fig.8, page 266).

To further support the hypothesis that the dorsomedial part of the hippocampus resembles to the dentate gyrus, serotonergic immunohistochemistry has revealed a dense 5-HT+ fibre network in this area (Metzger et al., 2002), as occurs in the mammalian dentate gyrus (Hornung et al., 1990). The entorhinal cortex in mammals receives afferents from the olfactory bulb, anterior olfactory nucleus and piriform cortex (Amaral and Witter, 1995). Electrophysiological studies (Siegel et al., 2002, Fig. 1.7.) have demonstrated different firing rates of units in the ventral and dorsocaudal in relation to dorsorostral HF.

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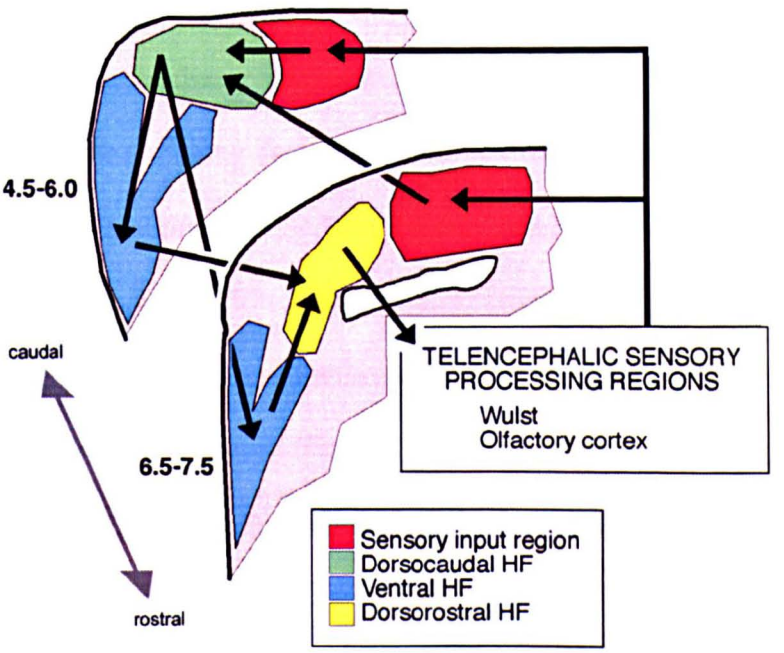


Fig.1.7. Model of subdivisions of the avian hippocampus proposed by Siegel et al., 2002 based on electrophysiological techniques. In this model, the "dentate gyrus" is limited to the dorsocaudal HF, the subiculum like region is restricted to the dorsorostral HF, whilst the ventral region is equivalent to Ammon's horn. Arrows show the flow of information assuming that the flow resembles that of the mammalian hippocampus (drawing from Siegel et al., 2002, page 266, modified by P.L.Gabbott).

Furthermore, the abovementioned authors showed that the dorsocaudal region is equivalent to the dentate gyrus whilst the dorsorostral part is comparable partially to the mammalian subiculum. In the pigeon the APH receives inputs from the olfactory bulb via the piriform cortex (CPi) (Reiner and Karten, 1985; Bingman et al., 1994). This finding indicates a possible equivalence of part of the APH to the entorhinal cortex. Studies after injections of neuronal tracers (biotinylated dextran amine – BDA and choline toxin B subunit-CTB) into the hippocampus at A. 6.50, have shown that in the medial part of the APH, BDA or CTB somata were located, whilst in the lateral part of APH, BDA fibres terminated (Atoji et al., 2002). Additionally, these authors demonstrated that the medial part of APH projects ventromedially and the lateral dorsolaterally as the APH projects rostrally. These findings, in conjunction with the studies of Siegel et al. (2002) indicate that the medial part of APH is comparable to the entorhinal cortex and the lateral part to the subiculum (Atoji et al., 2002).

The involvement of the mammalian hippocampus in spatial memory and other aspects of cognition (Squire, 1992; Wood et al., 2000) is controlled by a one-way feed-forward trisynaptic pathway (Amaral and Witter, 1995) that can be summarised as a series of projections running from the entorhinal cortex to the dentate gyrus to the CA3 region of Ammon's horn to the CA1 region and then to the subiculum and back to the entorhinal cortex (Andersen et al., 1971; Finnerty and Jefferys, 1993; Kahn et al., 2003). In the pigeon, Hough et al. (2002) by using stimulation evoked field potentials (EFPs) has suggested a circuit within the avian hippocampus (Fig 1.8.):

DL —> DM —> ipsi and contralateral ventral regions, VM and VL —> VM —> VL —> DLv

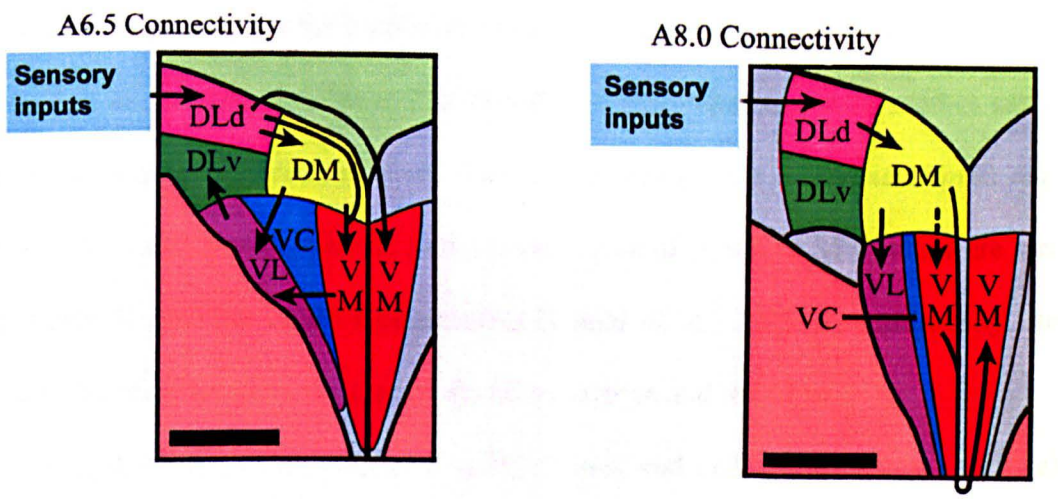


Fig.1.8. Summary of connectivity as seen at indicated levels of the pigeon brain. DLd: dorsal dorsolateral Hp, DLv: ventral dorsolateral Hp, DM: dorsomedial Hp, VL: ventrolateral Hp, VM: ventromedial Hp, VC: ventral core of Hp. Scale bar 1mm (drawing from Hough et al., 2002, pages 301 and 303)

This connectivity outline can be further supported by the fact that the pigeon dorsolateral Hp (DL) receives sensory input and contains two neuropeptides that exist in the

mammalian entorhinal cortex, preprotachykinin (substance P) and leucine encephalin staining (Amaral and Campbell, 1986; Erichsen et al., 1991). These data along with connectivity studies which show that the DL when stimulated results in evoked field potentials (EFPs) in dorsomedial Hp (DM) (Hough et al., 2002). The DM, which is efferent to the DL, appears to be equivalent to the dentate gyrus because it demonstrates choline acetyltransferase immunoreactivity (Krebs et al., 1991) as is found in the dentate gyrus of mammals (Amaral and Campbell, 1986), in addition to substance P and vasoactive intestinal polypeptide (VIP) (Erichsen et al., 1991; Krebs et al., 1991). These findings together indicate that the DL is homologous to the entorhinal cortex, since the latter also projects to the dentate gyrus in mammals (Andersen et al., 1971). The substance P field (SPf) shows a dense network of fibres and terminals which are immunoreactive for substance P (SP-ir). SP-ir was also found in the area called the medial substance P field (SPm), which is located lateral to the SPf. The latter constructs the borders of the hippocampus and covers a big part of the area parahippocampalis (Gould et al., 2001b). Substance P is probably species specific, since studies carried out did not show any immunoreactivity for three tit species (*Parus caeruleus, Parus major, Parus atricapillus*) unlike studies in the junko (*Junco hyemalis*), where SP+ cells were located along the ventricle and the entire hippocampus (Gould et al., 2001b). Additionally, there was a positive relation between the size of the hippocampus and the SPm.

Furthermore, neuropeptide Y (NPY) fibres and cell bodies (Gray and Morley, 1986) were also found in the avian hippocampus. Injection of NPY in the mouse hippocampus ameliorates performance and memory for training in the T-maze (Flood et al., 1989). Because it is believed that NPY helps memory improvement and food intake (Clark et al., 1984; Richardson et al., 1995), it is possible that there would be more NPY in the hippocampus of food storing birds during the peak of food storing season due to food collection and spatial memory formation (Gould et al., 2001b). The posterior ventral dorsolateral Hp (DLv) appears

to be equivalent to the subiculum since it receives output from a structure similar to CA1 as it happens in mammals (Witter et al., 1989b). Pyramidal cells in the mammalian Ammon's horn are surrounded by inhibitory basket-like terminals of cholecystokinin (Somogyi et al., 1984; Nunzi et al., 1985) in a similar way to that of the ventral layers of the avian hippocampal formation (Erichsen et al., 1991). Finally, other studies suggest that although the dorsal regions are implied to be equivalent to the dentate gyrus and the entorhinal cortex, they are displaced from each other along the anteroposterior axis (Siegel et al., 2002) with the dorsorostral areas (A 6.0-7.5) having subiculum like properties, contrasting with previous studies (Erichsen et al., 1991; Montagnese et al., 1996; Szekely and Krebs, 1996).

The avian hippocampus (and area parahippocampalis) is involved in homing (Lee et al., 1998a; Strasser et al., 1998; Gagliardo et al., 1999; Regolin and Rose, 1999), food storing (Sherry et al., 1989; Clayton and Krebs, 1994) and spatial learning (Lee et al., 1998a; Regolin and Rose, 1999). The hippocampus thus allows flying birds to perform long-distance migrations (Regolin and Rose, 1999) or to maintain recollection of the location of a disappeared object for a time period before starting to look for and locate it (Alerstam, 1990). In pigeons, it has been suggested that the hippocampal formation takes part in navigation map learning (Bingman et al., 1990). A navigation map is the sum of environmental landmarks or stimulus features that are combined into a single allocentric spatial representation that includes all the landmarks that characterize the target sites (Bingman et al., 2003). Food storing species choose spatial cues instead of non-spatial (although available) when storing food for retrieval, probably because in nature local characteristics of an area may change as weather conditions change, whilst spatial features remain unchangeable (Shettleworth, 2003). In contrast, non-storing birds use non-spatial cues for short term working memory duties (Macphail and Bolhuis, 2001).

Hippocampal lesions cause reduced ability to recall the location of hidden food (Krebs et al., 1989; Sherry et al., 1989) as well as homing (Bingman et al., 1984; Bingman et al., 1985) unlike hyperpallial lesions that do not cause any impairments. Pigeons with hippocampal formation lesions are impaired in discriminating conditional colours (Good, 1987) indicating possible loss of behavioural flexibility. However, Watanabe (2002), has shown that when the hippocampus was lesioned and birds were tested in a small space they could discriminate colours, so the test space itself is important in discrimination tasks. To support Watanabe's studies, a recent investigation has shown that lesions of Hp did not affect the ability of pigeons to learn non-spatial, conditional discrimination and novel stimulus presentation tasks (Strasser et al., 2004) unlike lesions in the rodent Hp which is believed to be involved in tasks other than of spatial nature (Dusek and Eichenbaum, 1997; Wood et al., 1999).

Studies from other groups have shown that the hippocampus also plays a significant role for the passive avoidance training in chicks; unilateral lesions to the left hemispheric hippocampus before passive avoidance learning caused amnesia for the acquisition and retention of the task (Sandi et al., 1992) whilst changes in synaptic plasticity were recently reported (Unal et al., 2002). The hippocampus has been shown to be less affected than the mesopallium intermediomediale (IMM, previously termed intermediate medial hyperstriatum ventrale-IMHV) in the presence of a stimulus (Nicol et al., 1998) and lesions of the avian hippocampal formation do not appear to affect memory for individual stimuli (Good and Macphail, 1994). Chick hippocampal neurones are affected by distance or approach movements (Nicol et al., 1998). McCabe and Horn (1994) have demonstrated an increase in Fos activity in the chick hippocampus after high level "movement" activity as with the "theta cells" in the mammalian hippocampus (Vertes and Kocsis, 1997; Buzsaki, 2002; Cantero et al., 2003).

Species that store food have been shown to have larger hippocampal formations in relation to non-storing species (Krebs et al., 1989; Sherry et al., 1989) which may be explained by the fact that food storers need a larger hippocampus to allow them to increase the time length during which they will remember spatial information (Biegler et al., 2001). Food-storing shows seasonal preference; chickadees and titmice store more food in the autumn and early winter (Biegler et al., 2001) preparing themselves for the forthcoming winter. Food storing can be also affected by changes in temperature, day length, or even the availability of food. Some researchers have shown that the size of hippocampus is larger relative to the telencephalon and the body size in October (Smulders et al., 1995) while others have demonstrated that there is increased neurogenesis at that time of year in free-ranging black-capped chickadees (Barnea and Nottebohm, 1994). It has been suggested that neuronal replacement must be taking place for the hippocampus to maintain a balance, so that cellular death of older neurones is compensated by new neurones which in turn will be incorporated into the circuit. Increased cell proliferation may help the young animals deal with the demands of environmental changes (Smulders et al., 1995). Another explanation could be that volume changes may be attributed to cell birth (increase of cell number) or cell death caused by enhancement of memory or lack of experience respectively (Clayton and Krebs, 1994). Furthermore, birds with reduced experience have less neurones and more apoptotic cells indicating probably that there is a minimum requirement of experience that is mandatory to cause alterations in the avian hippocampus (Clayton and Krebs, 1994). At the same time, under laboratory conditions, the stress of captivity may cause an increase in cell death or a reduction in cell proliferation due to stress dependent neurochemical signals (Hoshooley and Sherry, 2004). In addition, the latter authors support the idea that cells which are not functionally required fail to be recruited in the hippocampus. Laboratory photoperiodical experiments did not show any volume changes, which may, however, be attributed to the fact

that avian species need more than the food storing experience (probably environmental stimuli) for their hippocampal formations to show volumetric changes (Krebs et al., 1995).

Glutamate receptor binding studies (Stewart et al., 1999) showed that non-storers had higher binding levels per unit area of tissue in relation to storers, although the latter had larger hippocampal volumes but similar neuronal density (Krebs et al., 1989; Healy et al., 1994). The explanation for this finding is unclear, especially given that mammals need NMDA receptor activation for acquisition of memory for water maze training (Liang et al., 1994; Ylinen et al., 1995; Bannerman et al., 1997). A recent study has shown that NMDA receptor activity is essential for long term memory formation in black-capped chickadees (Shiflett et al., 2004). Additionally, other studies have shown the importance of NMDA receptors for accurate homing in pigeons (Riters and Bingman, 1994). NMDA receptors inactivation can lead to deficits during memory acquisition, but does not affect retrieval procedures, short term memory preservation or post-acquisition memory consolidation (Shiflett et al., 2004). Furthermore, the interval length between learning trials can determine if the presence of NMDA receptors is required or not. Only long intervals between training processes necessitate the activation of NMDA receptors for spatial learning tasks.

Nonetheless, Wieraszko and Ball (1993) have demonstrated that the avian hippocampus does not require activation of NMDA receptors for the induction of LTP. In contrast, studies in the avian hippocampus (Szekely, 1999) and other brain areas (Holscher and Rose, 1992) have shown the importance for nitric oxide synthesis for memory formation in the form of LTP as it also happens in mammals (Meyer et al., 1998; Okere and Kaba, 2000; Susswein et al., 2004). Studies in the mammalian hippocampus have demonstrated that the induction of LTP based on NMDA receptors activation varies between the different subregions (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). NMDA receptors activation is essential in CA3 for spatial recognition whilst dentate gyrus and CA1 require

them for the creation of long term memory (Lee and Kesner, 2002). Inactivation of NMDA receptors in the dorsocaudal region of the avian hippocampus prevents acquisition of long term memory formation (Shiflett et al., 2004).

Not many studies have been presented showing the spatial abilities of the domestic chick (*Gallus domesticus*). However, some older studies have demonstrated that chicks have an extraordinary ability to use spatial cues to retrieve food (Rashid and Andrew, 1989). Studies have shown hemispheric asymmetry, depending on the eye (hemisphere) they use for encoding of information; chicks are better at topographical learning (global spatial cues) when they use the left eye (right hemisphere-optic chiasma) (Rashid and Andrew, 1989; Tommasi and Vallortigara, 2001; Kahn and Bingman, 2004), whilst they use the right eye (left-hemisphere) for colour discrimination (Vallortigara et al., 1996; Tommasi et al., 2000), identification of local cues in the environment (Kahn and Bingman, 2004) and working memory (Vallortigara, 2000). There are differences between the avian species regarding hippocampal asymmetries. In pigeons, the left hemisphere regulates navigation map learning (Gagliardo et al., 2001) and object specific cue discrimination, whilst both hemispheres are responsible for position-specific cues (Prior and Gunturkun, 2001; Prior et al., 2002). In contrast, food storing birds use the right hemisphere for spatial cues and the left for object recognition cues (Clayton and Krebs, 1994) as observed in chicks.

Hemispheric asymmetry has also been demonstrated after passive avoidance training. Lesions to the left hippocampus before passive avoidance training caused amnesia for the task, whilst no effects were observed after lesions of the right hippocampus (Sandi et al., 1992). The authors concluded that the left hippocampus plays an important role in the acquisition and retention of memory, during at least the initial stages of memory formation. In addition, studies in the IMM and striatum mediale (Stm, previously termed lobus parolfactorius-LPO) have confirmed that blocking of the glycine site of NMDA receptors with the antagonist 7-

chlorokynurenate resulted in blockage of memory formation for the passive avoidance learning paradigm (Steele and Stewart, 1993). This finding was in accordance with previous studies that have shown the importance of the left IMM for the specific task (Rose, 1991; Sandi et al., 1993).

As mentioned above, the presence of VIP is apparent in the avian hippocampus. VIP in particular has been shown to be a prolactin (PLR)- releasing hormone in rats, monkeys (Kato et al., 1978; Frawley and Neill, 1981; Pecins-Thompson et al., 1996) and hens (Sharp et al., 1989; Talbot et al., 1991). PRL secretions cause a lactogenic response in mammals (Kuenzel et al., 1997). VIP similarities between mammals and avian species include vasodilation , increased blood flow, exocrine gland secretion in mammals (Shimizu and Taira, 1979; Andersson et al., 1982) and increased secretion from salt glands in birds (Lowy et al., 1987; Gerstberger et al., 1988). It is also implicated in energy metabolism in the mammalian brain (Magistretti et al., 1981) and is a marker for the chick visceral forebrain system (Kuenzel and Blahser, 1993), which is a system in the avian brain that dominates homeostatic mechanisms during stressful and emotional periods (van der Kooy et al., 1984). Concomitantly, the peptide is a modulator or metabolic component of circadian rhythm and is found in the suprachiasmatic nucleus (SCN) in rats, an area that can be influenced by photoperiod (Moore, 1983; Kafka et al., 1985; Aguilar-Roblero et al., 1994; Silver and Moore, 1998). It should be mentioned, though, that the SCN (Harmar et al., 2002) as well as the retina (Cavallaro et al., 1996) are also characterised by the presence of pituitary adenylate cyclase-activating polypeptide (PACAP), which is a novel member of the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily and is also involved in the regulation of the circadian rhythm (Arimura, 1992). It has been demonstrated that there are three types of PACAP receptors (Harmar et al., 1998), PAC1, VPAC1 and VPAC 2, the latter showing affinity also for VIP (Harmar et al., 1998), implying that there may be cross-reactivity between VIP and PACAP.

Similarly, the medial basal hypothalamus and septal region of the dove brain demonstrate VIP-immunoreactive neurones, which are suggested to be photoreceptor neurones (Silver et al., 1988). Apart from dense levels of VIP mRNA above the cell body region of neurones in the hippocampus, high VIP mRNA levels are present in the AI (intermediate arcopallium) and TnA (nucleus taeniae of amygdala), as well as in the bulbus olfactorius (BO) (Kuenzel et al., 1997).

It can be concluded that the avian hippocampus needs further investigation to fully elucidate its function, connectivity properties and structural changes. Studies have demonstrated clearly that it is homologous to the mammalian hippocampus (Kallen, 1962; Erichsen et al., 1991; Szekely and Krebs, 1996; Tommasi and Vallortigara, 2001; Atoji and Wild, 2004), which renders the avian hippocampus an interesting model for behavioural and anatomical studies.

Since the amygdala is a pivotal limbic structure processing sensory information about stress and fear (Akirav and Richter-Levin, 1999; Ebner et al., 2004) it will be discussed in some detail. Its general characteristics are described below as an introduction to stress and fear in the chick PAL response, since relatively little is known of equivalent regions in birds.

1.4 THE AMYGDALA

1.4.1 The mammalian amygdala

The mammalian amygdala is a complex structure in its organisation consisting of a number of different nuclei involved in a variety of information processing functions; central nucleus, basal nucleus and medial nucleus (Paxinos, 1995). The amygdala can be divided into pallial and subpallial amygdala (Puelles et al., 2000), where the cortical and the basolateral amygdala are characterized as pallial structures and in particular they derive from the ventral pallium (Johnston, 1923; McDonald, 1998; Swanson and Petrovich, 1998), whilst the

centromedial amygdala is considered to be a subpallial structure (Puelles et al., 2000). The amygdala is centrally placed in the neural circuitry responsible for emotion (LeDoux, 1992) and has been implicated to participate in conditioned fear to cues (Nader et al., 2001) and to environment (Roozendaal et al., 1993). It has been suggested that it is also involved in fear extinction (Marsicano et al., 2002; Walker and Davis, 2002) and reward learning (Hall et al., 2001; Parkinson et al., 2001). The human amygdala participates also in visual stimuli processing that are involved with emotional importance in social situations, one striking example being the recognition of facial expressions of humans (Darwin, 1827/1965; Kling and Brothers, 1992). Bilateral lesions to the human amygdala cause deficits in the recognition of facial fear expressions (Adolphs et al., 1994). The amygdala is part of the limbic system and is closely related to the autonomic nervous system, providing evidence for the participation of the amygdala in emotional situations (LeDoux et al., 1988; Amaral et al., 1992; Lee et al., 1998b). The limbic system consists of the cingulate gyrus, the parahippocampal gyrus, the hippocampal formation, the septal area, the nucleus accumbens, parts of the hypothalamus, neocortical areas and the amygdala (Maclean, 1955). The telencephalon consists of basal ganglia and cortical hemispheres. The basal ganglia link the thalamus and the cerebral cortex through diverse circuits including the limbic circuit and are responsible for a number of functions including voluntary movement, cognitive and emotional tasks.

The amygdala is filled with binding sites for substances that control fear and aggression, such as benzodiazepines (Niehoff and Kuhar, 1983) and serotonin (Hensman et al., 1991; Saudou et al., 1994). Furthermore, it has been shown that amygdalar neurones are activated during social interaction (Kling et al., 1979) in humans and communication in primates (Jurgens, 1982).

It takes part in paradigms where both learning and stress are concerned and especially when the training is highly emotional (Cahill and McGaugh, 1990). Induction of LTP has

been shown after fear conditioning studies (McKernan and Shinnick-Gallagher, 1997; Tsvetkov et al., 2002), indicating an association between learning and stress in the amygdala. The amygdala is the storage place of emotional events, fearful in particular (Cahill and McGaugh, 1998; Cahill et al., 1999; Vazdarjanova and McGaugh, 1999)

1.4.1.1 The basal nucleus of the amygdala

The basolateral nucleus (BLA) (Fig 1.9) is a very important nucleus in the amygdala because it modulates hippocampal memory procedures (Cahill and McGaugh, 1998; Roozendaal, 2000; Packard and Cahill, 2001) and electrical stimulation of BLA has biphasic effects on long term potentiation in the dentate gyrus, indicating participation of the amygdala in hippocampal associated events (Akirav and Richter-Levin, 1999). The connection between the hippocampus and the amygdala allows the former to receive information about odours and stress emotions, whilst the latter puts the events in the right context (Akirav et al., 2001).

The basolateral amygdala projects to the medial portion of the prefrontal areas, especially the orbital and medial prefrontal cortex (OMPFC) (Krettek and Price, 1977; Bacon et al., 1996), which are implicated in learning and memory procedures (Kesner et al., 1996; Ragozzino et al., 1998; Baldwin et al., 2002) and in emotional situations (Jinks and McGregor, 1997; Quirk et al., 2000). So, the amygdalar OMPFC [the OMPFC makes connections also with other limbic structures (Jay and Witter, 1991)], has been suggested to take part in classical fear conditioning (Garcia et al., 1999) and reward memory (Gaffan et al., 1993).

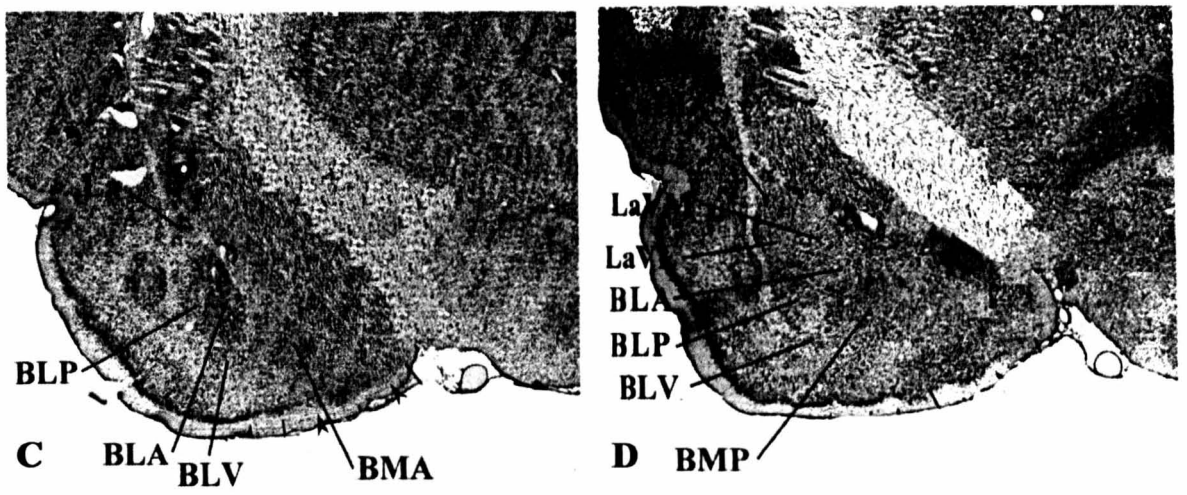


Fig.1.9. Nissl stained sections showing the cytoarchitecture of the rat amygdaloid nuclei. BLA: basolateral amygdaloid nucleus, anterior part, BLV: basolateral amygdaloid nucleus, ventral part, BMA: basomedial amygdaloid nucleus, anterior part, BMP: basomedial amygdaloid nucleus, posterior part, BLP: basolateral amygdaloid nucleus, posterior part, La: lateral amygdala, LaDL: lateral amygdala, dorsolateral part, LaVM: lateral amygdala, ventromedial part, LaVL: lateral amygdala, ventrolateral part, (from The Rat Nervous system, G. Paxinos, Second Edition, Academic Press, C, D: page, 512-3).

Noradrenergic activation of the BLA has been suggested to control memory acquisition and plasticity in the hippocampus (Ikegaya et al., 1997; Frey et al., 2001). At the same time, although the BLA does not have as highly dense glucocorticoids receptors as the hippocampus (McEwen and Sapolsky, 1995; Morimoto et al., 1996), activation of the BLA is necessary for excretion of adrenal steroids which will affect the hippocampal memory storage (Roozendaal and McGaugh, 1996; Roozendaal et al., 1996; Roozendaal and McGaugh, 1997; Ferry et al., 1999). Ipsilateral priming activation of the BLA has enhancing effects on DG-LTP (Akirav and Richter-Levin, 1999, 2002), whilst central amygdaloid nucleus (CeA) priming did not provoke the same effects, suggesting that it does not take part in hippocampal memory and plasticity (Ikegaya et al., 1994; Roozendaal and McGaugh, 1996, 1997).

Functional imaging studies have shown that the amygdala is greatly triggered during odour processing and especially if the odour is very intensive (Zald and Pardo, 1997; Anderson et al., 2003). BLA lesions caused impairments for olfactory memory (Cahill and McGaugh, 1990, 1998) and as mentioned above, the BLA adapts hippocampal plasticity

which may be implying that the BLA could influence memory by turning the olfactory stimulus into an encoding memory (Eichenbaum and Cohen, 2001).

The lateral nucleus (La) (fig 1.9) is a subdivision of the basolateral amygdala and is the main area that makes sensory input comprehensible and transmits it to the other nuclei of the amygdaloid complex for processing (LeDoux et al., 1990a).

Sensory information will then reach the hypothalamus and brainstem to control behavioural, autonomic and endocrine responses to sensory input (LeDoux, 1995). As connectivity is concerned, the basal and accessory basal nuclei project to the lateral nucleus of the amygdala (Savander et al., 1997). These projections are arranged in such a way, so that the basal nucleus projects to the ventrolateral part and the accessory projects to the medial lateral nucleus (Savander et al., 1997). Electron microscopy studies have demonstrated that the basal nucleus forms asymmetric and symmetric synapses with the ventrolateral part, implying the formation of both excitatory and inhibitory inputs respectively. The projections of the accessory basal nucleus to the lateral amygdala form only asymmetric synapses, indicating that this pathway is mainly excitatory (Savander et al., 1997).

The accessory basal nucleus could be a mediator passing data about the internal state of an organism as well as about past incidents and contextual issues (Savander et al., 1997). The accessory basal nucleus receives projections from the lateral hypothalamic area (Allen et al., 1991), which is responsible for behavioural responses to hunger, thirst, aggression and reproduction (Swanson, 1987) in mammals and the hippocampal formation via the ventral subiculum, which could pass on information from the medial temporal lobe memory system (Squire and Zola-Morgan, 1991) and spatial or contextual information (LeDoux, 1995) in rats and primates. The basal nucleus of the amygdala is a very important structure, because it is closely associated with stress (Akirav and Richter-Levin, 1999), whilst it also shows

homology to the chick arcopallium (Davies et al., 1997) as already mentioned in Table 1.1 (see page 13).

1.4.2 The chick arcopallium (A) (archistriatum) and nucleus taeniae of the amygdala (TnA)

The arcopallium (A) is a large heterogeneous area of the caudal, ventrolateral avian telencephalon. It can be divided at least in two major parts, one of which is homologous to the mammalian amygdala, whilst the other receives somatosensory input (Zeier and Karten, 1971). Some of the subdivisions of the arcopallium (Fig.1.10, 1.11) include the arcopallium dorsale (AD), the arcopallium intermedium (AI), and the nucleus taeniae of the amygdala (TnA) (Karten and Hodos, 1967; Reiner et al., 2004). The amygdala-equivalent part has been suggested to be part of the limbic system. In avian species the limbic system consists of the hippocampus, the intermediate arcopallium (ventral intermediate archistriatum), the septal nuclei, the preoptic area, the area ventralis tegmenti (AVT), the hypothalamus, the dorsomedial thalamus, prefrontal-equivalent pallial regions such as the caudolateral nidopallium (NCL) (Mogensen and Divac, 1982; Waldmann and Gunturkun, 1993) and the nucleus taeniae of the amygdala.

Electrical stimulation of the arcopallium has been demonstrated to provoke fear or escape responses in mallards (Phillips, 1964), pigeons (Goodman and Brown, 1966) and chickens (Phillips and Youngren, 1971). On the other hand, electrolytic lesions increase tonic immobility in chickens (Maser et al., 1973) and impair conditioned fear avoidance in pigeons (Dafters, 1975), whilst kainic acid lesions provoke reduction in fear responses in domestic chicks (Phillips and Youngren, 1986). Suction as well as bilateral electrolytic lesions to the arcopallium produce impaired ability for performance on the passive avoidance learning (PAL) task (Benowitz, 1972; Lowndes and Davies, 1994). Therefore, it has been suggested

that the arcopallium may belong to a memory circuit along with the IMM and StM that have been identified as key areas for their involvement in passive avoidance training (Rose and Csillag, 1985; Patel and Stewart, 1988).

Moreover, the arcopallium has been demonstrated to have reciprocal connections with the IMM (Bradley et al., 1985), whilst it also projects to the StM (Szekely et al., 1994; Csillag et al., 1997; Davies et al., 1997). For that reason, it has been implied that the arcopallium is a mediator area transferring the acquired information from the IMM to the StM (Lowndes and Davies, 1994). The arcopallium also receives afferents from the septal nuclei (Montagnese et al., 2004). Lesions of the part of the arcopallium which is considered a limbic area (eg. intermediate arcopallium, AI) have been shown to increase failure for the passive avoidance training (Lowndes and Davies, 1994). However, no signs of increased fear were observed after presentation of novel objects (Lowndes and Davies, 1995).

After passive avoidance training an upregulation of D1 receptors has been demonstrated without, though, increases in dopamine levels (Stewart et al., 1996). Since the arcopallium plays a role in passive avoidance training, it has been suggested that dopamine receptors are upregulated, possibly by enhanced arcopallial input caused due to fear or aversion. Thus, the output signal to reduce pecking behaviour is strong. Therefore, it is suggested that the arcopallium may be transmitting information to the StM not to peck for an extensive time period after training (Csillag, 1999).

1.4.2.1 Arcopallium dorsale (AD)

By using the anterograde pathway tracer *Phaseolus vulgaris leucoagglutin* (PHA-L) studies have demonstrated that afferents from the chick arcopallium project to parts of the Wulst, the mesopallium (M) and the nidopallium (N), as well as to the locus coeruleus (LoC) and the nucleus subcoeruleus ventralis (Davies et al., 1997). In particular, projections from the

dorsal arcopallium (AD) (Fig 1.10, 1.11) reach the ipsilateral hyperpallium densocellulare (HD), the mesopallium intermediomediale (IMM) and the mesopallium (M). The AD projects also to the StM, the lateral nidopallium (NL) and the antero-medial part of the nidopallium (Davies et al., 1997). The Stm along with the striatum laterale (StL) have been suggested to be equivalent to the mammalian caudate putamen (CPu) (Dubbeldam, 1991).

The StM has been subdivided according to its afferents, i.e. whether the projections come from limbic or non-limbic telencephalic areas (Veenman et al., 1995) as well as according to efferents originating from that area that target the ventral tegmental area and the substantia nigra indicating that the medial striatum contains striatal-equivalent and nucleus accubens parts (Mezey and Csillag, 2002). In domestic chicks (Davies et al., 1997), the lateral part of the StM (non limbic), receives afferents from the AD as in pigeons (Veenman et al., 1995), the latter projecting also to the visual thalamus, to the pontine reticular formation and cranial nerve motor nuclei as seen also in pigeons (Zeier and Karten, 1971), indicating possibly that the AD in chick is involved in the somatosensory system. The AD has been suggested to be homologous to the mammalian basal amygdala (B) (Martinez-Garcia et al., 2002), because it receives dopaminergic and cholinergic projections, and projects to the medial (StM) and lateral (StL) striatum (Veenman et al., 1995), projections that could be taken as amygdalostriatal which in mammals arise mainly from the basal amygdala (Martinez-Garcia et al., 2002).

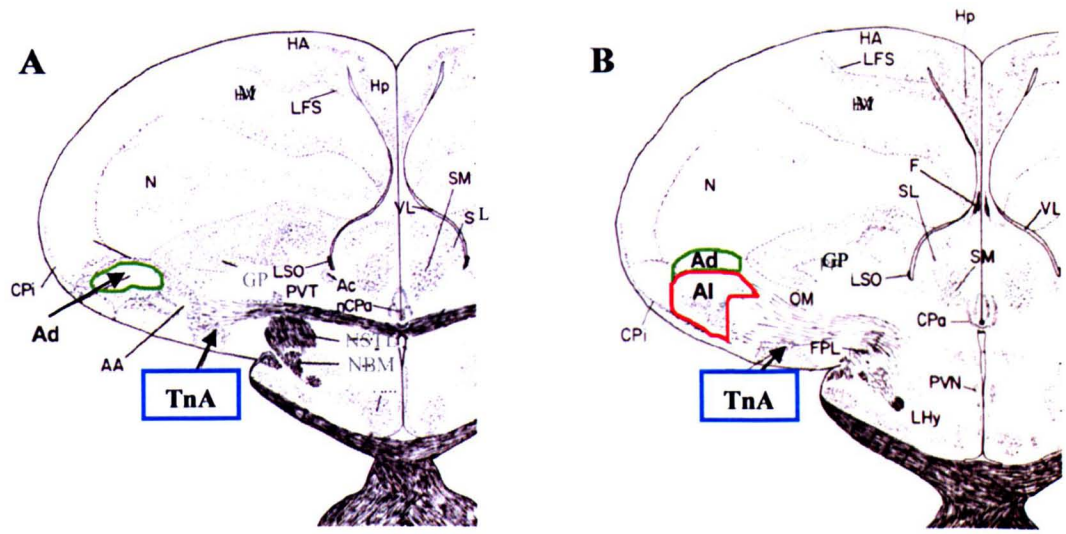


Fig. 1.10. Images representing the levels of arcopallium dorsale, nucleus taeniae of the amygdala and intermediate arcopallium. Anteroposterior coordinate A: 8.2, B: 8.0. AA: anterior arcopallium, AD: arcopallium dorsale, AI: arcopallium intermediale, M: mesopallium, N: nidopallium, HA: hyperpallium apicale, CPi: cortex piriformis, SL: nucleus septalis lateralis, AM: arcopallium mediale, NSTL: nucleus striae terminalis lateralis, SM: nucleus septalis medialis, GP: globus pallidus, Hp: hippocampus, LFS: lamina frontalis superior, PVN: nucleus parventricularis magnocellularis, VL: ventriculus lateralis, TSM: tractus septopallio-mesencephalicus, CPa: commissurae pallii, NBM: nucleus basalis magnocellularis, PVT: pallidum ventrale, OM: tractus occipito-mesencephalicus, LSO: organum septi laterale, LHy: regio lateralis hypothalami, nCPa: nucleus commissurae pallii (adapted from Kuenzel and Masson, 1988)

1.4.2.2 Arcopallium intermedium (AI)

The arcopallium intermedium (AI) (Fig. 1.10 B, 1.11) is the only telencephalic area that shows bilateral projections to the dorsocaudal nidopallium (Metzger et al., 1998), a region which shows neuronal activity after acoustic or visual stimuli (Bock et al., 1997). PHA-L labelled fibres reach the StM, the StL, the Hp (as in the pigeon (Casini et al., 1986)), the APH, the septal nuclei and the pallidum ventrale (PVt) (Davies et al., 1997). Projections were also found in the diencephalon, specifically in the tractus occipito-mesencephalicus (OM), ansa lenticularis (AL), fascicullus prosencephali lateralis (FPL) and tractus quintofrontalis. Fibres were also visible in the ipsilateral LoC, nucleus subcoeruleus, brainstem reticular formation and motor cranial nerve nuclei. It is clear that the AI has also connections with non-limbic areas (somatosensory) indicating heterogeneity.

According to Davies et al. (1997), the limbic part of the arcopallium includes the AI, the anterior arcopallium (AA) and the posterior pallial amygdala (PoA). In contrast, the non limbic part comprises the AD, and the arcopallium mediale (AM) due to the presence of sensory, somatosensory and motor efferents. The AI is thought to be equivalent to the lateral and accessory basal amygdala as well as to the amygdalo-hippocampal area of the mammalian amygdala (Davies et al., 1997; Martinez-Garcia et al., 2002) due to a projection (Davies et al., 1997) rich in heavy metals to the ventromedial hypothalamus (Dubbeldam et al., 1997). In particular, the basolateral amygdala in mammals shows zink-containing projections to the ventromedial hypothalamus via the stria terminalis which originates from ventral pallial structures, such as the accessory basal nucleus of the amygdala (Swanson and Petrovich, 1998), which supports the hypothesis of homology between the AI and basal accessory nucleus (Martinez-Garcia et al., 2002).

1.4.2.3 Nucleus taeniae amygdala (TnA)

TnA is located within the medial arcopallium (Fig. 1.10, 1.11). It has been suggested that it is homologous to the mammalian medial amygdala (Thompson et al., 1998; Cheng et al., 1999). In the Japanese quail and ring doves it shows high concentrations of estrogens and antrogens (Martinez-Vargas et al., 1976; Watson and Adkins-Regan, 1989; Foidart et al., 1999) and demonstrates aromatase activity (Foidart et al., 1995). A projection reaches the rostral part of TnA from the olfactory bulb (Reiner and Karten, 1985) as it also happens in hamsters (Lehman and Winans, 1982).

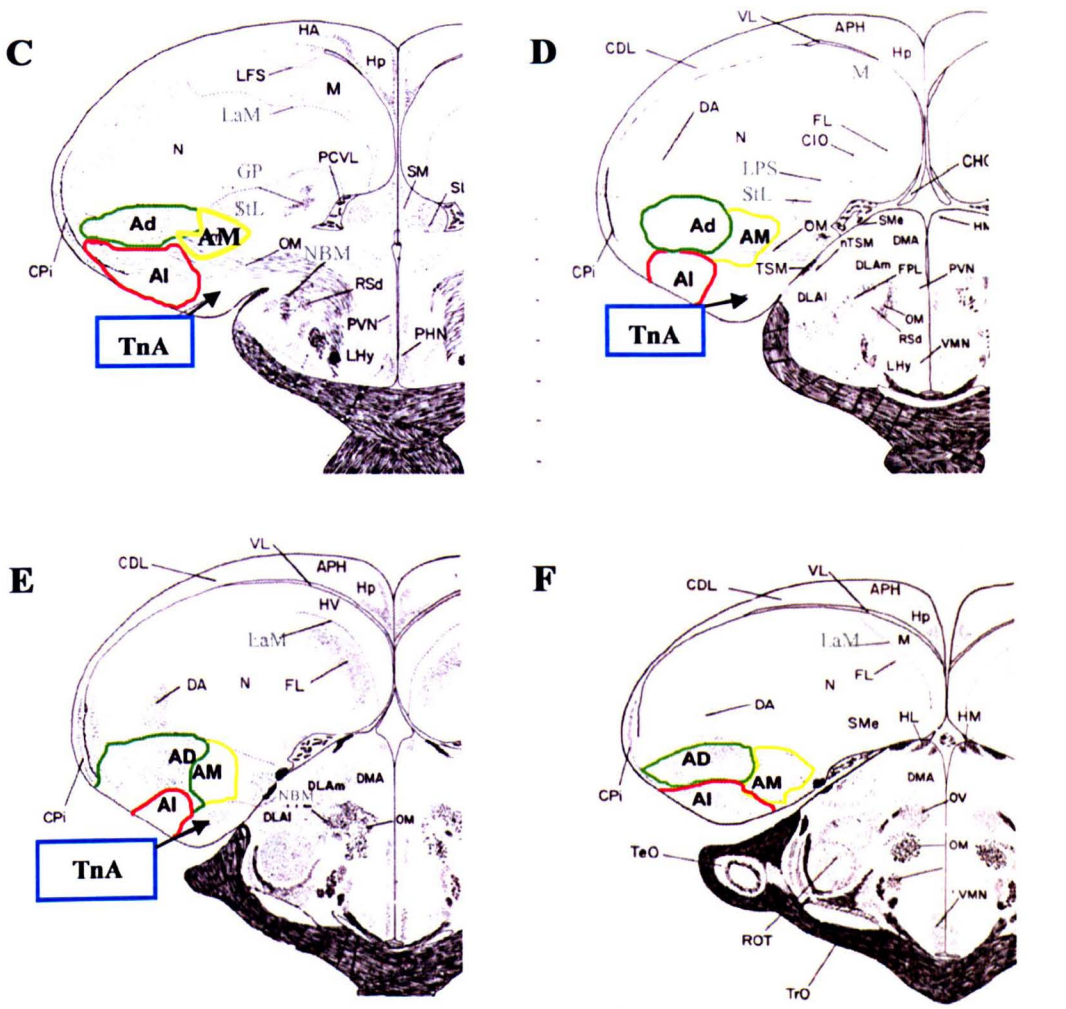


Fig. 1.11. Representation of levels where the arcopallium dorsale, intermediale and mediale are located together with the nucleus taeniae of the amygdala. C: A. 7.6, D: A 7.0, E: A.6.6, F: A.6.2. AD: arcopallium dorsale, AI: arcopallium intermediale, AM: arcopallium mediale, TnA: nucleus taeniae of the amygdala, CPi: cortex piriformis, TeO: tectum opticum, DA: tractus dorso-arcopallialis, ROT: nucleus rotundus, TrO: tractus opticus, Hp: hippocampus, APH: area parahippocampalis, VL: ventriculus lateralis, CDL: area corticoidea dorsolateralis, M: mesopalium, N: nidopallium, FL: field L, HA: hyperpallium apicale, LFS: lamina frontalis superior, SM: medial septum, SL: lateral septum, OM: tractus occipito-mesencephalicus, LHy: regio lateralis hypothalami, SMe: stria medullaris, TSM: tractus septopallio-mesencephalicus, PVN: nucleus parventricularis magnocellularis, NBM: nucleus basalis magnocellularis, LaM: lamina mesopallialis, StL: striatum lateralis, GP: globus pallidus, RSd: nucleus reticularis, superior, pars dorsalis, PHN: nucleus periventricularis hypothalami, PCVL: plexus choroideus ventriculi lateralis, DMA: nucleus dorsomedialis anterior thalami, DMAl: nucleus dorsomedialis anterior thalami, pars lateralis, DMAm: nucleus dorsomedialis anterior thalami, pars medialis, nTSM: nucleus tractus septopallio-mesencephalicus, VMN: nucleus ventromedialis hypothalami, LPS: lamina pallio-subpallialis, CIO: capsula interna occipitalis, HM: nucleus habenularis medialis, HL: nucleus habenularis lateralis, OV: nucleus ovoidalis (adapted from Kuenzel and Masson, 1988).

One type of male sexual behaviour is appetitive sexual behaviour (Balthazart and Ball, 1998; Pfaus et al., 2001), where the male looks for and flirts with the right female. Studies of sexual behaviour in rats have shown that the medial amygdala (Me) participates in male appetitive sexual behaviour (Everitt, 1990). Me is also highly activated by emotional stress as it has been demonstrated by *c-Fos* expression (Dayas et al., 1999). A similar situation may be taking place in the TnA, since serotonergic immunoreactivity studies have shown the presence of high concentrations of serotonergic fibres in this area (Metzger et al., 2002). Serotonin is enhanced after aggression and anxiety (Lucki, 1998) as well as after stressful situations (Gruss and Braun, 1997). Damage of the Me causes impairments in male sexual behaviour in rats (Kondo and Arai, 1995; Kondo and Yamanouchi, 1995) and hamsters (Lehman and Winans, 1982).

Lesions in TnA have caused delays to males to approach females and the contact lasted shorter time. In addition, the number of successful copulations was decreased and the time needed for males to copulate with females was longer. At the same time, vocalization was also affected in the presence of female quails (Thompson et al., 1998). The authors suggested that the delay in approaching females and the short contact time may be explained by lack of sexual excitement in the presence of sexual stimuli and sexual attraction (Holloway and Domjan, 1993; Thompson et al., 1998). No motor damage was noticed, so the lack of copulation was due to the damaged TnA (Thompson et al., 1998). In hamsters, abolition of copulation was observed after complete elimination of the olfactory input to the Me (Lehman and Winans, 1982).

Other studies, however, have not shown any deficits in sexual behaviour in male quails after lesions in TnA (Lehman and Winans, 1982). This discrepancy may be suggesting that there are subdivisions in the TnA (Parfitt and Newman, 1998). Lesions to the TnA of females have revealed that female doves have become more capable towards nest cooing (Cheng et al.,

1999) either due to reduction of fear emotions or lesions in the brain circuit regulating sexual behaviour.

Homology to the mammalian medial amygdala can be implied through numerous studies, of a) connectivity and b) hormonal expression. Tract-tracing studies have shown that both TnA and Me receive sub-cortical sensory inputs and establish connections with the hippocampal complex (Cheng et al., 1999). TnA projects to the hypothalamus and the preoptic area in pigeons and doves in a similar manner to the Me (Thompson et al., 1998; Cheng et al., 1999). Studies of the expression of mRNA for androgen receptors (AR), and estrogen type a (ERα) and type β (ERβ) receptors in TnA of the quail (Foidart et al., 1999) as well as of other species (Ball et al., 1999; Bernard et al., 1999) show a similar arrangement to that of the Me (Wood et al., 1992).

TnA projects to the hippocampus in pigeons (Casini et al., 1986) and receives efferents from the hippocampus both from medial and lateral routes (Casini et al., 1986; Atoji et al., 2002). The hippocampus in birds, as in mammals (Brown and Zador, 1990) does not receive direct input from the olfactory bulb, but this projection is mediated through the TnA in birds and the CeA in mammals. TnA sends efferents to the hypothalamus and in particular to the lateral and posterior medial hypothalamus and the paraventricular and preoptic areas in a bilateral way in doves and ipsilateral in starlings (Cheng et al., 1999). Additionally, it projects to the SL, the StM and the HD. TnA shares bilateral connections with the anterior commissure in ring doves and the tectal commissure in starlings (Cheng et al., 1999). Together with the AM it sends efferents to the mesopallium and the mesopallium intermediomediale, explaining probably the reason why the arcopallium plays a role in filial imprinting and passive avoidance training (Lowndes and Davies, 1994; Lowndes et al., 1994). Weak projections to the nidopallium caudolaterale (NCL) were also found in ring doves and starlings which is in agreement with other studies (Leutgeb et al., 1996). Finally, efferents from the TnA of the ring

dove reach the ovoidalis shell, the tractus nuclei ovoidalis, the bed nucleus of stria terminalis (BST) and the OM (Cheng et al., 1999).

Next, the olfactory bulb will be described, a structure which participates in odour recognition (Reinken and Schmidt, 1986; Buonviso and Chaput, 2000; McKeegan, 2002) and in chicks has been demonstrated to have connectivity with the TnA (Reiner and Karten, 1985). In rats, the olfactory bulb exhibits neurogenesis in adult life (Bayer, 1983; Winner et al., 2002), however little is known about the avian olfactory bulb and cell birth as an effect of PAL.

1.5 BULBUS OLFACTORIUS (BO)

The BO (in chicks, Fig. 1.12) is the area of the brain that receives olfactory signals directly from receptor neurones and then transmits the information to the rest of the central nervous system. The perception of olfactory sense needs a molecular machinery in order to transform a chemical stimulus into electrical signal. Most information comes from mammalian research; it has been demonstrated that olfactory chemoreceptors are neurones that detect chemical substances in the air and enhance axonal arborisation in order to make contacts with neurones in the olfactory bulb. They interact particularly with a given odour molecule and participate in odour identification (Lancet, 1986). These receptors belong to the superfamily of seven transmembrane domain proteins.

Odour stimulation causes neuronal activity to the olfactory neurones in rats (Lancet, 1986). The firing rates of neurones in the BO of hens (Steward, 2000a) show similar pattern with these of the mammals (Doving, 1987). Studies on odorant concentrations are controversial; some researchers suggest that concentration of an odorant substance affects neuronal activity (Reinken and Schmidt, 1986), while others have shown that activity remains

constant to concentration changes (Doving, 1987). It is noteworthy that the rat's olfactory neurones show reduced activity to recognizable odours (Buonviso and Chaput, 2000).

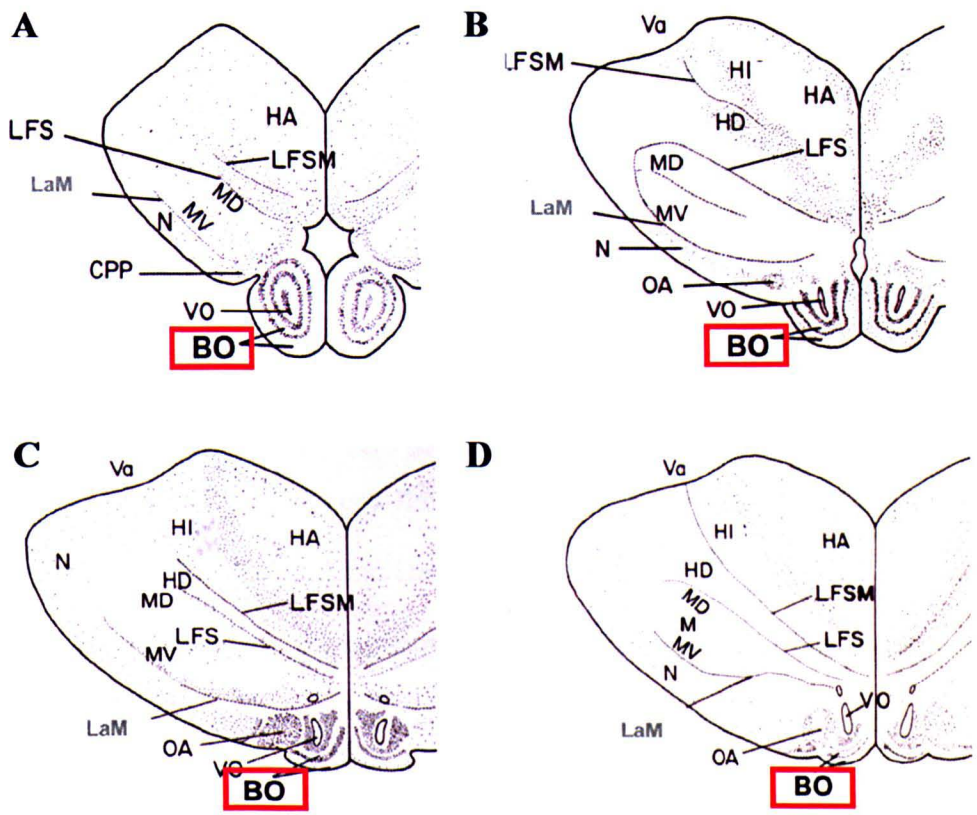


Fig 1.12. Representation of the levels that BO is located. Anteroposterior coordinates for A: 14.6, B:14.2, C:13.8, D: 13.6. BO: bulbus olfactorius, HA: hyperpallium apicale, HI: hyperpallium intercalatum, MD: mesopallium dorsale, MV: mesopallium ventrale, HD: hyperpallium densocellulare, VO: ventriculus olfactorius, N: nidopallium, M: mesopallium, LaM: lamina mesopallialis, LFS: lamina frontalis superior, LFSM: lamina frontalis suprema, CPP: cortex prepiriformis, OA: nucleus olfactorius anterior, Va: vallecula telencephali (adapted from Kuenzel and Masson, 1988).

In chicks, the left olfactory bulb has been suggested to be dominant, since studies have shown that chickens respond to odours when sniffing from the left but not the right nostril (Vallortigara and Andrew, 1994; Rogers et al., 1998). A number of odours appear to have an aversive effect for chicks such as blood (Jones and Black, 1979), odours of insects

(Roper and Marples, 1997) and cat fur (Fluck et al., 1996), even from the day of hatching (Burne and Rogers, 1996).

An interesting point for behavioural studies is that chicks can smell methylanthranilate (MeA) and take it for aversive odour (Marples and Roper, 1997) as observed from the latencies to peck the bead dipped in MeA. One day old chicks can associate odours with the colour of the bead in the passive avoidance learning (PAL) paradigm and can recall the smell of MeA. Therefore it has been suggested that MeA may be taking part in memory consolidation (Burne and Rogers, 1997). Other bitter but odourless substances (e.g. denatonium benzoate, quinine, Bourne et al., 1991; Marples and Roper, 1997) do not seem to be strong aversive stimuli for chicks resulting in decreased ability to remember PAL suggesting a strong link between limbic structures participating in PAL (e.g. mesopallium, arcopallium) and BO (Richard and Davies, 2000).

In the chick brain, the olfactory bulb sends afferents to the cortex piriformis (CPi), which is considered homologous to the mammalian CPi (Reiner and Karten, 1985; Bingman et al., 1994) and helps the animals associate atmospheric odours with navigation maps and discriminate odorant stimuli (Gagliardo et al., 1997), the medial septum, the TnA, the olfactory tubercle (Reiner and Karten, 1985) the StM and the mesopallium (Rieke and Wenzel, 1978).

1.6 SYNAPTOGENESIS

The functional units of the neuronal system are the neurone and the glial cells. The neuron consists of the cell body from which radiate dendritic processes that receive afferent synaptic information and the axon process that sends input to the dendrites and gives output of information. The nervous system operates by the creation of functional connections between its components so that neurones can communicate with each other. These connections are

called synapses, which are cellular junctions and their formation is associated with developmental events such as cell proliferation, neuronal survival, migration and death as well as dendritic and axonal arborisation (Goodman and Shatz, 1993).

During dendritic and axonal arborisation dynamic changes in branching occur, such as branch addition, elimination, lengthening and shortening (Pokorny and Yamamoto, 1981a; Cohen-Cory and Fraser, 1995; Baloyannis et al., 2000). During synaptogenesis, synapses are formed, mature, are stabilised and are eliminated by procedures that involve pre- and post-synaptic signal transmission (Katz and Shatz, 1996; Cohen-Cory, 2002), indicating that synapses can alter their function as well as their morphology by continuous shape changes demonstrating plasticity (Fischer et al., 1998), probably due to neuronal activity which leads to actin polymerisation (Fischer et al., 2000) or increases in intracellular calcium (Korkotian and Segal, 1999).

LTP, an enduring activity-dependent increase in synaptic efficacy believed to be the neural correlate for learning (Bliss and Lomo, 1973; Malenka and Nicoll, 1999), has been shown to cause the growth of filopodia (Maletic-Savatic et al., 1999), trigger the increase of synapses with perforated postsynaptic densities (PSDs) followed by multiple spine buttons where two spines develop from the same dendrite (Toni et al., 1999).

1.6.1 Dendritic spines

Most of the neuronal synapses occur on the surface of the dendrites. These synapses are formed either on the dendritic shaft or on spines that project from them (Harris, 1999). Dendritic spines are the main postsynaptic sites receiving glutamate from excitatory synapses and were probably evolved to bear the enormous number of synapses that take place between the neurones in the mammalian brain (Fig.1.13).

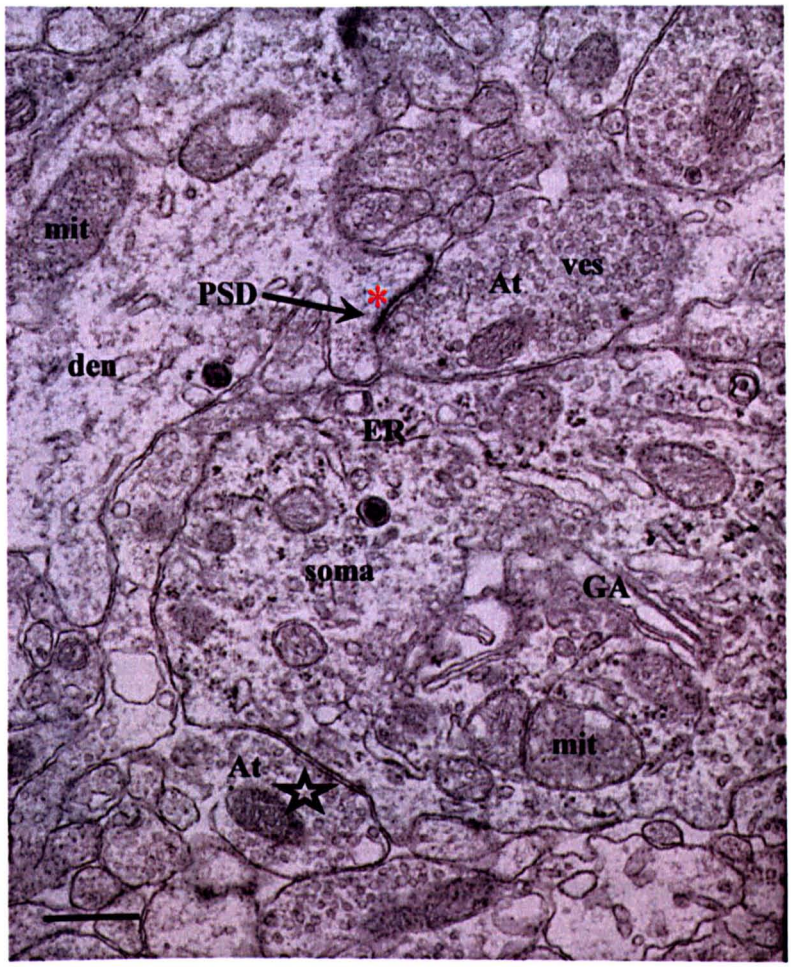


Fig 1.13. Image of chick ventral hippocampus. A mushroom spine synapse between a dendritic spine and an axon terminal (axo-dendritic synapse) is marked with a red asterisk. The post synaptic density (PSD) of this synapse can be clearly seen (labelled with an arrow). A symmetric synapse is marked with a star between an axon terminal (presynaptic part) and a soma (axo-somatic synapse). ER: endoplasmic reticulum, at: axon terminal, den: dendrite, mit: mitochondrion, GA: Golgi apparatus. (Nikolakopoulou 2004) Scale bar 200nm

It has been suggested that spines appear or disappear subject to the age and activity of a neurone (Harris, 1999). Spines are very sensitive to calcium concentration changes, so a small elevation of calcium causes elongation to spines, in contrast high elevations cause the collapse of a spine (Harris, 1999). Additionally, limiting the concentrations of calcium may be providing a protective mechanism for dendrites and the soma, whilst high concentrations may lead to dendritic swelling, microtubular breakdown and excitotoxicity (Choi, 1995). Spine shape influences calcium circulation within the spine independently from the dendrite (Korkotian and Segal, 1998).

There are different kinds of spines; thin spines, which dominate in the adult brain (Harris et al., 1992; Fiala et al., 1998) and they are characterised by a length greater than the spine neck diameter ending in a bulbous head, stubby spines which are short and wide with no

restriction on the neck diameter, sessile spines with greater length than diameter and the lack of bulbous head and mushroom spines (fig. 1.13) which have a constricted neck and a large irregular head (Sorra and Harris, 2000).

1.6.2 Postsynaptic density (PSD)

Synapses are characterised by a dense thickening, the postsynaptic density (PSD, see Fig. 1.13). It is attached under the surface of the spine membrane either on the top or the side of the spine head of the postsynaptic part, transversely from a presynaptic axon that contains vesicles (Sorra and Harris, 2000). It can form a simple disk (macular PSD), a perforated ring like shape or an extremely irregular or fragmented formation (Sorra and Harris, 2000). PSDs have been suggested to arrange adhesion between the pre- and postsynaptic parts and to regulate receptor clustering and function (Siekevitz, 1985) as well as calcium signal dependent systems. PSD is particularly thick and visible on asymmetric (excitatory) synapses (Landis et al., 1974, Fig. 1.14).

In the synaptic membrane there are at least four major categories of receptors including NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, metabotropic glutamate receptors (mGluRs) (Nusser et al., 1998; Racca et al., 2000). NMDA receptors can be found in the centre of the PSD (Racca et al., 2000), AMPA are located across the surface of the PSD and mGluRs are positioned on the outside surface of the PSD (Lujan et al., 1996)

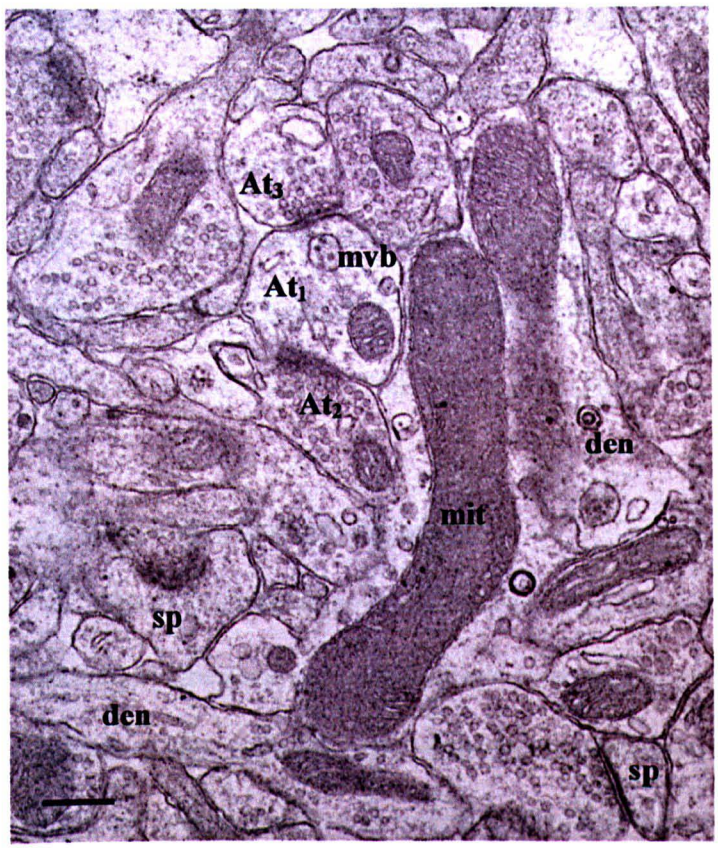


Fig 1.14. Image taken from the dorsal hippocampus of one day old chick. An asymmetric synapse is formed between At_1 and At_2 (axo-axonal synapse). A symmetric is formed between At_1 and At_3 (axo-axonal synapse). At: axon terminal, mvb: multi vesicular body, den: dendrite, sp: spine mit: mitochondrion. Notice the lack of mitochondria in spines. (Nikolakopoulou 2004) Scale bar =200nm

The PSD comprises tubulin, actin, fodrin and calmodulin (Kennedy, 1993), whilst the main protein present in the postsynaptic density is the α subunit of the Ca^{2+} calmodulin kinase type II (CaMKII) (Kennedy et al., 1983), which is a target of Ca^{2+} that passes through NMDA receptors and participates in synaptic plasticity (Silva et al., 1992). Protein kinase A (PKA) regulates plasticity by altering CaMKII activity (Blitzer et al., 1998). PKA phosphorylation of GluR1 AMPA receptor subtype although is essential for induction of LTP (Esteban et al., 2003), is not adequate since CaMKII is also needed. Possibly PKA makes AMPA receptors available for synapse stability.

The PSD also includes receptor binding proteins such as postsynaptic density protein of 95 kD MW (PSD-95) and guanylate kinase associated proteins (GKAPs) which group glutamate receptors on the spine head (Kim and Huganir, 1999). Glutamate receptor clustering proteins, for instance, PSD-95/SAP90, PSD-93/Chapsyn and synapse-associated protein of

102 kD MW (SAP-102) bind to NMDA receptors (Brenman et al., 1996a; Muller et al., 1996), whilst SAP97 binds to AMPA receptors *in vitro* (Leonard et al., 1998). PSD-95 binds tightly to NMDA receptors at synapses and they are both abundant in the PSD (Kornau et al., 1997). Other proteins that interact with the PDZ domains (repeated conserved segments for protein-protein interaction, Kandel et al., 2000) of the PSD-95 are the neuronal nitric oxide synthase (nNOS) (Brenman et al., 1996b), neuroligin (Irie et al., 1997) and SynGAP (Kim et al., 1998).

1.6.3 Organelles in dendritic spines

Spines contain smooth endoplasmic reticulum (SER), which is an elongated, flattened or enlarged structure consistent of cisternae and is present in almost half of the hippocampal dendritic spines, regulating the calcium concentration in spines (Spacek and Harris, 1997). It mainly appears in mushroom spines, whilst only a small proportion of thin spines exhibit SER (Spacek and Harris, 1997). In mushroom spines SER creates the spine apparatus (SA), which arranges the synthesis of the membrane bound proteins and their transport (Steward et al., 1996) as well as intracellular calcium increases (Capani et al., 2001). SER volume remains stable while spine size changes, however, SA becomes more plastic in larger synapses and increases its size (Spacek and Harris, 1997).

Polyribosomes are also present in the dendritic spine cytoplasm (Steward and Reeves, 1988) and it has been implied that when localised to the postsynaptic part of a synapse may supply a site for local and spine specific translation of proteins. Endosomes exist in dendritic spines which may be taking part in protein degradation.

Dendritic spines lack mitochondria (Fig. 1.13) unlike the dendrites where ATP is produced (Sorra and Harris, 2000). Hippocampal dendritic spines have a cytoskeleton that is actin based, whilst dendrites have also microtubules and intermediate filaments (Markham and Fifkova, 1986; Kaech et al., 1997). Intense staining for F-actin has been observed in the

dendritic spine head, whilst it disappeared or eliminated in the spine neck (Capani et al., 2001). Profilin is an actin-binding protein that participates in actin polymerization at the cell surface (Buss et al., 1992). It clusters in dendritic spine heads after activation of postsynaptic NMDA receptors and it participates in the control of actin function and spine shape stability (Ackermann and Matus, 2003), by steadying actin filaments and supporting the submembraneous actin network (Rothkegel et al., 1996).

1.6.4 Perforated PSD/synapses

Perforated synapses are those with a gap in the PSD (Calverley and Jones, 1990). They have a spine head area and a PSD area three times larger than the simple synapses (Toni et al., 2001) and can be fenestrated, horseshoe like or segmented (Hering and Sheng, 2001). Perforated synapses were increased during development after rats were exposed to an enriched environment (Greenough et al., 1978), after LTP (Toni et al., 1999) and kindling (Geinisman et al., 1990) and have been suggested to indicate synapse turnover (Harris et al., 1992). Since dendritic spines are extremely plastic, they can change their morphology within seconds (Fischer et al., 1998) and therefore perforated synapses can be created very quickly (Toni et al., 1999).

Another suggestion is that perforated synapses can actually alter the receptor turnover at the PSD (Maletic-Savatic and Malinow, 1998; Sorra et al., 1998). Since perforated synapses have a larger PSD, they probably contain more receptors (Sorra et al., 1998). Addition or removal of receptors from the neuronal network may be resulting in the discontinuation of the PSD. Perforated synapses could attribute to increased synaptic strength, because they have larger PSDs and since the PSDs are separated from their correlate by transition zones in the presynaptic terminal (Harris and Kater, 1994).

1.6.5 PSDs and LTP

Totally segmented, completely partitioned synapses (SCP) have been shown to experience a numerical increase after early phases of NMDA receptor dependent LTP (Toni et al., 2001), while shortly afterwards their numbers return to control levels (Geinisman et al., 1993; Toni et al., 2001). In particular, SCPs contain a wide variety of coated vesicles and have long spinules associated with the PSD after LTP (Toni et al., 2001), whilst multiple spine boutons consist of two spines, one similar to normal synapses and a much smaller one, probably newly formed and immature. The high presence of vesicles may be an indication of membrane-protein transport among the SA and the synaptic membrane (Spacek and Harris, 1997). The synaptic strength of the SCP has been attributed to the number of postsynaptic AMPA receptors (Malenka and Nicoll, 1999; Sheng and Kim, 2002). More AMPA receptors have been found in perforated than in non-perforated synapses (Desmond and Weinberg, 1998). The induction of LTP has been attributed to either the alteration of functional properties of AMPA receptors or the inclusion of new receptors (Liao et al., 1995). Other studies indicate that LTP mediates the production of AMPA receptor subunits (Nayak et al., 1998).

AMPARs arbitrate most of the brain's excitatory synaptic transmission, whilst NMDA receptors regulate the distribution of calcium and concomitantly they control the mechanisms underlying the trafficking and targeting of the AMPARs (Scannevin and Huganir, 2000; Sheng and Kim, 2002). The SCPs exhibit a significantly larger PSD in relation to any other synaptic subtype (Ganeshina et al., 2004) and express more AMPA receptors in comparison with other axospinous junctions. NMDARs activation is essential for early stages of synaptogenesis, such as localized and quick development of dendritic filopodia and new synapse formation (Maletic-Savatic et al., 1999). AMPARs are required at later phases, since blockage of AMPARs has been suggested to cause a decrease in axonal arborisation in mature

neurones (Zou and Cline, 1999; Cline et al., 2001). These studies indicate that NMDARs are developmentally needed before operating AMPARs (Lee and Sheng, 2000).

Dendritic spines usually make a single synapse, indicating probably a specialization in contacts rather than an expansion of the postsynaptic area (Shepherd, 1996). Studies have shown that synapses split under certain circumstances such as LTP in the dentate gyrus of the hippocampus (Trommald et al., 1990), not in CA1 though (Sorra et al., 1998), rearing in enriched environment (Jones et al., 1997) and chronic use of amphetamines (Robinson and Kolb, 1997).

During branching (splitting) the PSD of small synapses enlarges and perforates, whilst at the same time a division of the presynaptic bouton takes place by the intrusion of a spinule (narrow projections of the dendritic surface, Westrum and Blackstad, 1962) that projects from the perforation in the dividing PSD into the presynaptic bouton. As the synapse divides, the spinule is retracted (Sorra et al., 1998). The spinules derive from the head or the neck of a spine, never from the PSD, and they project into boutons. After the splitting of the synapse, a branched synapse occurs which has two heads originating from the same presynaptic part. Finally daughter synapses are created from each fragment of the earlier perforated synapse.

However, the results of LTP studies are quite controversial. Unlike studies demonstrating synapse doubling (Toni et al., 1999), others have shown that after LTP there are no increases in size of synapses (Toni et al., 1999). Newly developing synapses lack AMPA receptors exhibiting only NMDA receptors and therefore are silent (Gomperts et al., 1998; Petralia et al., 1999). Presynaptically silent synapses are those that show insufficient glutamate release to activate AMPA receptors, whilst postsynaptically silent are those that lack functional AMPA subunits relying only on NMDA receptor conductivity which in turn is blocked due to Mg^{2+} (Isaac, 2003). When NMDA receptors are blocked, the dendrites extend filopodia protrusions, returning probably to an immature state (McKinney et al., 1999).

Activations of NMDA receptors and calcium influx could mediate the release of nitric oxide in spines, which then could stimulate growth by local diffusion in presynaptic axons and boutons (Nikonenko et al., 2003). The axonal outgrowths could then initiate the production of shaft synapses (Fig. 1.15) which in turn would become spine synapses. Shaft synapses, in particular, that have originated from resorbed spines and protospines, may re-appear to form spines (Pokorny and Yamamoto, 1981b). Nitric oxide can influence synaptic growth by interacting with actin filaments (Hindley et al., 1997).

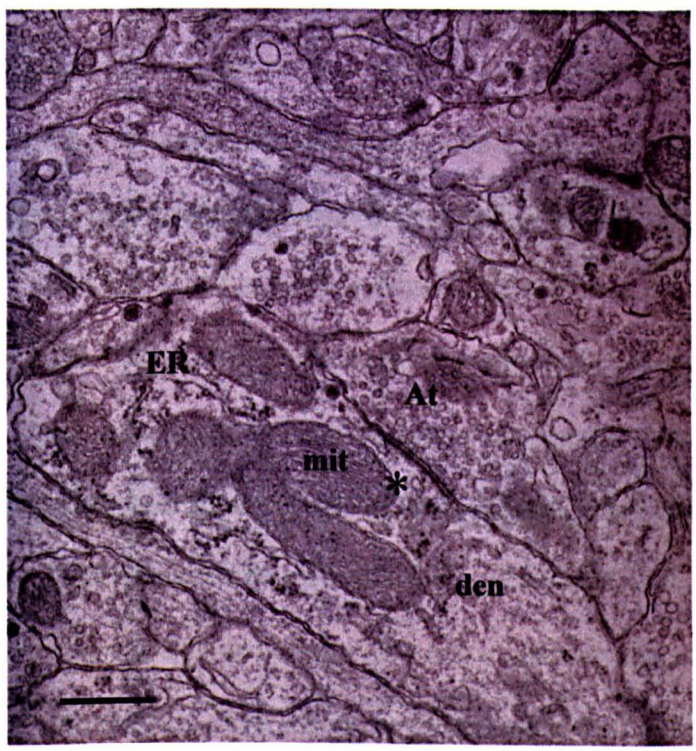


Fig 1.15. A symmetric axo-dendritic (shaft) synapse is formed between an axon terminal (presynaptic) and a dendrite (asterisk). ER (endoplasmic reticulum) can be seen on the top of the dendrite. (Nikolakopoulou 2004). Scale bar= 200 nm

1.6.6 New spines and synapse formation

Synaptic construction starts when axons come close to their targets and create connections with dendritic arbors or the soma of a neurone. Dendritic spines do not start to exist before the formation of synapses. Filopodia, which are long thin protrusions located at the periphery of migrating cells and growth cones, start to rapidly project from dendrites and axons, especially during the early stages of synaptogenesis (Ziv and Smith, 1996). Synapses can be found on filopodia during the first 2-3 postnatal weeks (Papa and Segal, 1996; Fiala et

al., 1998). Synaptic vesicles appear when axons and filopodia establish contacts (Ahmari et al., 2000) and accumulate at varicosities of the presynaptic sites (Ahmari et al., 2000). Filopodia have a darker cytoplasm than spines, they terminate in a pointed head and they are much longer than mature spines (Fiala et al., 1998). Filopodia are plentiful in the brain during the first postnatal week, (however, only 25% of synapses occur on them, the rest take place directly on dendritic shafts) afterwards they are replaced by shaft synapses and stubby spines, they decrease both in number and size to finally give their place to mushroom and thin synapses in the adult brain (Hering and Sheng, 2001). Therefore it has been suggested that filopodia are precursors to shaft synapses, in particular they do not seem to provide permanent support for synapses, instead they guide nascent synapses to dendritic shafts which finally mature to give dendritic spines (Harris et al., 1992; Fiala et al., 1998).

A hypothesis for spine synapses formation is based on the idea that spines originate from shaft synapses by a process of outgrowth (Pokorny and Yamamoto, 1981b). Another hypothesis for synapse formation is based on the creation of synapses on the tip of the filopodium which then retracts towards the dendritic shaft pulling the presynaptic axon along, an observation made after *in vitro* studies (Ziv and Smith, 1996). Spines can be also formed *de novo* (Engert and Bonhoeffer, 1999) due to activity and calcium concentration changes.

In addition, filopodia lack postsynaptic density protein (PSD 95) clusters which appear concomitantly with filopodia formation in the protospines to become copious in mature spines supporting even more the hypothesis of filopodia giving rise to glutamatergic synapses (Ziv and Smith, 1996). *In vitro* studies have shown that the presynaptic differentiation occurs earlier than postsynaptic in the developing hippocampal neurones (Okabe et al., 2001).

Astrocytes provide energy to the neurones by increasing the action potential-independent quantal release so that neurones can complete their functions as well as control the formation and efficacy of synapses (Pfrieger and Barres, 1996, 1997). They re-accumulate

(Rothstein et al., 1996; Danbolt, 2001), supply neurones with (Hertz et al., 1999) and arrange glutamate release via glutamate transporters (Hertz et al., 1999; Del Arco et al., 2003) and participate in the recycling of glutamate via glutamine and intermediates of the tricarboxylic acid (TCA) cycle (Schousboe et al., 1997). Their ability to clear excessive extracellular glutamate suggests that they protect neurones from excitotoxic glutamate concentrations which could result in neuronal cell death (Rosenberg and Aizenman, 1989). They also take part in cell-cell interactions through adhesion junctions (Spacek and Harris, 1998) and intercellular calcium influx (Vernadakis, 1996).

In addition, glia does not increase presynaptic activity only by calcium influx, instead it influences the number of vesicles released (Ullian et al., 2001). These authors have also demonstrated that removal of glia, causes reductions of the number of synapses indicating that it affects synaptic stability and is necessary for synapse preservation. *In vitro* studies have shown that the formation of synapses begins after astrocytes have started to appear suggesting that glia contributes dynamically in synapse increases (Ullian et al., 2001). Furthermore, glia offers steadiness postnatally, since during embryonic stages of an organism synapses are extremely plastic and immature and has been implied to take part in learning and memory procedures (Hyden and Lange, 1966). Cell swelling or gliosis would increase glia-spine contacts resulting in spine retraction, as it happens after chronic human epilepsy where the size and density of spines is decreased (Scheibel et al., 1974).

1.6.7 Cell Adhesion Molecules and their role in synaptogenesis

Cell adhesion molecules (CAMs) participate in synaptogenesis (Bruses, 2000) and members of the cadherin family have been shown to reside at synaptic junctions (Benson and Tanaka, 1998; Huntley and Benson, 1999), probably through homophilic/trans (on a different cell) interactions between identical cadherin molecules (Inoue et al., 1998; Boggon et al.,

2002). Cadherins are Ca^{2+} dependent (Hatta and Takeichi, 1986; Tomaselli et al., 1988) and are located at puncta adherentia which is linked to actin filaments (Spacek and Harris, 1998) and is located between the presynaptic axon and the postsynaptic dendritic spine at the edges of PSD (Spacek and Harris, 1997). Puncta adherentia contains apart from cadherins, neural adhesion molecules, catenins and nexilin (Uchida et al., 1996; Ohtsuka et al., 1998). Actin could act in this case as a scaffold and bind synapsin and other molecules to the presynaptic part, regulating in this way presynaptic activity. Cadherins are not necessary for the continuation of synaptic vesicle organization (Togashi et al., 2002).

Neuroligins, another type of transmembrane cell adhesion molecules, are located in the postsynaptic membranes of glutamatergic synapses and are considered to be taking part in the regulation of synaptic signals (Song et al., 1999). Neurexins are the cell-surface receptors of neuroligins (Ichtchenko et al., 1995). B-neurexins interact with PDZ proteins, controlling the gathering of presynaptic active zones (Dean et al., 2003). Endogenous neurexins are located in the synaptic terminals and their gathering causes generation of synaptic vesicles (Dean et al., 2003). Integrins on the other hand form cell-cell and cell-extracellular matrix adhesive connections (Springer, 1990) and play an important role during neural development (Bronner-Fraser, 1987).

Another CAM, the neural cell adhesion molecule (NCAM) has been shown to participate in LTP in the dentate gyrus of free moving rats (Fazeli et al., 1994). NCAM along with L1 (neuron/glial CAM (NgCAM) and NrCAM are members of the L1 family) belong to the immunoglobulin superfamily and are cell surface macromolecules which have the ability to regulate cell-cell interactions during development and in adult life due to their recognition and adhesion capacity and participate in synaptogenesis, neurite outgrowth and migration (Rutishauser et al., 1983; Covault and Sanes, 1986; Seki and Arai, 1993; Hu et al., 1996; Kuhn et al., 1996). NCAM makes Ca^{2+}-independent cell-cell connections and forms

homophilic/trans or/and heterophilic/cis interactions (Cunningham et al., 1987; Soroka et al., 2003). Post-translationally, 2-8-linked polysialic acid (PSA) chains are attached (Doherty et al., 1990) to NCAM -80 (Yang et al., 1992) and L1 (Acheson et al., 1991; Zhang et al., 1992). Addition of PSA causes a decrease of membrane-membrane binding rate (Hoffman and Edelman, 1983; Rutishauser et al., 1988) and promotes neurite outgrowth (Doherty et al., 1990). PSA-NCAM has been implicated in learning (Seki and Rutishauser, 1998; Solomonia et al., 1998; Knafo et al., 2004; Sandi et al., 2004) whilst an increase in polysialation of NCAM -180 has been demonstrated in the rat hippocampus after passive avoidance training (Doyle et al., 1992). The expression of PSA-NCAM can be also affected by stress (Merino et al., 2000; Nacher et al., 2004)

1.6.8 Neurotrophins

Neurotrophins such as BDNF, NT-3, NT4/5 and NGF have been suggested to regulate synaptogenesis and affect presynaptic neurones as well as postsynaptic targets (von Bartheld et al., 2001). Neurotrophins can change dendritic and axon morphology as well as dendritic spine stability in developing neurones (McAllister et al., 1999). BDNF has been suggested to control axonal and dendritic branching and remodelling (Cohen-Cory and Fraser, 1995; Lom and Cohen-Cory, 1999), it increases the efficiency of synaptic communication (Boulanger and Poo, 1999), participates in the maturation process of inhibitory and excitatory synapses (Rutherford et al., 1998) and possibly plays an essential role in synapse formation and stability (Katz and Shatz, 1996; Poo, 2001) as has been observed for axonal arborisation after *in vivo* imaging (Alsina et al., 2001). Previous studies have shown that it participates in the conversion of immature silent synapses into functional AMPA receptor synapses (Itami et al., 2000). Additionally, BDNF controls synaptic vesicle protein expression and the density of synaptic innervation (Gonzalez et al., 1999; Pozzo-Miller et al., 1999). Studies have shown

that BDNF modulates synaptic connectivity (Causing et al., 1997) and alters synaptic density without changing the axon number (Causing et al., 1997).

1.6.9 Other factors affecting synaptogenesis and spine formation

1.6.9.1 Ageing

Many different factors affect synaptogenesis. Ageing has been shown to decrease the number and the surface area of synapses, whilst the synaptic size increases. Additionally, pre and postsynaptic elements become thicker with age, in contrast, vesicle size decreases (Schulpis et al., 2001). Synaptic efficacy is reduced due to decreased transmitter release and receptor density (Scheuer et al., 1995). Synaptic proteins are also decreased in axons and dendrites (Hatanpaa et al., 1999) as well as dendritic branches, their length and their cell soma size (Anderson and Rutledge, 1996).

1.6.9.2 Learning and stress

Learning paradigms and stress have been demonstrated to alter synaptic plasticity. Water maze training causes mossy fibre synaptogenesis in CA3 stratum oriens (Ramirez-Amaya et al., 1999). An enriched environment can also provoke increases in synaptic density in the CA3 (Altschuler, 1979) even after ageing (Arnaiz et al., 2004). The stratum radiatum of CA1 has also demonstrated synaptic increases after conditioning (Wenzel et al., 1980). Finally, LTP as already mentioned has been shown to increase the number of spine boutons 1h after LTP as well as the number of synaptic contacts between axons and dendrites (Toni et al., 1999). It must be mentioned, though, that in birds although LTP is present in the avian brain, it does not require NMDA receptor activation, indicating probably different mechanisms involved in synaptic plasticity (Wieraszko and Ball, 1993; Margrie et al., 1998).

Many studies have been focused on synaptogenesis in the chick brain mainly after passive avoidance training and imprinting. Increases in spine density have been demonstrated particularly in the left StM (Stewart et al., 1987; Lowndes and Stewart, 1994) and IMM (Patel and Stewart, 1988) 24 hours after PAL in the MeA trained group in relation to controls. Furthermore, a decrease in postsynaptic density length in the right IMM and an increase in the number of synaptic vesicles in the left hemisphere have been observed in the MeA trained group (Stewart et al., 1984). On the other hand, 1h after PAL studies have shown an increase in the number of asymmetric spine synapses in the right IMM whilst there is a concomitant decrease of the synaptic height (size of postsynaptic density) in the same area (Doubell and Stewart, 1993). These authors have also shown an increase of numerical density in the left IMM and StM 1 hour after PAL, which had disappeared 24 afterwards. A recent study has also demonstrated a numerical increase in shaft synapses in the dorsolateral Hp after PAL, as well as a decrease in mean synaptic height (Unal et al., 2002).

In contrast to these studies, auditory filial imprinting has been suggested to reduce the number of spine synapses in the mediorostral nidopallium/mesopallium (MNM) and dorsocaudal nidopallium (NDC) (Bock and Braun, 1998; Braun et al., 1999). Auditory filial imprinting requires the activation of NMDA receptors for synapse elimination, indicating probably a different activation mechanism for emotion-related learning tasks (Bock and Braun, 1999a, b). Another factor that can alter synaptic plasticity is ischaemia, which causes a significant reduction in synaptic density of asymmetric synapses in the dorsal (Horner et al., 1996) and the ventral Hp (Horner et al., 1998) 7 days after ischaemia. These authors argue that the uncontrolled release of glutamate (since asymmetric synapses are presumed to be excitatory) following ischaemic episodes (Rothman and Olney, 1986) is responsible for cell death (Szatkowski and Attwell, 1994) due to activation of NMDA receptors (Ghribi et al., 1994) and the increases of Ca^{+2} influx.

Learning has been demonstrated to cause structural changes to the mammalian (Rogan et al., 1997; Ramirez-Amaya et al., 1999; van Praag et al., 1999) and avian brain (McCabe and Horn, 1988; Horn, 1998). In the chick brain PAL has been exhibited to cause synaptic remodelling (Stewart et al., 1987; Stewart and Rusakov, 1995), whilst recent studies have shown an increase in cell proliferation in IMM and StM (Dermon et al., 2002). However, little is known about the effects of PAL on cell proliferation and neurogenesis. Below, the origin of newborn cells is described along with mechanisms that can influence neurogenesis in the vertebrate brain.

1.7 CELL PROLIFERATION-NEUROGENESIS

Adult neurogenesis has been identified in the brain of rodents (Altman and Das, 1965; Cameron et al., 1993; Kempermann et al., 1997a; Gould et al., 1999b; van Praag et al., 1999; Dayer et al., 2003), birds (Nottebohm, 1989; Alvarez-Buylla, 1990a; Patel et al., 1997; Lee et al., 1998a), fish (Zikopoulos et al., 2001; Zupanc, 2001; Zupanc and Clint, 2003), amphibians (Chetverukhin and Polenov, 1993; Polenov and Chetverukhin, 1993), lizards (Perez-Canellas and Garcia-Verdugo, 1996), snakes (Wang and Halpern, 1988), primates (Gould et al., 1999c; Gould et al., 1999a; Rakic, 2002a) and humans (Eriksson et al., 1998; Roy et al., 2000; Haughey et al., 2002; Curtis et al., 2003). Studies in monkeys and humans have indicated that hippocampal neurogenesis is lower when compared with rodents (Eriksson et al., 1998; Kornack and Rakic, 1999), which is due probably to different maturation stages examined..

Neurogenesis can be studied by 5-bromo-2-deoxyuridine (BrdU) immunohistochemistry (Gould et al., 1998; see Fig. 1.15 for examples of BrdU labelled cells from Nikolakopoulou, 2001; Dermon et al., 2002; Dayer et al., 2003), [^{3}H]thymidine (Alvarez-Buylla et al., 1990; Barnea and Nottebohm, 1994; Cameron and Gould, 1994), retroviruses or green fluorescent protein (GFP), the latter often introduced with the help of a

retrovirus (van Praag et al., 2002) and endogenous Ki-67 (Del Bigio, 1999; Dayer et al., 2003). BrdU immunohistochemistry has demonstrated that the total length of the cell cycle in twenty day old mice lasts for almost 16 hours, the S phase lasts for 8 hours and a maximum of 24% of the cells in the hilus are proliferating (Nowakowski et al., 1989). The rate of neurogenesis has been identified as 1 neuron/2000 granule cells per day in 3 month old mice (Kempermann et al., 1997b).

In the hippocampus, newborn neurones (precursors) derive from stem cells that are located in the subgranular zone of the dentate gyrus and differentiate into granule neurones (Altman and Das, 1965; Cameron et al., 1993; Kuhn et al., 1996) which survive for 8 months in rodents (Altman and Das, 1965), 12 months in primates (Gould et al., 2001a) and 2 years in humans (Eriksson et al., 1998). Stem cells are cells that can self renew and are multipotent, whilst progenitors are cells that can be either unipotent or multipotent and show less self renewal ability and precursors are those that are a mixed or unidentified population (Weiss et al., 1996). Studies have demonstrated that neural stem cells exist only in the ventricular subependyma, whilst the dentate gyrus contains only progenitors in the subgranular layer (Chiasson et al., 1999; Seaberg and van der Kooy, 2002). Neural stem cells give rise to neural or glial progenitors which in turn induce the production of precursors (Gage, 2000). The former have the ability to differentiate into functional neurones with mature neurones properties (Song et al., 2002). There was an assumption that in mammals neuronal cells are not born in the sub- or ventricular zone, but they rather arise from precursor cells that are located at these regions (Altman and Das, 1965; Kaplan and Hinds, 1977) unlike birds where neurones are born in the ventricular zone (Alvarez-Buylla et al., 1990, examples of BrdU+ cells originating from the VZ are presented in Fig. 1.16).

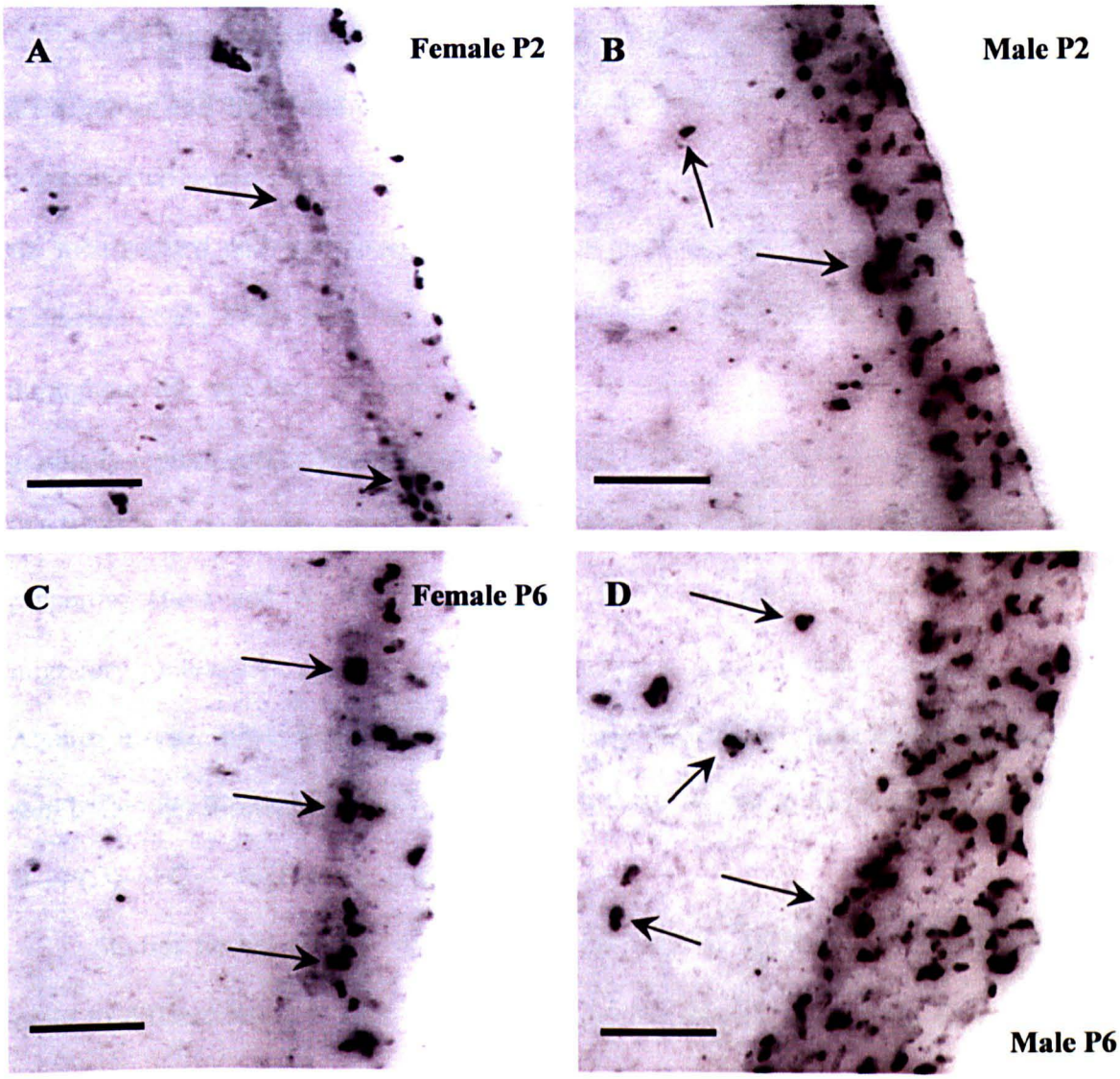


Fig. 1.16. Ventricular zone of the mesopallium of a female (A) and a male (B) 2 day old quail (Coturnix japonicus). These animals were injected with BrdU the first day post hatching and were sacrificed the next day. The BrdU labelled cells (arrows) can be easily visualised in both cases. It is interesting to mention that the male quail in this case shows more newborn cells in relation to the female animal. Images C and D represent the mesopallium of a female and male quail respectively. These animals were injected on P5 and were sacrificed the next day (P6). Again the male shows more BrdU positive cells, however more cells are also present in relation to the P2 male, indicating probably that at five days cell proliferation is more intense (Nikolakopoulou, 2001). Scale bars =50μm

Lois and Alvarez (1993) have demonstrated *in vitro* that cells located in the subventricular zone are precursors that can actually give rise to neurones or glia after proliferation.

After precursors divide, they migrate along radial glia (Schlessinger et al., 1975) into the granular cell layer and start to show neuronal processes along with the expression of polysialylated neural cell adhesion molecule (PSA-NCAM) (Seki and Arai, 1993) where they are incorporated and start to express neuronal markers (eg neuron-specific enolase, NSE) (Cameron et al., 1993; Jin et al., 2001). The newly formed neurones are added in the brain throughout life and they concentrate in the deeper layers of granule cell layer where they gradually replace earlier formed neurones (Crespo et al., 1986). Four types of cells have been identified in the subventricular zone (SVZ) based on immunohistochemical and morphological properties (Lois and Alvarez-Buylla, 1994; Garcia-Verdugo et al., 1998). Type A are migratory neuroblasts, type B are astrocytes, type C are precursors and type D (Lois and Alvarez-Buylla, 1994; Seri et al., 2001) originate from type B cells and are an intermediate step before the formation of C type cells. It has been suggested that the line of progression is B—>(D)—>C—>A (Doetsch et al., 1999).

Studies further support that astrocytes provoke the proliferation of types B and C in order to differentiate into type A cells (Lim and Alvarez-Buylla, 1999). Other studies have demonstrated that neurones can originate from astrocytes and radial glia, implying that neurones and glia may be derived from the same lineage and that astrocytes in the SVZ are neural stem cells (Lim and Alvarez-Buylla, 1999; Noctor et al., 2001). In addition, astrocytes can affect the number of newborn neurones that are produced from neuronal precursors (Song et al., 2002) as well as control the phenotype of neurones based on the area of origin (Denis-Donini et al., 1984; Skogh et al., 2001). Astrocytes that give rise to neurones are limited to specific areas of the CNS and don't affect neighbouring astroglial cells (Malatesta et al., 2003).

Neurogenesis can be influenced by learning (Alvarez-Buylla et al., 1988b; Kempermann et al., 1997a; van Praag et al., 1999) and stress (Lemaire et al., 2000; King et al.,

2004). Below, the effects of stress in the vertebrate brain are described and associated with PAL in one-day old chick brain.

1.8 STRESS

Stress is one of the factors that can cause morphological alterations to the brain and provoke pathological conditions to the organism (Munck et al., 1984; Brindley and Rolland, 1989). In addition, stress has been suggested to be responsible for depression, possibly through an existing neurochemical relationship (Trolle, 1955; Iny et al., 1994) and Cushing's syndrome (von Werder et al., 1971; Spencer and Hutchison, 1999; Drapeau et al., 2003b).

The HPA axis is activated after exposure to stimuli that threaten homeostasis and is considered part of the defence system of an individual (Spencer and Hutchison, 1999). It receives positive feedback from the amygdala (arcopallium in birds), but negative from the hippocampus (Bouille and Bayle, 1973; Sapolsky et al., 1985a; Schulkin et al., 1994; Mizoguchi et al., 2003). The HPA is affected by corticotrophin-releasing-factor (CRF), which is involved in stress responses in rats (Rivier et al., 1982; Herman and Cullinan, 1997) and birds (Launay et al., 1993) and elevated corticosterone levels in birds (Furuse et al., 1997).

Lipopolysaccharide (LPS) has also been shown to increase HPA activity, by increasing the concentration of circulating glucocorticoids (Zuckerman et al., 1989) as well as plasma corticosterone levels in the avian brain (Sell et al., 2003). Adrenocorticotropic hormone (ACHT) (Rivier, 1999), corticosterone (CORT) and cortisol (in rats and humans respectively) are adrenal hormones secreted from the adrenal medulla and especially CORT and cortisol have been implied to participate in metabolism after stressors in order to regulate energy requirements (Nagra and Meyer, 1963; Feller and Neville, 1966; Remage-Healey and Romero, 2001).

The hippocampus expresses high levels of adrenal steroid receptors (McEwen et al., 1994; van Steensel et al., 1996), type I (mineralcorticoid, MRs) and type II (glucocorticoids, GRs) that take part in synaptic plasticity and neuronal excitability (Reul and de Kloet, 1985; De Kloet et al., 1998; de Kloet, 2000). The two types of receptors have different binding affinities, MRs bind corticosterone with high affinity, whilst GRs have one tenth of the affinity of MRs (Reul and de Kloet, 1985). When corticosterone circulation is in basal levels, the MRs are occupied, on the contrary, when the levels of CORT begin to elevate, the GRs get gradually occupied (Reul and de Kloet, 1985; Reul et al., 1987). The GRs concentration is increased between the 9^{th} and 15^{th} postnatal day as has been demonstrated by autoradiographic studies (Meaney et al., 1985). The GRs are involved in memory formation whilst MRs take part in perceiving the environmental stimuli and navigate the behavioural action to be adopted, in particular blocking of GRs didn't allow consolidation of the water maze task, whereas inhibition of MRs changed the search strategy (Oitzl and de Kloet, 1992; Roozendaal and McGaugh, 1997).

LTP has been shown to be affected by stress in CA1, CA3 and DG, where high levels of corticosterone or chronic stress caused suppression of LTP (Pavlides et al., 1993; Pavlides et al., 2002). Furthermore, CORT elevation has been suggested to increase glutamate release in the hippocampus of adult rats (Gilad et al., 1990; Moghaddam et al., 1994) and binding increases have been observed in NMDA receptors as well as elevation of NR2A and NR2B mRNA subunits (Weiland et al., 1997). It has been shown that NMDA receptors are downregulated in the amygdala during fear conditioning in order for the brain to be protected from excitotoxicity (Zinebi et al., 2003), indicating a possible connection between elevated glucocorticoids, calcium increases and activation of NMDARs by glutamate.

Chronic stress has been demonstrated to attenuate glucocorticoid negative feedback, and it has been demonstrated that in depressed patients the HPA axis function is disrupted and

does not respond to cortisol secretion levels (Kalin et al., 1982; Holsboer, 1983). High corticosterone levels have been shown to downregulate BDNF and NT-3 in the hippocampus (Smith et al., 1995; Ueyama et al., 1997; Nitta et al., 1999), implying that synapse stabilization or cell survival may be affected, since addition of BDNF in culture suppresses cell death (Nitta et al., 1999).

During stress, cell interactions may also be disturbed due to decreased expression of NCAM in the prefrontal cortex (Sandi and Loscertales, 1999) and hippocampus (Sandi et al., 2001) after increases in plasma CORT concentrations. Finally, volume loss has been suggested to be caused as a result of neuronal death and neurogenesis suppression (Lee et al., 2002a). In rhesus monkeys it has been suggested that prenatal treatment with dexamethasone resulted in 30% reduction of hippocampal size in adult life (Uno et al., 1994), whilst patients with Cushing's disease and elderly people have also demonstrated volume reductions due to elevated cortisol probably because of dendritic tree shrinkage (Starkman et al., 1992; Convit et al., 1995). At a molecular level, it has been indicated that glucocorticoids can downregulate the expression of early genes (Yin and Howells, 1992).

Corticosterone controls learning and memory procedures in animals and humans (Sandi and Rose, 1994a, b; Wolkowitz, 1994; Lupien and McEwen, 1997). However, chronic stress or high quantities of CORT can be verified to be harmful for memory (Bodnoff et al., 1995; Conrad et al., 1996). Studies have shown that continuous blockage of glucocorticoid receptors resulted in facilitation of memory and learning processes unlike phasic blockage that impaired memory formation (Oitzl et al., 1998). It has been well documented that moderate levels of corticosterone enhance learning and memory (Sandi et al., 1997), whilst very high levels disrupt memory acquisition and learning (Diamond et al., 1999); the performance of animals under stress follows a U shaped distribution, low levels of corticosterone do not facilitate learning due to lack of motivation (Anderson, 1976), high levels disrupt memory as described

above, whilst moderate stress helps the animal concentrate on the factors providing information in order to memorize the environmental stimuli (Selden et al., 1990) in view of the fact that corticosterone improves passive avoidance behaviour (McEwen and Sapolsky, 1995).

In one day old chick, studies have shown that corticosteroid receptor antagonists cause amnesia for passive avoidance learning (Sandi and Rose, 1994b) and corticosterone facilitates longer memory preservation for the weak passive avoidance training task (10% MeA in absolute ethanol) until 24 h post training (Sandi and Rose, 1994a), indicating that a considerable amount of stress is essential for memory retention and the transition from short term to long term memory (Sandi and Rose, 1997). Corticosteroid inhibitors also caused amnesia for the passive avoidance learning paradigm in a dose dependent way (Loscertales et al., 1997). Corticosterone has been proposed to promote long term memory by affecting glycoprotein synthesis (Sandi et al., 1995). NCAM antibodies during the second wave of post translational glucosylation 5.5 hours after training reduced the beneficial effects of corticosterone, whilst corticosterone injections enhanced glycoprotein synthesis for the same time point. Corticosterone has been implied to participate in the development of an animal because studies have shown that injections of CORT in E20 dark-incubated chick embryos facilitated memory retention for the weak passive avoidance training, whilst the same phenomenon was not observed if animals were injected earlier or later (Sui et al., 2001).

Accumulation of CORT has been suggested to cause neuronal loss in later years, implying a role for corticosterone in ageing (Sapolsky et al., 1985b). Injections of CORT on mid-aged rats caused effects similar to those of ageing such as cell loss, CORT receptor depletion, probably as a result of concentrating CORT cell death and increased glia. Therefore, Sapolsky and colleagues (1985) concluded that stress experience throughout lifetime and the resulting accumulation of CORT may be responsible for increasing the effects

of ageing. Additionally, HPA dysfunction and hypersecretion of glucocorticoids that have been observed during ageing are considered some of the causes for neuronal loss (Coleman and Flood, 1987), since adrenalectomy reversed the effects of ageing, in particular neuronal loss and memory impairment (Landfield et al., 1981), nonetheless PSA-NCAM expression was not upregulated (Montaron et al., 1999).

Other studies do not agree with these results, instead they suggest that elevated glucocorticoids during ageing may play a role in hippocampal atrophy, synaptic loss and reduction of neurogenesis, but not in neuronal loss (Lupien et al., 1998; Lemaire et al., 2000). Reduced neurogenesis during ageing may also result from maternal deprivation (Mirescu et al., 2004) or prenatal stress (Lemaire et al., 2000). Glial fibrillary acidic protein (GFAP) mRNA levels are much higher in adult animals in relation to younger rats (Morgan et al., 1997). Another assumption is that the perseverance of excitatory amino acids release in the aged brain after stress may render the aged hippocampus more susceptible to damage (Lowy et al., 1995).

1.9 CELL DEATH

Apoptosis is the process during which the cell uses ATP from its stores to activate the appropriate intracellular flow of actions with the intention that the cell dies under controlled conditions. This will eliminate the toxic effects of local inflammation and cellular spillage (Lowy et al., 1995). Apoptosis needs gene activation for these events to take place and is a natural way of cell death for cell populations (Kerr et al., 1972; Wyllie et al., 1980; Gould et al., 1994). Some of the genes known to affect apoptosis are the *bcl-2, bax,* interleukin-1β converting enzyme (ICE), *bcl-x* and the suppressor tumour gene p53 (Reagan and McEwen, 1997). *bcl-2* and *bcl-x* are negative regulators of cellular death, *bax* is a promoter of cell death which suppresses its function when it creates dimmers with *bcl-2,* ICE is a protease that turns

the inactive pro-interleucin-1 into active IL-1β and its overexpression causes apoptotic death, whilst p53 mediates DNA repair after cell damage. However, if DNA damage is very severe, p53 induces apoptosis to prevent the formation and expansion of mutated DNA (Reagan and McEwen, 1997). Cell membrane and organelles are preserved until late apoptosis phases constructing apoptotic bodies (Reagan and McEwen, 1997). Cells that die from apoptosis are phagocytosed or digested by neighbouring cells or macrophages (Wyllie et al., 1980). The final signs of apoptosis are regulated by caspases, which are apoptotic cysteine proteases, and involve chromatin condensation and nuclear fragmentation that lead to DNA laddering, cleavage of cytoskeletal proteins, survival proteins and cellular substrates (Chan and Mattson, 1999). Necrosis, on the other hand, is a degenerative procedure which is characterized by irregular cellular signals that lead to cell lysis (Fawthrop et al., 1991).

Thyroxine (T_4) can induce cell death in the High Vocal Center (HVC) and other telencephalic areas of the adult zebra finch brain that experience neuronal turnover (Tekumalla et al., 2002). Chronic treatment with T_4 can cause reduction in the total number of HVC neurones and hyperthyroidism can increase neuronal death which is not compensated by neuronal substitution.

Excessive acetylcholinesterase (AChE) can trigger neuronal death and astrocytic hypertrophy in the dentate gyrus of rats (Chacon et al., 2003) accompanied by learning impairments.

Energy restriction is another cause of reduced cell proliferation and increased cell death (Dunn et al., 1997). It has been reported that *in vivo* energy restriction and corticosterone mediate cell death probably due to the reduced circulation of survival factors that these agents regulate, conversely the same effect has not been demonstrated after *in vitro* studies (Jiang et al., 2002).

Finally, low doses of kainic acid can provoke hippocampal CA1 and CA3 delayed cell lose by apoptosis (Humphrey et al., 2002), since p53 is increased in CA3 pyramidal cells (Sakhi et al., 1994), without causing seizures (Montgomery et al., 1999). This delayed apoptotic death has been shown to be accompanied by neurogenesis, probably in order for lost neurones to be replaced (Dong et al., 2003).

During apoptosis the cell undergoes death under controlled conditions that inhibit the creation of toxicity due to cellular destruction (Roy and Sapolsky, 2003). Apoptosis is a type of cell death that requires gene activation to take place and has been suggested to be the normal way of loss of cell populations (Wyllie et al., 1980; Gould et al., 1994). Cells may experience apoptosis as a natural process during development.

Cell death can be increased or decreased according to the milieu that surrounds the cell. Increases in the levels of adrenal steroids by administration of corticosterone and aldosterone reduce neurogenesis (Gould et al., 1991a) as well as cell death (Gould et al., 1991b). In contrast, adrenalectomized rats show increased cell death after adrenalectomy (ADX) (Sloviter et al., 1993a; Cameron and Gould, 1994, 1996). New cells that are born in the hippocampus have been observed to die before they reach maturity between 6 and 28 days after labelling with BrdU (Cameron et al., 1993; Gould et al., 1999b; Dayer et al., 2003); almost 50% of newborn cells have been identified as apoptotic. Most of the apoptotic cells were found in the borders of the hilus with the granular cell layer, indicating that neuronal death starts in the neurogenic zone of the dentate gyrus (Gould et al., 1990; Bengzon et al., 1997; Biebl et al., 2000).

Apart from neurones, glia have been suggested to experience apoptosis at different developmental stages (Soriano et al., 1993; Dalmau et al., 2003) as well as in CNS pathologies (Nguyen et al., 1994; Vela et al., 2002). One possible reason for apoptosis may be the competition for survival for both neurones (Cowan et al., 1984; Reynolds and Wilkin, 1988)

and astrocytes (Reynolds and Wilkin, 1988; Krueger et al., 1995) and the use of neurotrophic factors.

Apoptosis of newborn cells has been also observed in the proliferative zone of the cerebral cortex (Thomaidou et al., 1997), particularly 1 in 14 cells die at embryonic day 16, whilst the number increases at newborn rats reaching 1 cell in every 1.5. In the olfactory bulb apoptosis has been demonstrated in the layers (mitral and granule cell layer) where new cells innervate (Fiske and Brunjes, 2001) indicating possibly that older neurones die to make space for newer ones to incorporate in the circuitry (Kaplan et al., 1985). Cell death has been also identified in the BO after the newly formed cells have extended branches and have created synaptic contacts, implying that the survival of neurones depends on their activity (Petreanu and Alvarez-Buylla, 2002). Therefore, an assumption from these studies could be that cell death regulates neuro- and gliogenesis during all stages of neonatal and postnatal development into maturity.

Stress and high levels of glucocorticoids have been suggested to trigger neuronal death (Sapolsky et al., 1985b; Sapolsky, 1996), probably by apoptosis in rats (Sapolsky, 1996) and tree shrews (Lucassen et al., 2001). Studies in the electron (Sloviter et al., 1993a) and light microscopes (Sloviter et al., 1993b) have demonstrated that glucocorticoids cause apoptosis, as has been demonstrated by observation of morphological characteristics.

To strengthen this hypothesis, studies on gene expression have been conducted after ADX or cerebral ischaemia. These studies have demonstrated that p53 is expressed in the dentate gyrus after ADX (Schreiber et al., 1994), *bax* increases and *bcl-2* and *bcl-x* reduction have been found in CA1 cells after ischaemia (Krajewski et al., 1995; Chen et al., 1996).

Inhibition of excitatory amino acid (EAA) transporters can lead to increased levels of glutamate at the synapse and consequently to excessive activation of EAA receptors. Excessive glutamate can elevate the concentrations of Ca^{2+} causing apoptotic death through

the generation of oxygen free radicals, the buffering capacity of mitochondria and the activation of endonucleases sensitive to Ca^{2+} concentrations (Reagan and McEwen, 1997). The latter can regulate DNA strand cleavage, leading to apoptosis (Montague et al., 1994). Furthermore, elevated calcium concentrations and excitotoxicity can cause decreases of mitochondria dependent respiration (Sun and Gilboe, 1994) leading to the creation of oxygen free radicals.

Oxidative stress has also been suggested to induce neuronal apoptosis (Linnik et al., 1993) during stroke, however the gene kallikrein could reverse the damage caused by ischaemia by increasing the levels of NO, which is considered an antioxidant (Xia et al., 2004). In cortical cultures oxygen-glucose deprivation have been shown to induce both necrosis and apoptosis (Gwag et al., 1995). Free oxygen radicals can cause DNA damage leading to neuronal death (Liu and Martin, 2001).

Other data, however, do not agree with the assumption that glucocorticoids lead to apoptosis, they rather speculate that glucocorticoids do not cause DNA cleavage, a characteristic of apoptosis and further support that more studies need to be performed in order to prove that apoptosis could be a side effect of elevated glucocorticoids (Masters et al., 1989; Roy and Sapolsky, 2003).

In aged rats (Liu et al., 2003), mitochondrial oxidative stress is taking place at the initial steps of apoptosis (Esteve et al., 1999), during which glutathione oxidation opens the permeability transition pores of the mitochondria by making the hepatocytes and brain cells susceptible to apoptosis (Mather and Rottenberg, 2000; Minana et al., 2002). Another cue for apoptosis is the increased release of cytochrome *c* in the heart of aged rats (Phaneuf and Leeuwenburgh, 2002).

Neurogenesis in the rat hippocampus is frequently followed by apoptotic death; in particular, newborn cells may die before they mature (between 6 and 28 days after birth,

Cameron et al., 1993; Dayer et al., 2003). Almost half of the newborn cells have been also recognized to be apoptotic and the majority are located in the neurogenic zones of the rat hippocampus (Gould et al., 1990; Bengzon et al., 1997; Biebl et al., 2000). Glial cells also undergo apoptosis (Dalmau et al., 2003), indicating that apoptosis may occur as a consequence of competition for energy and space resources between neurones (Cowan et al., 1984) and astrocytes (Reynolds and Wilkin, 1988; Krueger et al., 1995).

Stress and ageing may be some of the causes for the induction of apoptosis (Sapolsky et al., 1985b; Sapolsky, 1996; Lucassen et al., 2001; Schulpis et al., 2001; Phaneuf and Leeuwenburgh, 2002). Excess levels of corticosterone may cause an increase in glutamate release (Gilad et al., 1990; Moghaddam et al., 1994), resulting in elevated Ca^{2+} concentrations which could lead to apoptosis via the generation of oxygen free radicals (Reagan and McEwen, 1997). ADX has been shown to enhance the expression of tumor gene p53, which promotes cellular death when DNA damage is severe and may lead to the formation of mutated DNA (Reagan and McEwen, 1997), additionally ADX increases cell death (Sloviter et al., 1993b; Cameron and Gould, 1994).

In chicks, studies have shown that apoptosis takes place in the HVC (High Vocal Center) during song learning by adult songbirds (Alvarez-Buylla and Kirn, 1997) and it has been suggested that older neurones die in favour of newborn neurones which will become incorporated into the brain and participate in the formation of new memories and synaptic remodelling.

CHAPTER 2

MATERIALS

AND

METHODS

In this chapter the methodological details are provided of the two different aspects of studies on plasticity after passive avoidance training in the chick brain. The first part describes synaptogenesis where the objective was to investigate the formation of new synapses after passive avoidance training and the second part describes a cell proliferation project where the number of new born cells was counted and then characterized for cell type in each area of interest.

Animals

Commercially obtained Ross Chunky eggs (domestic chick-*Gallus domesticus*) were incubated and hatched in our own brooders until 18±6 hours old. Chicks were placed in pairs in small aluminium pens illuminated by red bulbs at a temperature of 25-30^{0}C. One of the birds was marked with a black marker on the head so that it could be distinguished from the other. The animals were then left undisturbed for 45 minutes so that they could familiarize with each other and adapt to the novel environment.

2.1 SYNAPTOGENESIS

2.1.1 Animal training

The animals were separated into three groups, naïve (undisturbed, developmental control), water-trained (appetitive task) (W) and methylanthranilate-trained (MeA). Chicks were first pretrained by being presented a small white bead 3mm in diameter 3 times with intervals of 5 minutes between presentations. Animals that successfully pecked the bead were noted as 'peck' whilst those that did not peck were noted as 'no peck'. In order for the chicks to be included in the study, they should have pecked the white bead 3 times. Ten minutes after the last presentation of the white bead, a chrome bead 4mm in diameter that was either dipped in water or in methylanthranilate (MeA) was presented to the animals (training). Chicks peck

once and if they have tasted MeA they should exhibit a disgust response by shaking their heads, emitting stress sounds and beating their beaks on the ground. Chicks are tested 6 hours (naïve group n=7, water group n=5, MeA group n=5) (it is known that between 5.5 and 8 hours there is a second wave of glycoprotein synthesis, involving neural cell adhesion molecules, which are invilved in the formation of long-term meory, Rose, 1995b) or 24 hours (naïve group n=6, water group n =6, MeA group n=5) after training by the presentation of a dry chrome bead.

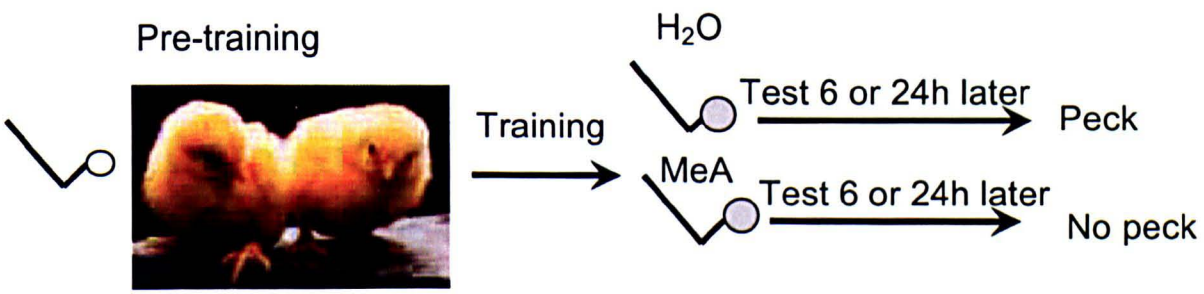


Fig. 2.1. Representation of passive avoidance learning (PAL). One-day old chicks were pre-trained by the presentation of a white bead, then trained by the presentation of a chrome bead dipped either in water or methylanthranilate (MeA). Six or twenty four hours after training, animals were tested by the presentation of a dry chrome bead and their response was recorded.

If they had tasted water during the training, they should have pecked the dry bead whilst if they had tasted MeA they should have remembered the bitter taste and therefore avoid the chrome bead. Animals that failed to remember the task (~20%) were excluded from the study. All experimental procedures took place under UK Home Licence and were also in agreement with the European Communities directive (86/609/EEC) for the care and use of laboratory animals.

2.1.2 Tissue fixing and postfixing

Animals were anesthetized by intraperitoneal (i.p) injection with 0.2 ml of sodium pentobarbital after testing. They were then transcardially perfused with heparin in 0.9% saline

to avoid blood clotting followed by 20 ml of 3.75% acrolein in 2% paraformaldehyde in 0.1M PB, pH=7.4 (speed 33 rpm/min, 8.25ml/min), which helps to preserve the ultrastructure of the tissue. Then 150ml of 4% paraformaldehyde was perfused with a perfusion pump at speed of 30 rpm/min. Brains were removed from the skull and were postfixed at 4^0C overnight in the same fixative (Dong et al., 2003).

2.1.3 Tissue processing

The brains were washed in 0.1 M PB for 10 minutes and the cerebellum was removed with a razor blade. A hole was made along the horizontal axis in the left hemisphere with a syringe needle and the brains were then stuck with cyanoacrylate to the base of a vibrating microtome (VT 1000, Leica, UK) and dipped into a bath filled with 0.1 M PB pH=7.4. The brains were cut at 100 μm thickness with the use of a blade at speed scale 6 (1mm/sec) and frequency scale 10 (100Hz). All the sections of the levels of the brain that contained the hippocampus (Hp) were kept sequentially in 6-well plates filled with 0.1M PB. They were then transferred into cryoprotectant solution (keeps the tissue from freezing) and kept in the freezer at -20^0C until they were processed for electron microscopy (Dong et al., 2003).

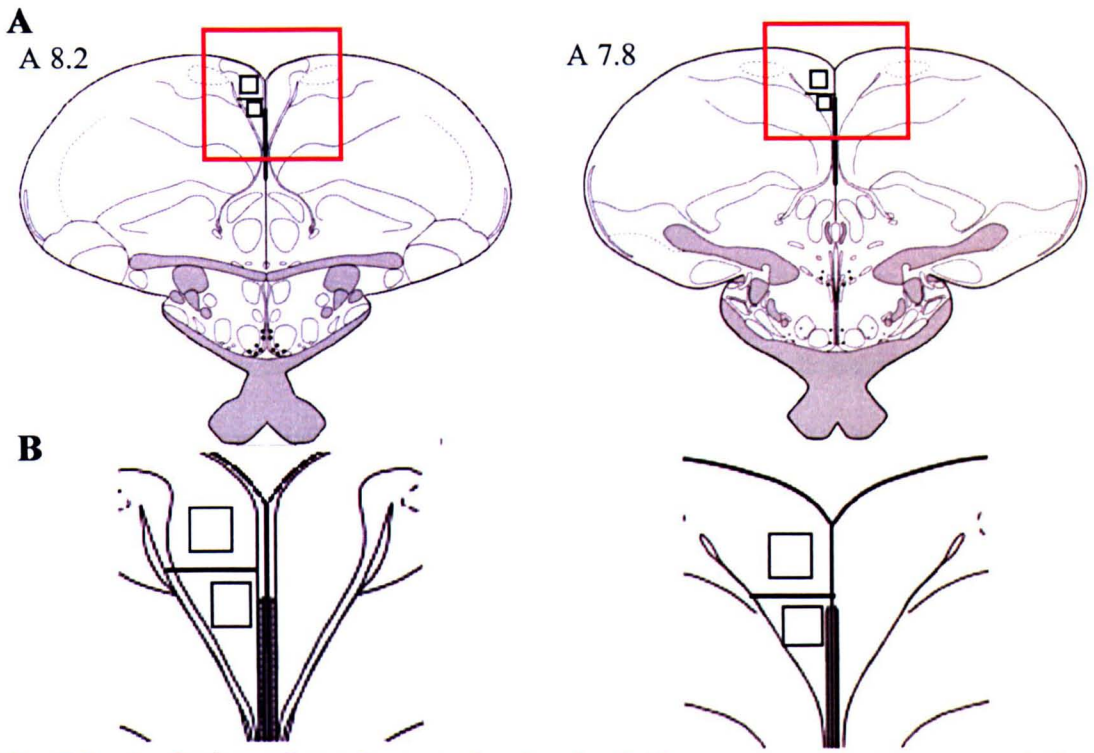


Fig 2.2. A. Series of sections at the level of hippocampus that were used for electron microscopy studies. Small squares show the areas examined in the ventral and dorsal Hp. B. Enlargement of the dorsal and ventral Hp.

Two sections of each experimental sample (antero-posterior coordinate A.7.8 and 8.2, fig.2.2) were removed from cryoprotectant solution and washed in PB 0.1M for 15 minutes before they were further processed. The sections were flattened in 0.1M PB in Coor's dishes and then the PB was removed using glass pipettes and was replaced with 2% osmium tetroxide in 0.1M PB. The dishes were covered and were left to incubate in a fume cupboard for 1 hour. Osmium was then removed and the sections were rinsed by aspiration in 0.1M PB three times 3 minutes each. The tissue was dehydrated with a graded ethanol series of 30%, 50%, 70%, and 95% ethanol for 5 minutes each. Sections were then transferred to capped vials and dehydration continued with 100% ethanol plus molecular sieve twice for 10 min each, next propylene oxide followed twice 10 minutes each time and after that sections were incubated in 1:1 propylene oxide:Epon overnight at room temperature rotating. Next day the solution was replaced with fresh 100% Epon and the sections were further rotated for 2 hours. Trays with

fluorhalocarbon film (Aclar) on which the sections were going to be flat embedded were thoroughly cleaned with alcohol. The sections were then transferred onto Aclar with flattened cocktail sticks. The excess Epon was removed from sections with tissue by pressing carefully on tissue and bubbles of Epon were removed from the edges of the tissue with cotton buds. Five drops of Epon were dropped among tissue and then the sections were covered with clean Aclar that served as cover. Aclar was pushed hard to remove bubbles from the tissue and the excess that came out from the edges was wiped off with tissue. Heavy weights were put on top of the tissue and the sections were put in oven at 60^0C for 48 hours to polymerize.

After two days the sections were removed from the oven and the top cover of the Aclar was carefully removed. With the use of a stereoscope, the hippocampus was divided into ventral and dorsal (fig 2.3, Székely and Krebs, 1996; Székely, 1999).

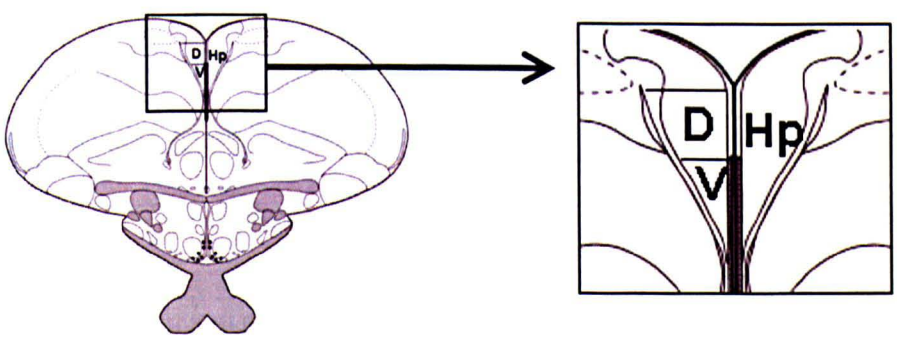


Fig. 2.3. Division of ventral and dorsal hippocampus (Hp) is shown, antero-posterior coordinate 8.2 mm to ear bars.

With a scalpel blade the areas of the hippocampus were removed and the small pieces of processed tissue were stuck separately onto numbered blank Epon blocks with cyanoacrylate. The numbers were coded so that the investigator was blind to the origin of the blocks and this was similar in all further experiments described in this thesis. The blocks were then put in the oven at 60^0C overnight so that the tissue pieces were firmly stuck to the resin stubs.

2.1.4 Tissue cutting and staining for electron microscopy

The blocks with tissue were cut on an ultramicrotome (Leica, UK). The excess Epon around the tissue was removed with a razor blade and the tissue cut into a mesa (perfect square created with a glass knife) so that when the tissue was then cut with a diamond knife, the sections adhered to each other in a perfectly aligned ribbon. The mesa is first aligned with the diamond knife and then the knife bath is filled with water. Two hundred nm thick sections were cut in order to determine if they were at sufficient depth in the tissue and then ribbons of 70 nm thick sections were cut and collected carefully with the use of an eyelash mounted on a cocktail stick on carbon coated thin film made of 2% formvar in chloroform which rests in the middle of Cu/Ni slots. The slots were left to dry and then placed in a grid box until stained.

The slot holder, where slots were placed to be stained, must be very clean in order not to leave any dirt on the film or the sections. The sections were first placed in uranyl acetate in dark for 30 minutes, washed throughout 3 times in distilled water and then dipped into Reynolds lead citrate surrounded by NaOH pellets for 7 minutes. Sections were again washed and dried with filter paper.

2.1.5 Electron Microscopy

The beam current was normally set at 74 μA which was required for thickness estimation, spot size was 2, condenser aperture was set to 200 μm, the objective aperture was set at 50 μm and the current density was set at 80 KV. The slot holder takes two grids, the first of which was the diffraction grating for magnification check (2160 lines per mm) whilst the other was the grid for analysis. Images were collected from the electron microscope (EM) via a Gatan BioScan camera. The eucentric height of the diffraction grating was adjusted at magnification of 12K. The section was focused by using the image wobbler. The next step was

to acquire a digital image at 12K, which was then analyzed by an image analysis software for Macintosh computers (NIH image).

In order to determine section thickness, the section of interest was located at low magnification (x300). The thickness estimation is based on the difference in electron scattering in pA/cm^2 of unexposed areas of the support film and of the support film plus resin without tissue (De Groot, 1988). The relative electron transmission (RET) at a magnification 12K was used to measure the pA/cm^2 of the film close to the edge of the section. adjusted to 50 ± 0.5 on an unexposed area and take at least two readings. Then, five readings from capillaries are taken. To ensure that the area was truly unexposed the pA/cm^2 should increase at least 5. In order to calculate the thickness of the section it is necessary to calculate the RET from the formula: $RET = ES_{section}/ES_{film} \times 100$. A standard curve (fig. 2.4) was created for RET for sections of different thicknesses. Small's minimal fold technique (de Groot and Bierman, 1986; De Groot, 1988; Tigges et al., 1996) is a direct measurement of section thickness from deliberately introduced folds.

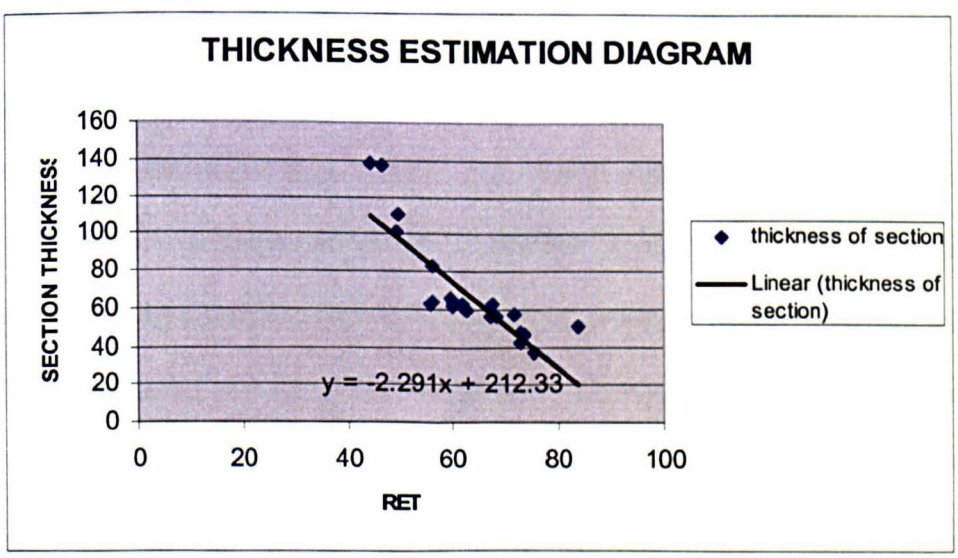


Fig. 2.4. Thickness estimation diagram made by Small folds from sections 40nm to 120nm thick. The equation is used to calculate the thickness of a section where x = RET

At low magnification, the sections from which the images of disectors are going to be obtained are located and the black dot is placed on some point of the section that is going to serve as the 'look up' section (usually close to the top edge or top side). This place is saved with the use of a specimen relocation system, Deben Sprite computerized stage controller. Then, I move to the next section of interest that is going to be the 'reference section', and the same place is located on this section and saved. The eucentric height is set (it must be set every time a new slot is analyzed) and an image of the look up section is taken. Then, with the use of Gatan Digital Micrograph v 3.4.4 software, an image at the same point of the reference section is captured. These two sections are a pair of sections that make up the disector pair. This procedure is repeated until 20 disector pairs are acquired.

2.2 NEUROGENESIS

2.2.1 LIGHT MICROSCOPY

2.2.1.1 Animal training

The pretraining was the same as described above for the synapse counting experiment. Immediately after the pretraining, control and trained animals are injected intraperitoneally with a single dose of a BrdU normal saline solution (0.1mg/g of body weight; Sigma, UK). Animals were left for 30 minutes and then trained as described before. Animals were tested 24 hours after the training and sacrificed immediately after training (control n=6, water trained n=8, MeA trained n=8), and 9 days (control n=5, water trained n=5, MeA trained n=5) post BrdU injection. The animals sacrificed at 9 days after injected with BrdU were taken back to the brooders after having spent 48 hours in the pens, under the care of the Animal House personnel.

2.2.1.2 Tissue fixing and postfixing

Animals were anesthetized by being injected with 0.2 ml to 0.4ml of sodium pentobarbital depending on body weight. They were then transcardially perfused with heparin (600 USP/l) in 0.9% saline to avoid blood clotting followed by 120 ml to 200 ml (determined by age and body weight) of 4% paraformaldehyde in 0.1M PB, pH=7.4 (speed 30 rpm/min, 7.5ml/min). Brains were removed from the skull and were postfixed at 4^0C overnight in 4% paraformaldehyde and 10% sucrose for cryoprotection. Next day the solution was replaced with 20% sucrose in 0.1M PB, pH=7.4 and the brains were further cryoprotected overnight. Then the brains were frozen in a beaker containing isopentane that was surrounded by dry ice to keep temperature around -50^0 C. The brains were dipped in isopentane for 20 minutes with constant agitation to avoid sticking to the walls of the beaker and then wrapped in foil and kept in a -80^0 C freezer until immunocytochemistry (de Groot and Bierman, 1986).

2.2.1.3 BrdU immunocytochemistry

Brains of animals sacrificed 24 hours and 9 days post BrdU injection were cut using a cryostat microtome (Leica, UK) and 50μm thick free-floating sections were collected in phosphate buffer (PB 0.1M, pH=7.4) (de Groot and Bierman, 1986). In a pilot study, the sampling distance was determined taking into consideration the fact that the sections should be a safe distance apart to avoid sampling of the same cell profile twice. Furthermore, the optimum sampling distance depends also on the size of the telencephalon which in turn is determined by the developmental stage (Bolam, 1992). Therefore, in this case one series of sections every 300μm (1/6) was used for BrdU immunocytochemistry. Sections were kept from the point where the Hp first appeared.

Sections were incubated for 2h at 65 ^{0}C in 1:1 formamide/2XSSC, rinsed in 2XSSC for 5 min and then incubated in 2N HCl at room temperature for 30 min to denaturate DNA.

Tissue was rinsed 3x5 min and 1x10min in 0.1M PB and then was transferred in 3% (v/v) hydrogen peroxide in 0.1M PB, pH=7.4 for 10 min to block the endogenous peroxidase. Three washes in 0.1M PB followed and then the tissue was blocked with 1.5% (v/v) horse normal serum, 0.1% (v/v) Triton X-100 in 0.1M PB blocking solution for 20 min at room temperature. The sections were incubated overnight at 10 ^{0}C with a mouse anti-BrdU monoclonal antibody solution (Becton Dickinson, UK) diluted 1:100 in 0.1% Triton X-100 in 0.1M PB, pH=7.4. On the second day, the sections were rinsed in 0.1M PB 3x5 min and then incubated with a biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories, UK) diluted 1:200 in 0.1M PB for 2h at room temperature. Tissue was again rinsed in phosphate buffer and then transferred in an avidin/biotin/peroxidase solution (Vector Laboratories, ABC kit, diluted 1:50 A and 1:50 B in 0.1M PB, pH= 7.4) for 1h in the dark at room temperature. Tissue was rinsed successively in 0.1M PB, pH=7.4 and 0.1M Tris buffer saline, pH=7.5. BrdU containing cells were visualized by the polymerization of 0.05% diaminobenzidine (DAB kit, Sigma) in 0.01% hydrogen peroxide in Tris buffer saline for 2 min at room temperature. The reaction was stopped by dipping the sections in Tris buffer saline (0.1M TBS, pH=7.5) and they were next mounted on gelatinated slides and left to dry. For light microscope observation they were dehydrated in graded series of ethanol and cleared with xylene and were coverslipped using Entellan rapid mounting media (Merck, Germany).

2.2.2 IMMUNOFLUORESCENCE

All of the steps of the first day including the step of blocking were repeated for fluorescence immunocytochemistry. The sections were incubated overnight at 4 ^{0}C with a rat anti-BrdU monoclonal antibody (Abcam, UK, diluted 1:100 in 0.1% Triton X-100 in 0.1M PB). On the second day, after rinsing the tissue 3 times in 0.1M PB, sections were incubated with Alexa Fluor 647 goat anti rat IgG secondary antibody (to bind onto the rat a-BrdU)

diluted 1:200 in 0.1M PB for 2h at room temperature to visualize BrdU positive cells. Next, the tissue was washed in 0.1M PB 3x10 min and then incubated in 2% goat normal serum, 0.1% Triton X-100 in 0.1M PB and finally incubated for 48h at 4 ^{0}C with a marker for mature astrocytes, glial fibrillary acidic protein (GFAP, diluted 1:500), or neuronal nucleus marker (NeuN, diluted 1:1000) (all from Chemicon International,UK), 0.1%Triton X-100 in 0.1M PB, pH=7.4. The sections were afterwards rinsed in phosphate buffer and after that they were incubated in an Alexa Fluor 488 goat anti mouse IgG1 secondary antibody (to bind onto mouse anti-NeuN or mouse anti-GFAP). In order to detect non-specific labelling, adjacent sections were incubated in the absence of the primary or the secondary antibody and in each case there was no detectable labelling. Then the tissue was throughout rinsed and the sections were mounted on gelatinated slides and left to dry in dark at room temperature. Then, they were coverslipped using fluoromount mountant (BDH, UK) and stored in the fridge.

2.3 QUANTIFICATION

2.3.1 SYNAPTOGENESIS

2.3.1.1 Stereology

i) Disector

Two sections separated by one section from each block were chosen for estimation of synapse density (number of distinct synapses in a unit reference volume) using the 'disector' technique (Sterio, 1984). The disector can take a 3-dimensional measurement in space without taking into account the volume, the shape or height of the object or particle being counted. The idea of the physical disector is to take two serial sections a known distance apart, with an unbiased counting frame on the reference section, transects (2D profile or set of profiles through a particle, -a particle is a discrete three dimensional object in 3D space, whilst a profile is a 3D object cut by a 2D section) are counted if seen in one section and not in the

other. In reality the disector is a clever strategy for counting the number of times a continuous scan would have hit particles for the first time. In stereology it is important to create random samples that are related to spatial position. Uniform random sampling means that every object has the same likelihood of being sampled as all other elements. Selected areas were used for synaptic density estimation that occupied one third of the disector image captured by the Gatan camera. The mathematical type for calculation of the numerical density (N_v) of synapses is:

$$\mathbf{Nv}_{\mathbf{synapse}} = \Sigma \mathbf{Q}^{-}_{\mathbf{syn}} / \mathbf{t.A}$$

where ΣQ^{-}_{syn} is the total number of counted synapses in the sections, t is the section thickness (distance between the two sections) and A is the area of the counting frame see fig. 2.5).

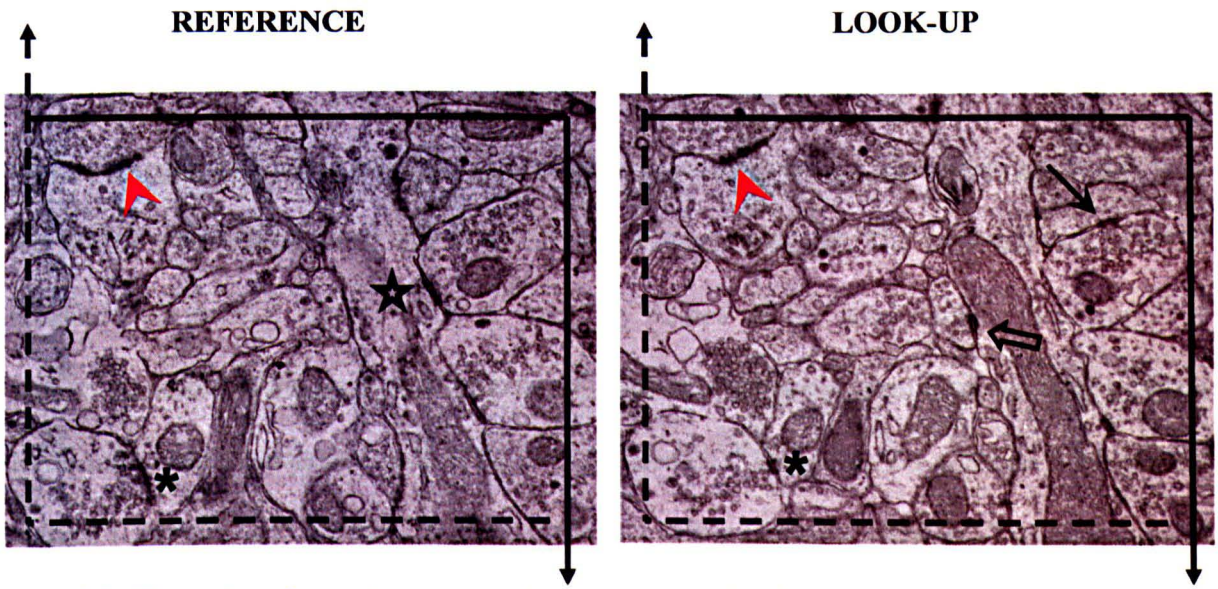


Fig. 2.5. Example of two images used for synapse density estimation with the disector method. The image on the right is the 'look-up' image whilst the left is the 'reference' image. Only synapses that are located within the borders of the lines are counted. The dashed lines are the forbidden lines, so if a synapse touches the dashed lines is not counted. An asymmetric spine on a dendrite is marked with an asterisk in both images so it is not counted. The block arrow in the look up section indicates an asymmetric synapse onto a dendrite which is counted since it does not appear in the reference section. The black arrow indicates a symmetric synapse onto a spine (look up section). The red arrowheads show an asymmetric synapse onto a spine in both images and therefore are not counted. In the reference image the star indicates a symmetric axodendritic synapse. In this case three synapses would have been counted, one symmetric axodendritic and one asymmetric axodendritic synapse in the reference section and one symmetric axospinous in the look-up section.

ii) Synapse counting

Synapses are counted only if they exist in one of the two sections. They are not counted if they touch the forbidden lines (left side and bottom lines, fig 2.4). They are identified as 'asymmetric shaft' if the postsynaptic density (PSD) is thicker at the postsynaptic side and if the postsynaptic part is a dendrite and 'asymmetric spine' if the postsynaptic structure is a spine. Symmetric shaft and spine are synapses where both the pre- and postsynaptic parts have the same density. In general a synapse is identified by the pre- and postsynaptic apposition zones and the presence of at least 3 synaptic vesicles at the presynaptic element.

2.3.2 CELL PROLIFERATION

Cell profile counting was confined within neuroanatomical borders of the chick forebrain regions as well as the ventricular zone adjacent to there areas where applicable with the use of a stereoscope and the areas of interest were outlined. The total number of BrdU labelled cell profiles was counted in counting frames of the total surface throughout the section thickness of the total areas of interest. Images of the areas were captured from a Nikon E800 microscope connected with a Pentium IV personal computer via a Nikon 5 Megapixel camera (DX1200) and the images were measured using analySIS software. The total number of cells was divided by the whole area of each region and the ratio of the number of cells/area (mm^2) calculated. At the same time the volume of each area can be calculated if the area of each anatomical structure is multiplied with the thickness of the section (50 μm).

2.3.3 IMMUNOFLUORESCENCE

For immunofluorescence five images/section of a known surface area were captured using Leica TCS confocal microscope from 5 serial sections of each brain and all the BrdU

positive cells in the hippocampus and area parahippocampalis were counted and then identified if they presented double labelling. Percentages were determined by dividing the number of each type of double labelled cells (ND), by the total number of BrdU positive cells present in the brain (NB) which is then multiplied by 100,

$$\%=(ND/NB) \times 100$$

All the measurements were made per mm^2.

2.3.4 VOLUME ESTIMATION

The estimation of the volume of the individual areas is very important, because it will reveal any volume changes caused by stress. These volumes can be calculated using the Cavalieri method (Oorschot, 1996; Wulfsohn et al., 2004). From the experiments used for cell proliferation, the total area of each anatomical structure area is known since it has been measured with the use of analySIS software. The total area is then multiplied with the thickness of the section, which is 50μm, and then multiplied x6, since the sampling distance used is one section every 300 μm (1/6). The equation for total volume estimation for each area in mm^3 is

$$V_{area} = \text{total area } (mm^2) \times 0.05mm \times 6$$

2.3.5 STATISTICAL ANALYSIS

Three- and four- way analyses of variance (ANOVA) for cell proliferation and synaptogenesis respectively were used for testing the statistical differences between the different training groups and experimental conditions. All of the analyses were conducted using STATISTICA 6.0 software and values $P<0.05$ were taken as statistically significant. If the P value was less that 0.05, then a least significant difference (LSD) Fisher test was

performed to specify the differences present. Again, formal statistical significance was set at $P<0.05$

2.4 CORTISOL STUDIES

2.4.1 Animal training

Animals (n=80) were pre-trained as described in part 2.1 i. Five (n=40) and twenty minutes (n=40) after pre training, the animals were decapitated and the hippocampus, striatum mediale and arcopallium were immediately dissected with a scalpel blade and the removed tissue was transferred in foil and frozen in isopentane kept in dry ice. The procedure of tissue dissection needed to be fast in order to avoid tissue decomposition. Samples were kept in -80^0C until cortisol extraction studies were performed.

2.4.2 Cortisol extraction

Cortisol extraction was performed as described in de Jesus et al. (1991). In particular, tissue samples were homogenized in 5 times v/w phosphate buffer saline (PBS, 0.01M, pH=7.3, containing 0.14M NaCl). 250 μl of the homogenate were extracted twice with diethyl ether by strong vortexing for 1 min. The aqueous phase was frozen at -80^0 C in isopentane and the ether layer was transferred to another tube. Combined extracts were dried in a water bath at 45^0C in an atmosphere of nitrogen. Extracts were resuspended in 250 μl PBS containing gelatin (0.1%) and 100 μl aliquots (in duplicate) were used for radioimmunoassay (RIA). Extraction efficiencies were monitored for each sample by addition of tritiated cortisol to homogenates extracted in the same manner as samples that were used for RIA. The average recovery for the samples was 92%.

2.4.3 Radioimmunoassay (RIA)

Cortisol was measured by radioimmunoassay (Sufi et al., 1994). Samples were analysed in duplicate in 100 µl of homogenate. The first step of the procedure is the preparation of reagents (tracer, antiserum and dextran-charcoal reagent). The working tracer solution is prepared by removing 150 µl from the stock solution, pouring the solution into a tube and then evaporating the solvent. The solution is then redissolved in 15 ml of assay buffer for 30 minutes. This solution contains 3.7 KBq/ml (100 nCi/ml) of tritiated cortisol (1,2,6,7-[^{3}H] cortisol) (Amersham). The antiserum (generated in rabbits against cortisol-21-hemisuccinate-BSA, Chemicon, UK) is prepared by mixing the contents of one antiserum vial with Buffer S and allowing them to stand for 10 minutes. The antiserum used cross-reacts with cortisol (100%), cortisone (<0.1%), corticosterone (9.2%), 11-deoxycortisol (27.1%), progesterone (0.8%), 17a hydroxyprogesterone (0.8%), 11a hydroxyprogesterone (0.07%) and testosterone (0.08%) measured at 50% zero binding. The standards were then prepared by setting up a rack of 5 x10ml test tubes in which 1ml of buffer S is poured. Solution B is prepared from 100 µl of ethanol mixed with 10ml of Buffer S. Solution B is heated at 40^{0}C for 30 minutes and is then left to cool at 4^{0}C before use. Solution B contains cortisol at a concentration of 60nmol/l. In tube 1 1ml of solution B is added with a pipette; then by using the same pipette and tip, 1ml of solution from tube 1 is transferred into tube 2. The solution in tube 2 is mixed carefully and then 1 ml is removed from tube 2 into tube 3 and the content is well mixed. The same procedure is continued until 1ml of tube 4 has been transferred into tube 5.

This assay is for 100 tubes which should be arranged as:

a) tubes 1-2: total counts tubes (TC)

b) tubes 3-4: non specific binding tubes (NSB)

c) tubes 5-6; 49-50; 99-100: zero antigen tubes (B0)

d) tubes 7-18: standard tubes

e) tubes 19-98: unknown samples including sets of quality control samples

In TC tubes 100 μl of the working solution of 1,2,6,7-[^{3}H] cortisol and 400 μl of Buffer S are added. In these tubes no charcoal reagent will be added after the end of the incubation. In NSB tubes 100 μl and 600 μl of buffer (PB 0.1M) are added. In B0 tubes, 500 μl of buffer (PB 0.1M), 100 μl of tracer and 100 μl of antiserum are added and finally in the standard and unknown sample tubes 100 μl of standard or sample, 100 μl of tracer, 100 μl of antiserum and 400 μl of Buffer S are added. The tubes are incubated overnight (18 hours) at 4^0C and then 200 μl charcoal reagent is added where applicable. The tubes are vortex mixed and left to stand for 30 minutes at 4^0C. Then, the tubes are centrifuged at a minimum of 1500g for 15 minutes. After centrifugation, the supernatant was carefully transferred into scintillation vials. In order to avoid drift at the separation stage, it is important to keep all the tubes at 4^0C when adding charcoal and the addition must take place rapidly so that the time of contact of the incubation medium is not too different across the assay.

Scintillation cocktail is added in every scintillation tube and the tubes are left to stand for 1 hour and are then transferred to the counter, where the scintillation counting occurred for each sample. The results were calculated from a dose-response curve of plots of bound counts vs. log dose. Results are presented in ng g^{-1} brain, following recovery correction.

RESULTS

CHAPTER 3

SYNAPSE FORMATION

IN THE CHICK HIPPOCAMPUS

6 AND 24 HOURS

AFTER

PASSIVE AVOIDANCE LEARNING

3.1 INTRODUCTION

As mentioned earlier, the avian hippocampus has been suggested to be homologous to the mammalian hippocampus (Kallen, 1962; Erichsen et al., 1991; Atoji et al., 2002). Previous studies have shown that as in mammals the bird hippocampus is responsible for spatial memory (Bingman et al., 1990; Regolin and Rose, 1999; Kahn and Bingman, 2004) and demonstrates synaptic plasticity in the form of LTP (Margrie et al., 1998). In the domestic chick *Gallus domesticus*, previous studies have proposed that the hippocampus is affected by the passive avoidance learning paradigm (Sandi et al., 1992; Unal et al., 2002).

The present study has examined the effects of passive avoidance training 6 hours after testing, because it has been shown that at this time point a protein cascade takes place in order for short term memory to be consolidated into long term memory (Rose, 1991, 1995a, b) when synaptic changes occur (Rose and Stewart, 1999). Furthermore, studies in the chick IMM and StM 24 hours after passive avoidance training have shown synaptic remodelling and formation in the MeA trained group in relation to controls (Stewart et al., 1987; Patel and Stewart, 1988; Hunter and Stewart, 1993; Lowndes and Stewart, 1994). The hippocampus was divided into two separate regions, ventral and dorsal, and these areas were studied individually, since previous studies from our group have shown different responses after ischaemia in these two regions of the chick hippocampus (Horner et al., 1996). In the rat brain the dorsal and the ventral hippocampus have been confirmed to play different roles in learning tasks (Moser et al., 1993; Hock and Bunsey, 1998; Moser and Moser, 1998). Here, each hemisphere was studied separately, since it is believed that chicks show hemispheric asymmetry (Stewart et al., 1987; Gagliardo et al., 2001) possibly due to the occlusion of the left eye while *in ovo* (Rogers, 1990). At the same time, comparisons were made between 6 and 24 hours in order to investigate any possible transient increases or reductions in the synaptic density among the different synapse types, since it is known by now that synapses belong to a very dynamic

system and can change their shape within seconds (Hering and Sheng, 2001). More importantly, 6 hours is the beginning of the protein synthesis required to enable structural changes necessary for long term memory formation and by 24h this process should be well advanced (Rose and Stewart, 1999).

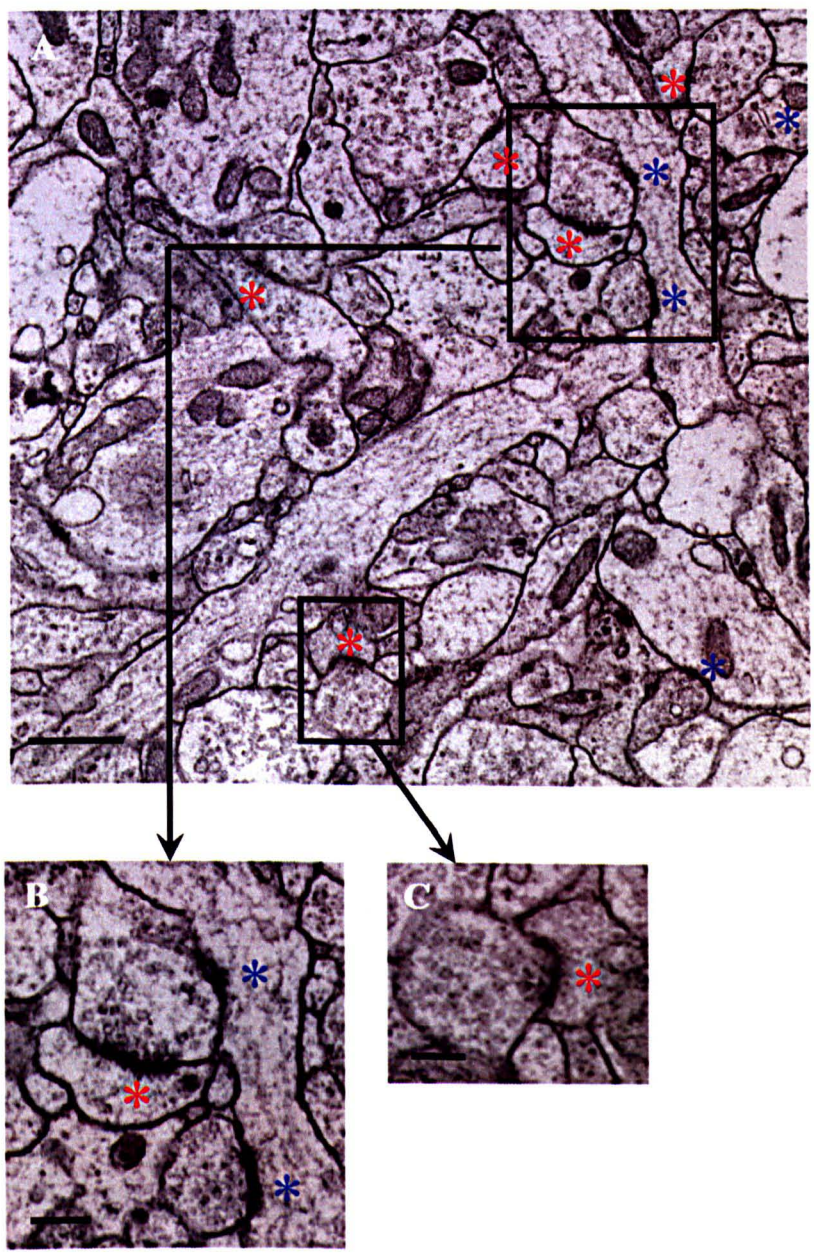


Fig 3.1. The majority of synapses in the chick hippocampus are asymmetric (presumed excitatory) as can be seen in image A. Images B and C show magnified examples of asymmetric axospinous and axodendritic synapses. Red asterisks represent axospinous asymmetric synapses, whilst blue asterisks indicate axodendritic asymmetric synapses. Scale bar in image A=1μm, in image B and C=250nm

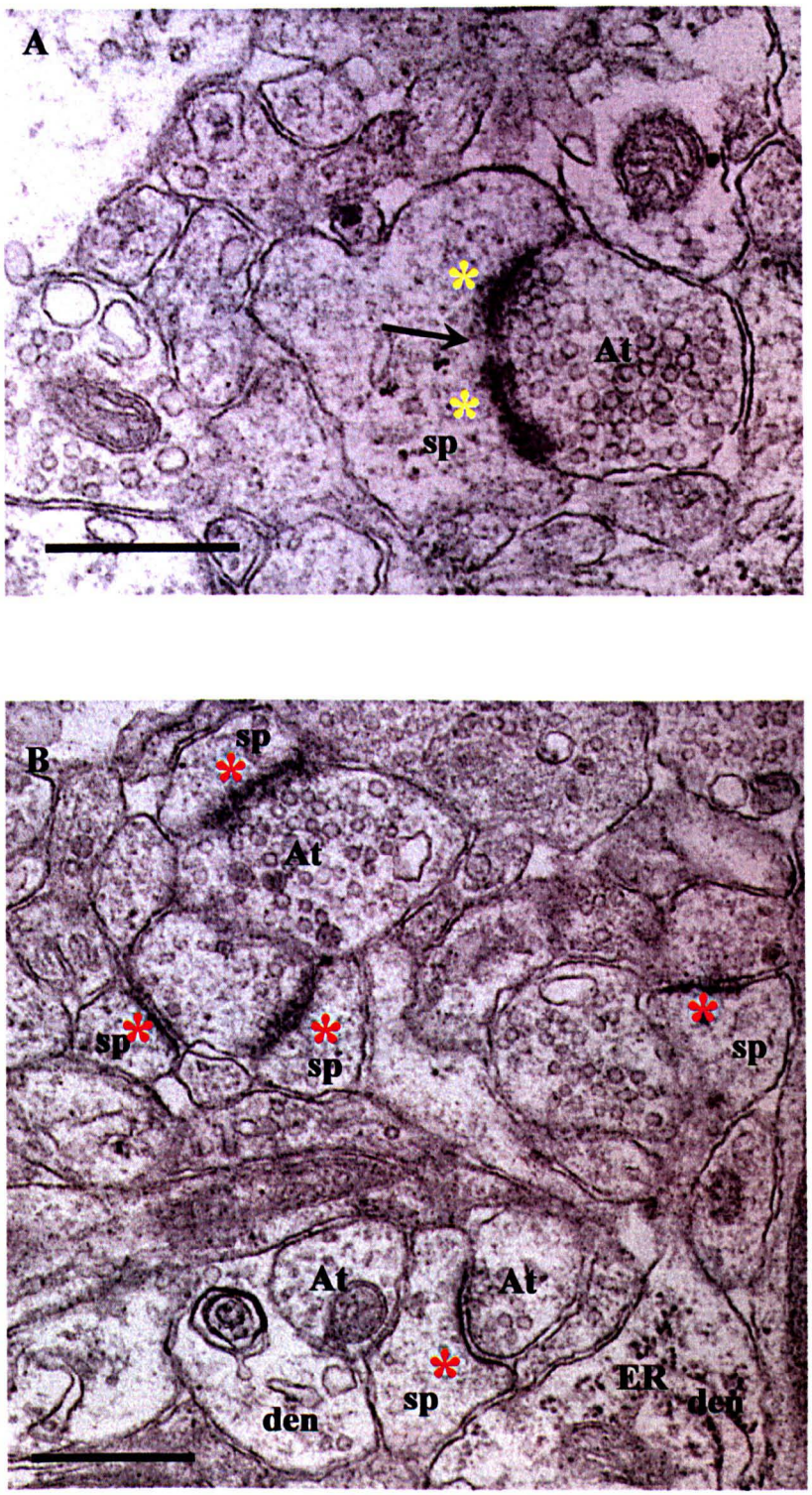


Fig 3.2. A. A perforated asymmetric synapse in chick hippocampus onto a dendritic spine (sp) is marked with yellow asterisks. In image B all the asymmetric spine synapses are marked with red asterisks. ER: endoplasmic reticulum, den: dendrite, At: axon terminal. Scale bars= 200 nm

3.2 SYNAPSE ULTRASTRUCTURE

In this study four different types of synapses were examined; asymmetric shaft, asymmetric spine, symmetric shaft and symmetric spine synapses. The asymmetric shaft or asymmetric axodendritic synapses are presented in figs 3.3 and 3.4. The asymmetric spine or asymmetric axospinous synapses are demonstrated in figs. 3.1, 3.2 and 3.5. Synapses are termed as symmetric when the pre- and the post-synaptic density have the same thickness. Symmetric shaft synapses are shown in figure 3.6.

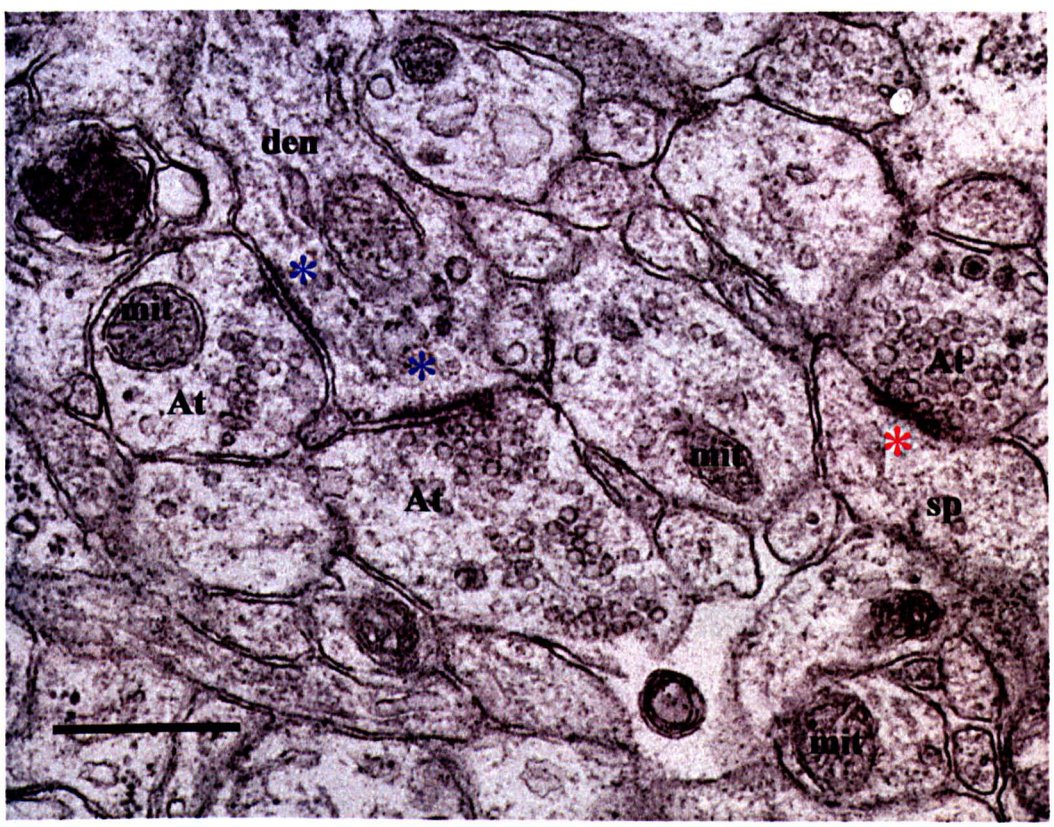


Fig 3.3. A dendrite in ventral hippocampus of a control bird 24h post training receiving two asymmetric synapses from presynaptic axon terminals (At) resulting in axodendritic synapses. Asymmetric shaft synapses are marked with blue, whilst red asterisks show asymmetric spine synapses. Mit: mitochondrion, den: dendrite. Scale bar=200 nm.

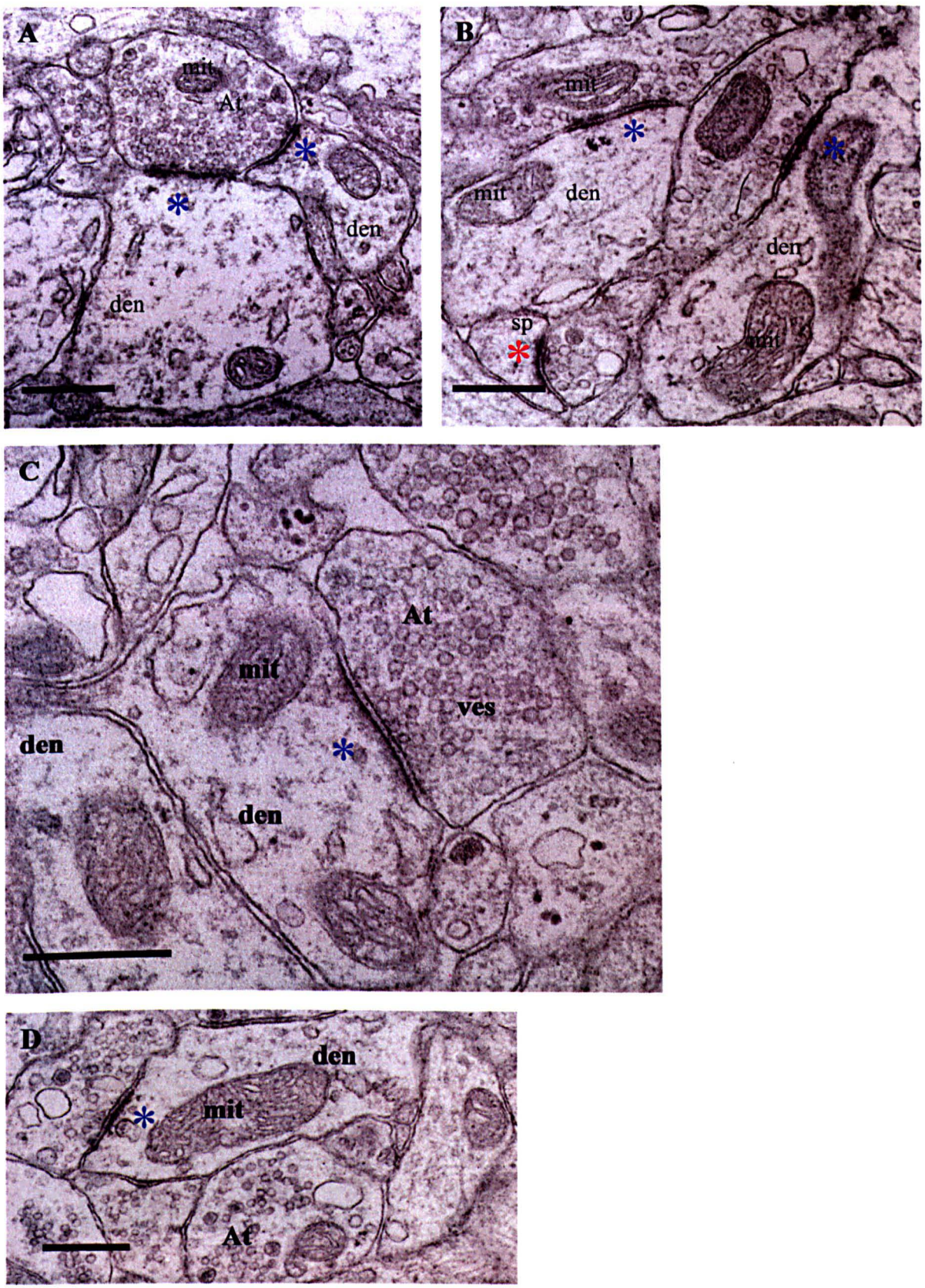


Fig 3.4. Images of asymmetric shaft synapses (on dendrites) in dorsal one day old chick hippocampus marked with blue asterisks. Red asterisk shows an asymmetric spine (sp) synapse. The presynaptic part can be clearly distinguished by the presence of vesicles (ves), den: dendrite, mit: mitochondrion, At: axon terminal, ves: vesicles. Scale bars=200 nm

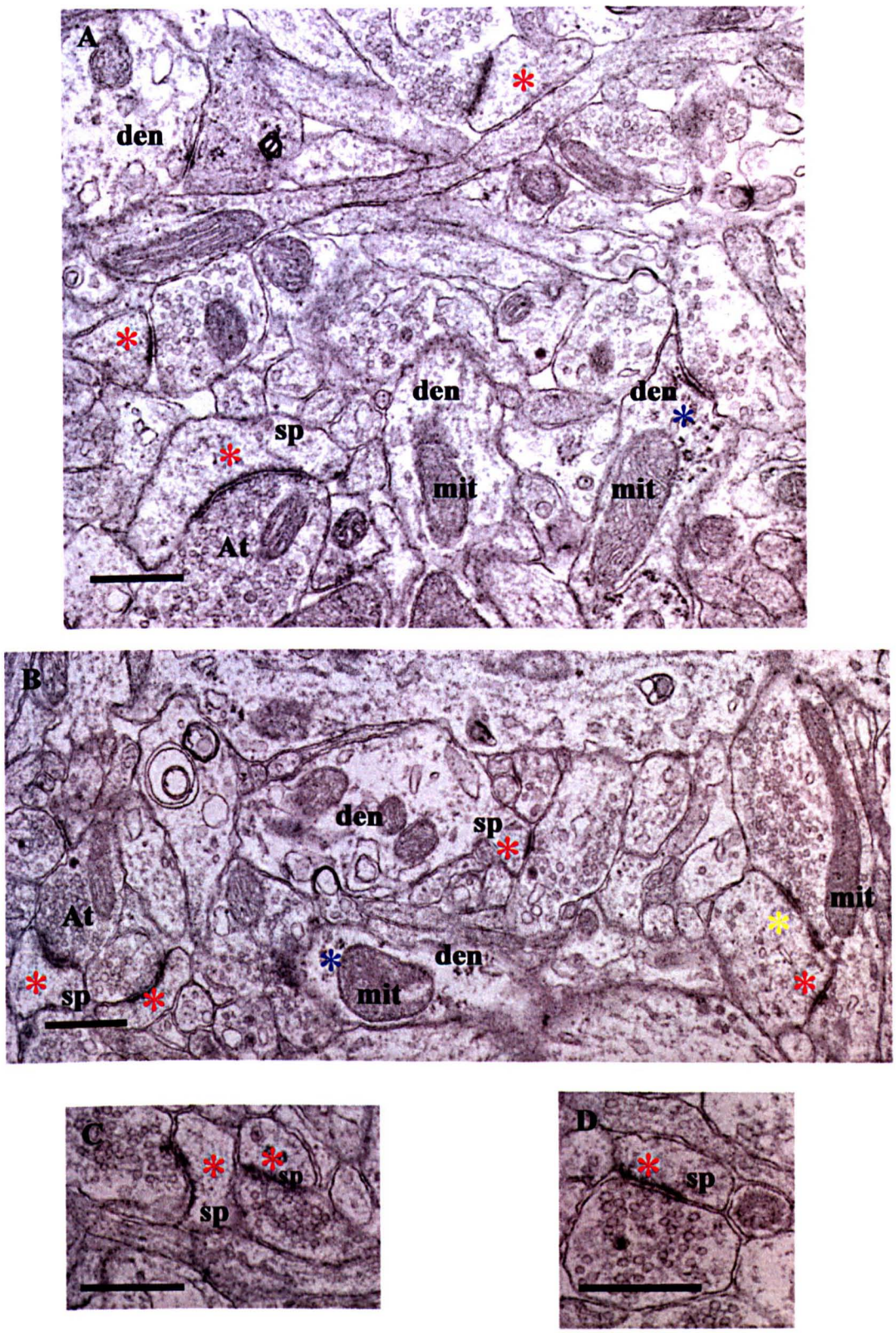


Fig 3.5. Representation of asymmetric spine synapses marked with a red asterisk from the right hemisphere of the ventral Hp of water trained group. The blue asterisk indicates asymmetric shaft synapses whilst the yellow shows a perforated asymmetric spine synapse. Den: dendrite, At: axon terminal, mit: mitochondrion, sp: spine. Scale bars=200 nm

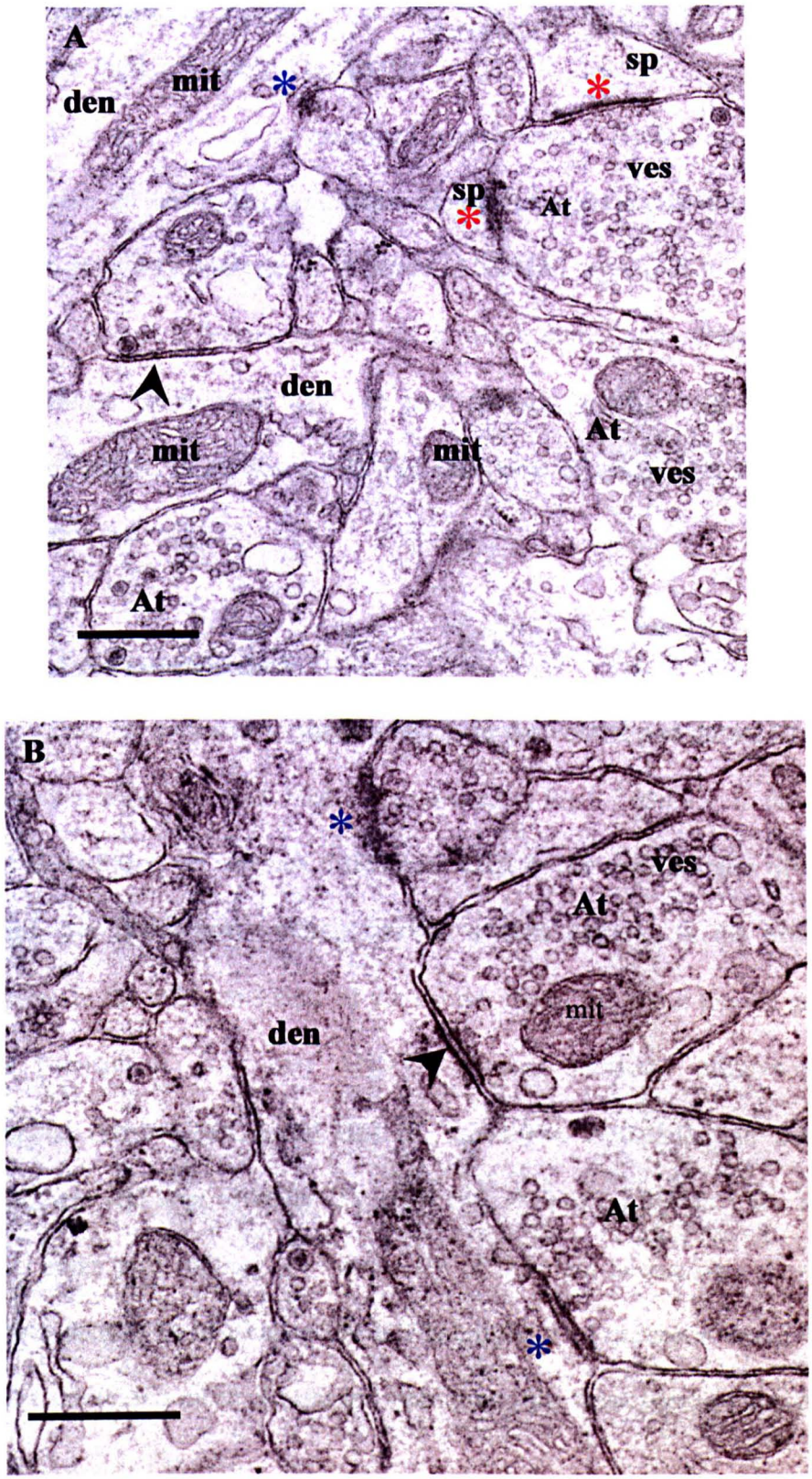


Fig 3.6. Representation of a symmetric shaft synapse marked with arrowheads from the dorsal Hp of MeA trained group. Red and blue asterisks indicate asymmetric spine and asymmetric shaft synapses respectively. Den: dendrite, mit: mitochondrion, At: axon terminal, ves: vesicles, sp: spine. Scale bars=200 nm

3.3 RESULTS

3.3.1 6 HOURS POST TRAINING

Four sets of data are presented below, ventral Hp-left hemisphere, ventral Hp- right hemisphere, dorsal Hp-left hemisphere, dorsal Hp- right hemisphere.

A four way analysis of variance (ANOVA) was used to test differences in synaptic density (dependent factor), as a result of hemisphere, area, training and time after training (comparisons between 6h and 24h post training groups were conducted which are also presented below, in section 3.3.2). For each type of synapses a separate four way ANOVA was carried out.

3.3.1.1 Asymmetric shaft

Data for asymmetric shaft synapses in the ventral and dorsal Hp 6 hours post training for control, water and MeA birds are presented in Fig 3.7 and 3.8. In the left hemisphere or the right hemisphere, no obvious differences exist in the total number of synapses between the groups. Six hours post training there is a 48% increase in the asymmetric axodendritic synapses in the left hemisphere of the ventral Hp of the water trained group in relation to the right hemisphere. Four way ANOVA showed statistical differences for brain regions examined (ventral and dorsal Hp) ($F_{1,\ 109}$=4.648, P=0.033), and the interaction between hemisphere and area of study ($F_{1,\ 109}$=5.546, P=0.02). LSD post hoc analysis demonstrated that 6 hours post training the ventral Hp of the right hemisphere shows significantly lower synapse density in relation to the left hemisphere of the same area (P=0.045) in the water trained group. No other differences were found for these types of synapses at this time point (tables 3.1, 3.2, Fig 3.7).

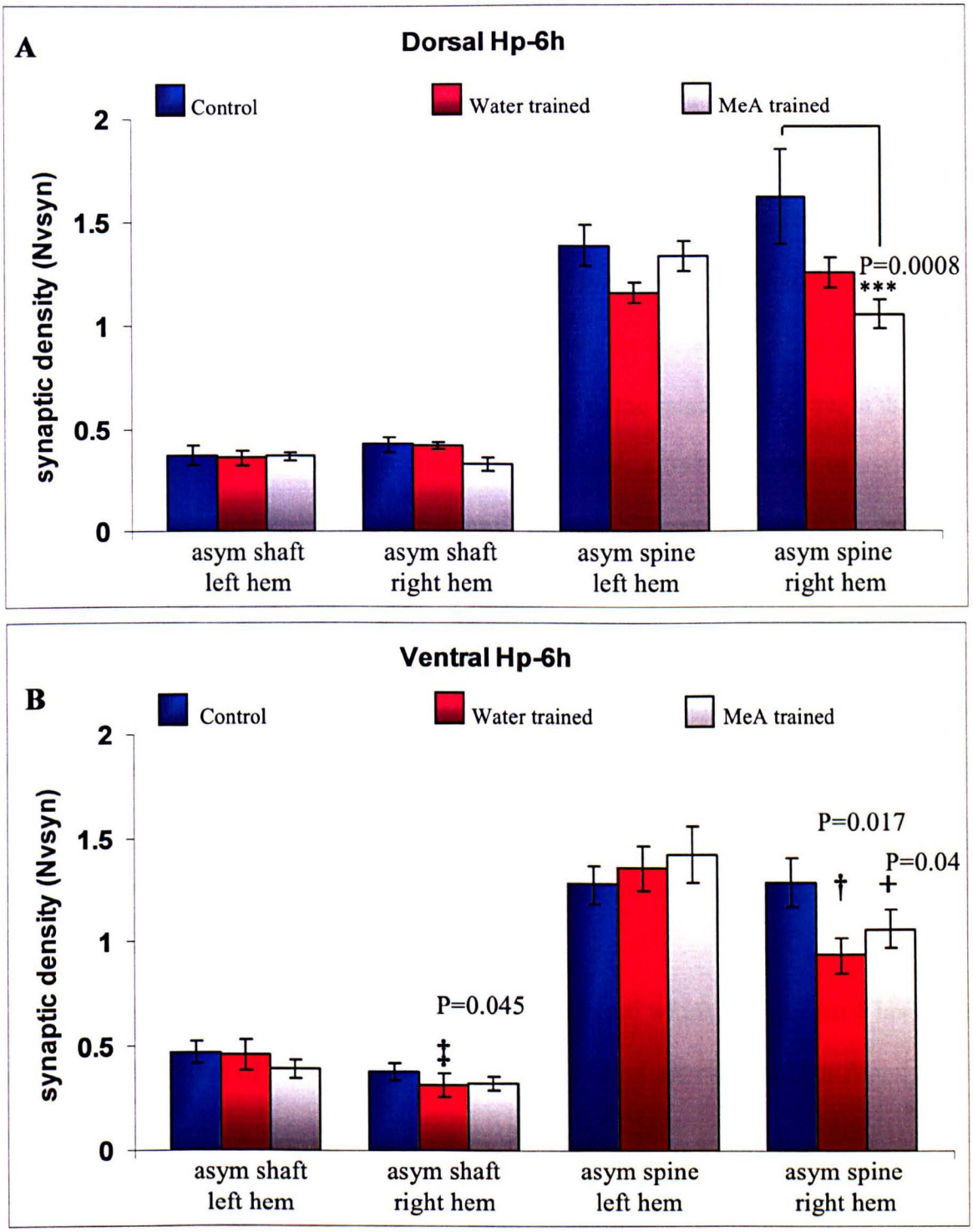


Fig 3.7. Graph showing the results for asymmetric synapse types in the ventral and dorsal hippocampus both in the right and left hemisphere (control n=6, water n=5, MeA n=5). In graph 3.7A the asterisks indicate the reduction of asymmetric spine synapses in the MeA trained group in the dorsal Hp of the right hemisphere (P=0.0008). In graph B the ‡ represents the difference between the right and the left hemisphere in the water trained group (n=5) (P=0.045) for asymmetric shaft synapses, whilst the † and + show the differences of right and left hemisphere of water and MeA trained groups for asymmetric spine synapses (P=0.017 and P=0.04 respectively). Columns represent means of data and vertical bars show standard error means (S.E.M).

3.3.1.2 Asymmetric spine

Data for asymmetric axospinous synapses are demonstrated in Fig. 3.7. In the ventral Hp, there is a 44% increase in asymmetric axospinous synapses in the left hemisphere of water trained birds in comparison with the right hemisphere. Additionally, in the ventral Hp of the left hemisphere, there is a 33% increase for this type of synapses in the MeA trained group in relation to the right hemisphere. In the right hemisphere, the dorsal Hp of the control group shows a 26% increase in comparison with the ventral Hp of the same hemisphere. In the dorsal Hp of the right hemisphere, the MeA trained group shows a 36% decrease in asymmetric axospinous synapses in comparison with control animals 6 hours after training. Four way ANOVA revealed statistically significant differences for the interaction of time after training and training group ($F_{2, 109}$=3.377, P=0.038). Therefore, a three way ANOVA (training group, hemisphere, area) was performed between the different groups at 6 hours, which revealed that 6 hours post training there is a statistically significant difference between the training groups ($F_{2,52}$=3.856, P=0.027) and the interaction between hemisphere (LSD post hoc analysis demonstrated that only the right hemisphere shows differences between the groups) and group ($F_{2,52}$=3.738, P=0.03).

After four way ANOVA post hoc tests were performed which showed that 6 hours post training the dorsal part of the right hemisphere of the control animals has significantly more asymmetric synapses on spines in relation to the MeA trained group (P=0.00084) and in comparison with the ventral part of the right hemisphere of controls (P=0.04). Furthermore, the water and the MeA trained groups show hemispheric dissimilarities after 6 hours of training, where the ventral part of the right hemisphere shows less asymmetric spine synapses in relation to the left hemisphere (P= 0.017 and P=0.04 respectively) (tables 3.1, 3.2, 3.4, Fig 3.7).

Table 3.1. Synaptic density expressed as Nv syn/μm3 in the left hemisphere of the ventral Hp 6 hours post training ± standard error means (S.E.M) (control n=6, water n=5, MeA n=5). All control animals are developmental controls.

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.47±0.05	1.28±0.09	0.012±0.007	0.02±0.01	1.78±0.11
WATER TRAINED	0.46±0.07	1.36±0.11	0.01±0.0048	0.03±0.019	1.65±0.2
MeA TRAINED	0.39±0.04	1.42±0.14	0.035(*)±0.009	0.02±0.009	1.92±0.2

Table 3.2. Synaptic density expressed as Nv syn/μm3 in the right hemisphere of the ventral Hp 6 hours post training ± standard error means (S.E.M) (control n=6, water n=5, MeA n=5). All control animals are developmental controls

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.37±0.04	1.29±0.012	0.02±0.012	0.014±0.006	1.7±0.15
WATER TRAINED	0.31±0.05 (‡)	0.94±0.08 (‡)	0.025±0.018	0.033±0.027	1.3±0.15
MeA TRAINED	0.32±0.03	1.07±0.092(‡)	0.016±0.0068	0.025±0.005	1.4±0.12

(differences between groups are indicated by asterisks (*), the double crosses (‡) show differences between different hemispheres of the same group. The underlined control group indicates differences between the ventral and dorsal part of the same group-see table 3.4)

3.3.1.3 Symmetric shaft

The results for symmetric shaft synaptic density are presented in Fig 3.8 and tables 3.1, 3.2, 3.3, 3.4. Statistically significant differences were found for the interaction between hours and training group ($F_{2, 109}$= 3.125, P=0.048). No differences were found between the main factors. Therefore, a three way ANOVA was carried out (hours post training, hemisphere and area) where statistically significant differences occurred for the main factor of time ($F_{1, 34}$=13.16, P=0.0009), which show that at 6h post training there are significantly more symmetric axodendritic synapses in the MeA trained group in relation to 24 hours after training. Fisher LSD post hoc analysis was performed for all the factors (training group, hours post training, hemisphere and area) that showed that the ventral part of the left hemisphere of the MeA trained group shows a 192% increase in symmetric axodendritic synapses in relation to the control group (P=0.023).

Table 3.3 Synaptic density expressed as Nv syn/µm3 in the left hemisphere of the dorsal Hp 6 hours post training ± standard error means (S.E.M) (developmental control n=6, water n=5, MeA n=5).

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.37±0.05	1.38±0.1	0.009±0.0045	0.0025±0.002	1.76±0.13
WATER TRAINED	0.36±0.036	1.15±0.05	0.015±0.005	0.012±0.0046	1.54±0.08
MeA TRAINED	0.37±0.02	1.33±0.07	0.026±0.0076	0.023±0.005	1.75±0.07

Table 3.4 Synaptic density expressed as Nv syn/µm3 in the right hemisphere of the dorsal Hp 6 hours post training ± standard error means (S.E.M) (control n=6, water n=5, MeA n=5).

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.42±0.035	1.62±0.23	0.025±0.008	0.04±0.02	2.1±0.26
WATER TRAINED	0.41±0.016	1.25±0.07	0.02±0.012	0.03±0.02	1.6±0.12
MeA TRAINED	0.33±0.033	1.05 (*)±0.07	0.026±0.008	0.036±0.02	1.4±0.11

Asterisk (*) shows significant differences between control and MeA trained groups for asymmetric spine density (P= 0.0008)

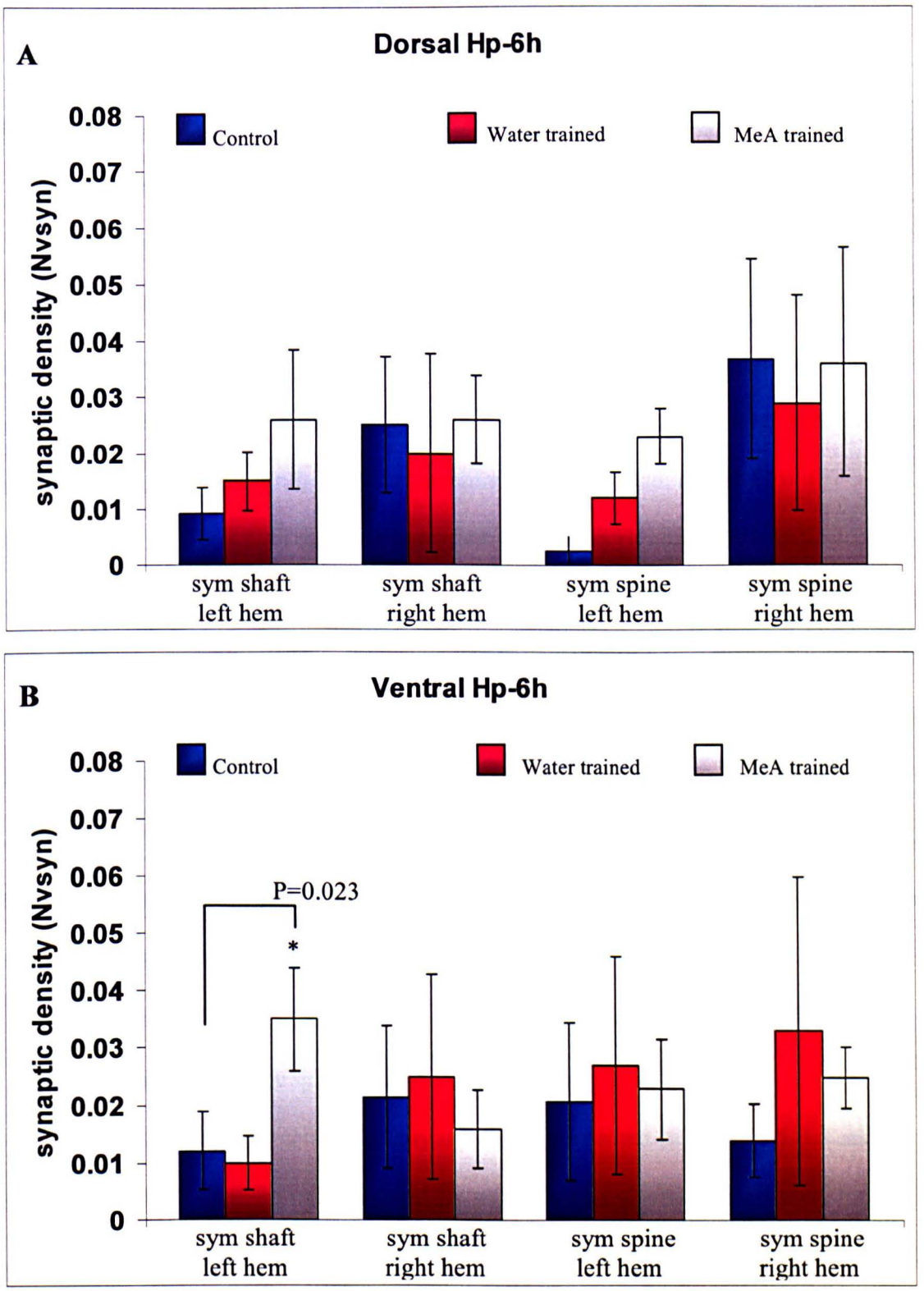


Fig. 3.8. Data showing symmetric synapse types examined in the dorsal and ventral hippocampus, both left and right hemisphere (control n=6, water n=5, MeA n=5). In graph B, the MeA trained group shows an increase in the symmetric shaft synapses in the left hemisphere of the ventral Hp. Columns represent means of data of six control, five water and five MeA trained birds and vertical bars represent standard error means (S.E.M). Note difference in y axis scale compared with fig. 3.7

3.3.1.4 Symmetric spine

No statistical differences were found in any areas or hemispheres after statistical analysis for symmetric synapses on spines (tables 3.1, 3.2, 3.3, 3.4, Fig. 3.8)

3.3.2 24 HOURS POST TRAINING

3.3.2.1 Asymmetric shaft

Data for asymmetric axodendritic synapses in control, water and MeA trained birds 24h post training are shown in Fig. 3.9, whilst the values for each area (ventral, dorsal) and hemisphere (right, left) are displayed in tables 3.5, 3.6, 3.7, 3.8. Four way ANOVA showed statistical differences for brain regions examined (ventral and dorsal Hp) ($F_{1,\ 109}$=4.648, P=0.033), and the interaction between hemisphere and area of study ($F_{1,\ 109}$=5.546, P=0.02), so Fisher LSD post hoc was performed. No differences have been found for the synaptic density of total synapses between the groups or the areas and hemispheres examined. In the dorsal hippocampus of the right hemisphere there is a 33% decrease in Nv_{syn} in the MeA trained group in comparison with control birds (P= 0.038) (fig.3.10). Furthermore, the right hemisphere of the dorsal hippocampus demonstrates a 29.4% increase in axodendritic synapses (i.e. asymmetric shaft) in comparison with the left hemisphere (P= 0.031). In the left hemisphere of the ventral hippocampus synapses show a 29.4% transient increase in relation to the dorsal part (P=0.042) (tables 3.6, 3.7 Fig. 3.9 and 3.10).

3.3.2.2 Asymmetric spine synapses

The results for asymmetric spine synapses are presented in tables 3.5-3.8 and fig. 3.9. Nv_{syn} shows a 9.5% increase in the ventral Hp of the left hemisphere in the MeA trained birds in comparison with controls. In the ventral Hp of the right hemisphere the water trained group shows 8% and 13% increase in Nv_{syn} in relation to MeA trained and control birds respectively. Post hoc analysis showed no significant differences for this type of synapses among the 24 hours groups.

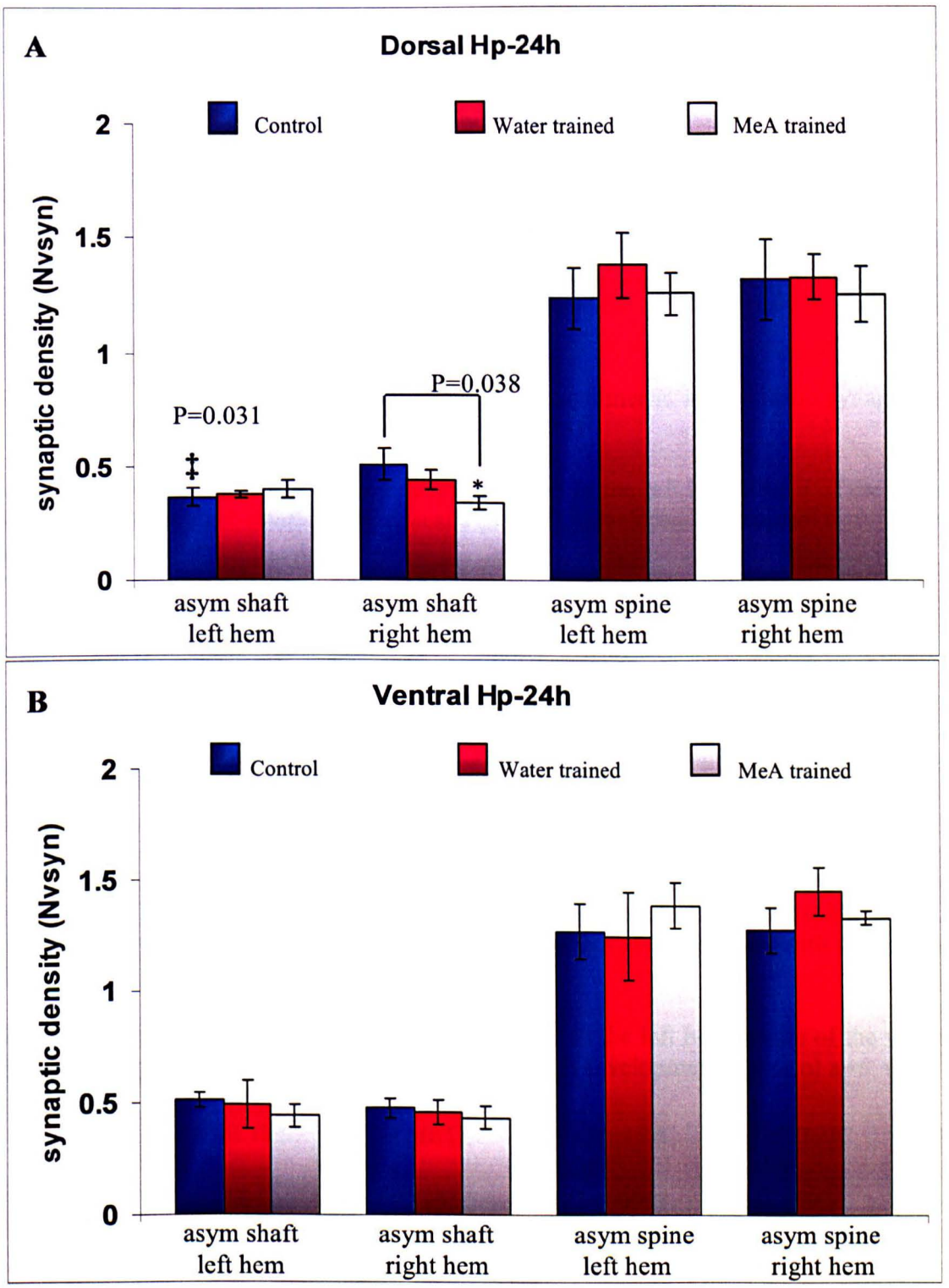


Fig 3.9. Data showing the effect of passive avoidance training on synaptic density (Nv/μm3) in the dorsal and ventral hippocampus of the right and left hemisphere. The ‡ in graph A marks the difference shown between the asymmetric shaft synapses in the dorsal hippocampus of the control animals, where the left hemisphere has significantly less asymmetric shaft synapses in relation to the right (P=0.031). In the same graph the MeA trained group has significantly less asymmetric synapses onto dendrites in comparison with the control group (P=0.038). Columns show mean values (control n=6, water n=6, MeA n=6) of birds and vertical bars represent standard error means (S.E.M).

Table 3.5. Synaptic density expressed as Nv syn/μm3 in the right hemisphere of the dorsal Hp 24 hours post training ± standard error means (S.E.M) (developmental control n=6, water n=6, MeA n=6)

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.51±0.069	1.31±0.18	0.013±0.005	0.012±0.006	1.85±0.23
WATER TRAINED	0.44±0.045	1.32±0.1	0.02±0.0066	0.021±0.013	1.8±0.11
MeA TRAINED	0.34(*)±0.03	1.25±0.12	0.01±0.005	0.004±0.0038	1.6±0.13

(The asterisk (*) shows the significant decrease in asymmetric axodendritic synapses in the MeA trained group in relation to control birds, P=0.038)

Table 3.6. Synaptic density expressed as Nv syn/μm3 in the left hemisphere of the dorsal Hp 24 hours post training ± standard error means (S.E.M) (developmental control n=6, water n=6, MeA n=6)

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.36±0.038 (‡)	1.23±0.13	0.012±0.004	0.012±0.006	1.62±0.17
WATER TRAINED	0.37±0.014	1.37±0.14	0.01±0.008	0.016±0.009	1.68±0.14
MeA TRAINED	0.4±0.037	1.25±0.09	0.01±0.004	0.004±0.002	1.66±0.11

(Double cross (‡) indicates that the dorsal Hp of control animals in the left hemisphere has significantly less asymmetric shaft synapses in relation to the right hemisphere (P=0.042), whilst concomitantly it has less asymmetric shaft synapses in comparison with the ventral part of the left hemisphere.

Table 3.7. Synaptic density expressed as Nv syn/μm3 in the left hemisphere of the ventral Hp 24 hours post training ± standard error means (S.E.M) (developmental control n=6, water n=6, MeA n=6)

	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.51±0.032	1.27±0.123	0.023±0.0037	0.02±0.003	1.83±0.15
WATER TRAINED	0.49±0.11	1.25±0.195	0.015±0.0055	0.03±0.024	1.79±0.28
MeA TRAINED	0.45±0.05	1.39±0.1	0.01±0.004	0.009±0.003	1.86±0.11

Table 3.8. Synaptic density expressed as Nv syn/μm3 in the right hemisphere of the ventral Hp 24 hours post training ± standard error means (S.E.M) (developmental control n=6, water n=6, MeA n=6)

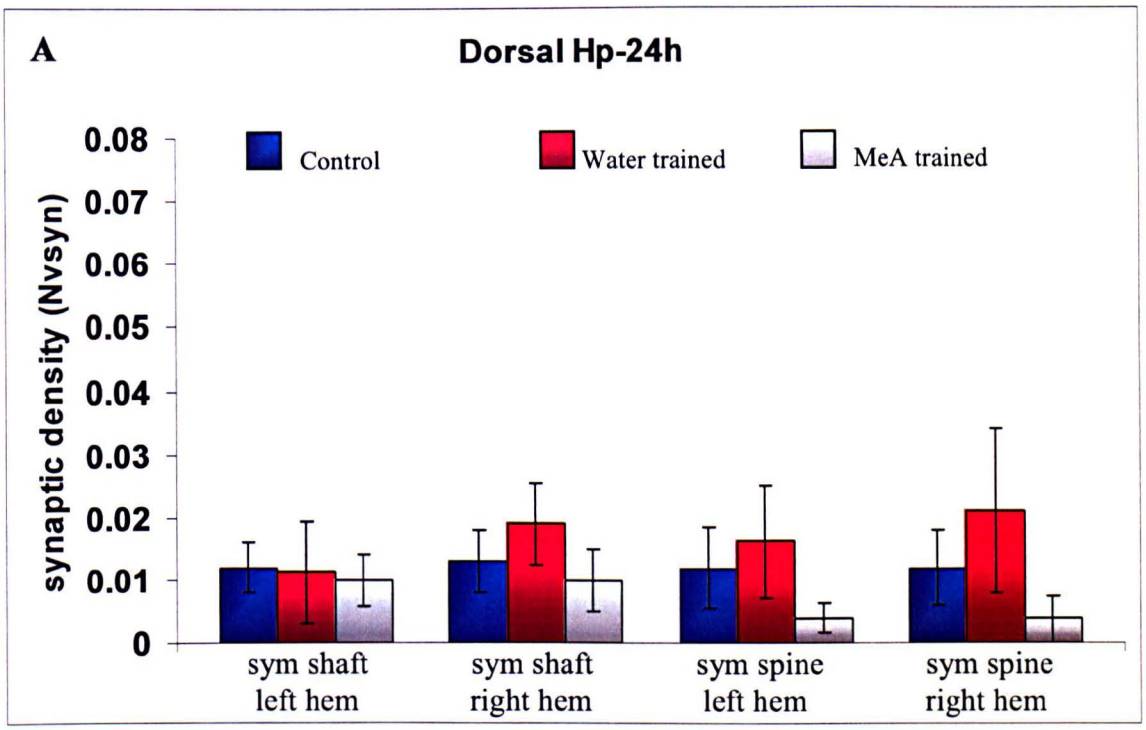
	ASYMMETRIC SHAFT	ASYMMETRIC SPINE	SYMMETRIC SHAFT	SYMMETRIC SPINE	TOTAL
CONTROL	0.48±0.04	1.28±0.1	0.02±0.0055	0.02±0.005	1.8±0.116
WATER TRAINED	0.46±0.05	1.45±0.11	0.03±0.008	0.016±0.0058	1.96±0.14
MeA TRAINED	0.43±0.05	1.34±0.03	0.012±0.006	0.012±0.004	1.77±0.09

3.3.2.3 Symmetric shaft

The results for symmetric spine synapses are presented in tables 3.5, 3.6, 3.7, 3.8 and fig. 3.10. The ventral Hp of the left hemisphere shows reduced numerical synaptic density in the MeA trained group in comparison with control (53% decrease) and water trained groups (27% decrease). In the ventral Hp of the right hemisphere the decreases are 43% and 54% for control and water trained groups respectively. No differences exist for the symmetric shaft synapses between the 24h groups after Fisher LSD post hoc was carried out.

3.3.2.4 Symmetric spine

The results for symmetric spine synapses are presented in tables 3.5, 3.6, 3.7, 3.8 and fig. 3.10. In the dorsal Hp of the left hemisphere, the MeA trained group shows a 75% decrease in relation to the water trained group and a 67% decrease in comparison with control birds. In the ventral Hp of the left hemisphere, the MeA trained group shows decreased synaptic density in relation to control (53% decrease) and water trained (71% decrease) animals. Finally in the dorsal Hp of the right hemisphere the control and water trained animals show 200% and 400% increases in relation to the MeA trained group. However, ANOVA had shown no differences, so post hoc test was not performed.



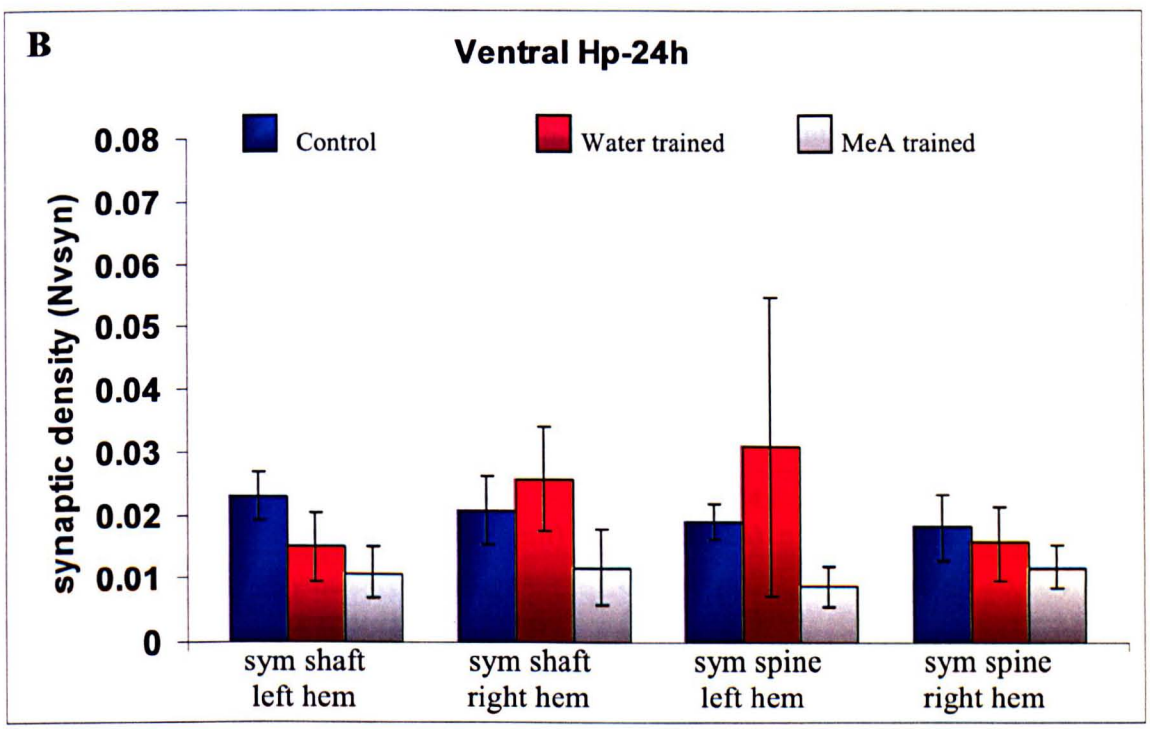


Fig 3.10. Data showing synaptic density of symmetric shaft and spine synapses (Nv/μm3) in the dorsal (A) and the ventral (B) hippocampus of the left and right hemisphere 24 h after training in control (n=6), water (n=6) and MeA trained (n=6) birds. Columns indicate means of data and vertical bars standard error means (S.E.M). Note difference in y axis scale compared with fig. 3.9.

3.3.3 DIFFERENCES IN SYNAPTIC DENSITY AMONG 6h AND 24h POST TRAINING GROUPS

3.3.3.1 Asymmetric shaft

A summary of data showing significant differences in synaptic density between 6h and 24h after training which occurred after Fisher LSD post hoc test for the four synapse types is presented in table 3.9. There is 48% increase in asymmetric shaft synapses in the ventral hippocampus of the right hemisphere (P=0.012) of the water trained group (table 3.9) 24h in comparison with 6h after training. No other differences in asymmetric axodendritic density (asymmetric shaft) were revealed after post hoc analysis between 6h and 24h post training.

Table3.9 Differences in synapse density between 6h (control n=6, water n=5, MeA n=5) and 24h post training ± standard error means (S.E.M) (developmental control n=6, water n=6, MeA n=6).

TRAINING GROUP	SYNAPSE TYPE	6 h POST TRAINING	24 h POST TRAINING
WATER	Asymmetric shaft (ventral Hp, right hem)	0.31±0.055 (P=0.012)	0.46±0.054
CONTROL	Asymmetric spine (dorsal Hp, right hem)	1.61±0.23 (P=0.033)	1.31±0.176
WATER	Asymmetric spine (ventral Hp, right hem)	0.94±0.0845 (P=0.0024)	1.45±0.11
MeA	Symmetric shaft (ventral Hp, left hem)	0.035±0.008 (P=0.0135)	0.011±0.004

3.3.3.2 Asymmetric spine

Post hoc test showed a contrasting pattern 6h after training for the control and the water trained groups. Six hours post training the control group formed more asymmetric spine synapses (23% increase) in the dorsal hippocampus of the right hemisphere (P=0.033) in

relation to the group of 24h post training. In contrast, the water trained group 6h after training had less asymmetric synapses onto spines (35% decrease) in relation to the 24h water trained group in the ventral Hp of the right hemisphere (P=0.0024). No other changes were observed between 6h and 24h for asymmetric spine synapses in the different experimental conditions (table 3.9).

3.3.3.3 Symmetric shaft

Fisher LSD post hoc analysis revealed that 24h after training there is a 69% reduction in the symmetric shaft synapses in the ventral hippocampus of the left hemisphere of the MeA trained group in relation to 6h post training (P=0.0135) (table 3.9). Although the control group shows 92% increase in synaptic density in the ventral Hp of the left hemisphere 24h after training in comparison with 6h post training, this difference is not statistically significant. The MeA training group shows reduction in symmetric shaft numerical density in the dorsal Hp of the right and left hemisphere (62% decrease) 24h after training in relation to 6h. However, none of these reductions is statistically significant.

3.3.3.4 Symmetric spine

Although 24h post training the MeA trained group shows reduced synaptic density in the ventral Hp of the left (55% decrease) and right (52% decrease) hemisphere as well as the dorsal Hp of the left (83% reduction) and right (89% reduction) hemisphere, no post hoc test was performed due to lack of differences after four way ANOVA statistical analysis.

3.4 DISCUSSION

The data presented in this study support earlier research which has shown that passive avoidance training affects hippocampal plasticity (Sandi et al., 1992; Unal et al., 2002). Interestingly, as has also been demonstrated previously in the chick brain (Stewart et al., 1984; Sandi et al., 1992; Sandi et al., 1993) this study supports the finding of an hemispheric asymmetry in some synaptic parameters of the hippocampus. However, unlike the findings of Sandi and collaborators (1992), the left hemisphere was not solely affected; changes were also found in the right hemisphere 6 hours after training. The ventral hippocampus of the right hemisphere exhibited reduced synaptic density in relation to the left hemisphere for asymmetric shaft synapses in the water trained group and for asymmetric spine synapses in both the water and MeA trained groups.

Concomitantly, there is an increase in the ventral hippocampus of the left hemisphere of symmetric shaft synapses of the MeA trained group in comparison with controls. This increase in symmetric shaft synapses in the MeA trained group may be an indication of inhibition of information or signal transmission in the ventral hippocampus. Symmetric synapses (type II) are presumed inhibitory, unlike asymmetric (type I) (Gray, 1959) which are presumed excitatory (Gray, 1959; Steward, 2000b).

In the dorsal hippocampus of the right hemisphere, surprisingly, there is a reduction in synaptic density in the MeA trained group in comparison with the control group. Studies in the rat so far have indicated that water maze training increases dendritic spine density in hippocampal CA1 (Moser et al., 1994) and enriched environment in has similar effects in CA3 (Altschuler, 1979). In contrast, auditory filial imprinting in the chick has been shown to cause a reduction of spine density in the dorsocaudal nidopallium (Ndc) (Bock and Braun, 1999b) and mediorostral nidopallium/ mesopallium (MNM) (Bock and Braun, 1998). One may argue that passive avoidance training is not a spatial task and therefore is affected differently,

resulting in reduced synaptic connectivity in the MeA trained group. However, studies in the IMM 1h after passive avoidance learning have shown increases in spine density in the right hemisphere in relation to the left in MeA trained group as well as in comparison with untrained animals (Doubell and Stewart, 1993). At 1h post training the biochemical cascade for memory formation is probably different than after 6h (Rose, 1995a; Rose and Stewart, 1999), since cell adhesion molecules, which are essential for memory formation, are activated 5-8 hours post training (Scholey et al., 1993; Scholey et al., 1995). c-Fos and c-jun proteins show a peak in expression 1-2 hours after passive avoidance training (Freeman and Rose, 1995). Consequently, the 6h may be the time point when the procedures for long term memory formation start to take place and as a result synaptogenesis data will be different from those at 1 h post training.

Another issue arising is the differences seen between the dorsal and the ventral hippocampus. In rats, lesions of the dorsal hippocampus impair spatial learning and memory preservation after passive avoidance training (Black et al., 1977; Cogan and Reeves, 1979), whilst ventral lesions have no effects (Moser et al., 1993; Moser et al., 1995; Moser and Moser, 1998). The ventral hippocampus in contrast has been suggested to take part in autonomic, emotional and social procedures (Moser and Moser, 1998) due to its connections with the amygdala and the hypothalamus (Witter et al., 1989a; Risold and Swanson, 1996, 1997).

In the chick brain it has been suggested that the dorsal hippocampus is equivalent to the dentate gyrus, whilst the ventral is homologous to Ammon's horn based on electrophysiological (Siegel et al., 2002), immunocytochemical (Erichsen et al., 1991) and connectivity (Casini et al., 1986; Szekely, 1999; Atoji et al., 2002) data. The dorsal hippocampus in this study corresponded to area 3 and 4 of Erichsen et al. (1991) work (dorsomedial Hp and part of dorsolateral as in Szekely and Krebs, 1996), which are suggested

to be homologous to the dentate gyrus and hilus respectively, whereas the ventral hippocampus related to area 2, which shows homology with Ammon's horn. Furthermore, studies have also shown that the dorsal hippocampus has been proposed to have bilateral connections with the mesopallium and StM (Bradley et al., 1985; Szekely and Krebs, 1996; Atoji et al., 2002) which have been demonstrated to be involved in passive avoidance learning (Stewart et al., 1987; Stewart and Rusakov, 1995; Dermon et al., 2002), whilst the ventral hippocampus sends efferents to the medial septum and the contralateral ventral hippocampus and arcopallium.

The reduction in the axospinous synapse density in the MeA trained group in the dorsal hemisphere of the right hemisphere could be explained firstly either by late spine formation or secondly by branch elimination. In the first case, it is known from mammalian studies that shaft synapses first appear and then give rise to dendritic spines (Mates and Lund, 1983; Fiala et al., 1998). The present data, however, have not shown any differences in the number of shaft synapses in the MeA trained group in relation to controls. Perhaps synaptogenesis is delayed in the MeA trained group and at a later time point more shaft synapses are formed which will eventually become spine synapses, though further data would be necessary to determine this.

The second hypothesis could be branch elimination that would result in decreased dendritic spine formation (Alsina et al., 2001). Many explanations could be given for this phenomenon; apoptosis may occur to eliminate dendrites and therefore although shaft synapses actually increase in number in order to form dendritic spines, cell death could counterbalance this increase resulting in the reduced formation of spine synapses. Thus, apoptosis may modulate synaptic remodelling probably by inducing the death of old or newly formed neurones after training so that new contacts may take place to transform short to long term memory and at the same time keep the synaptic balance in the chick brain.

Another perhaps more plausible assumption based on prior data could be that passive avoidance training is a stressful experience. Older studies (Sandi and Rose, 1997) have demonstrated that plasma corticosterone levels are increased 5 min after MeA tasting, but return to basal levels by 15 min. Although the levels of corticosterone return to normal levels, the arousal of corticosterone may be affecting synaptic plasticity by acting on BDNF, which has been demonstrated to show reduced expression after stress (Smith et al., 1995; Ueyama et al., 1997). BDNF has been shown to participate and possibly enhance synaptic plasticity since its levels are increased after LTP (Castren et al., 1993). Furthermore, it has been shown to regulate axonal remodelling and branching (Inoue and Sanes, 1997; Lom and Cohen-Cory, 1999; McAllister et al., 1999), synapse formation and stability (Poo, 2001) and synaptic transmission (Boulanger and Poo, 1999). An alternative possibility may be that NMDA-mediated Ca^{2+} overexpresses, eventually becoming toxic for the cell after corticosterone increases (Takahashi et al., 2002) resulting in cell death (Reagan and McEwen, 1997). Additionally, since it has been shown that stress reduces cell proliferation and induces apoptosis (Gould et al., 1991b; Gould et al., 1992; Gould et al., 1998; Gould and Tanapat, 1999), it could also affect glial cells that are essential for glutamate levels regulation protecting the cell from Ca^{2+} excitotoxicity (Vernadakis, 1996).

Twenty four hours post training, a reduction in asymmetric shaft synapses in the MeA trained group was demonstrated in relation to control animals in the dorsal part of the right hippocampus. The most obvious hypothesis would be that shaft synapses turn to spine synapses and therefore their number is reduced, resulting in equal levels of spine synaptic density between the untrained animals and the MeA trained. However, the overall synaptic density is again slightly reduced in the MeA trained group since this significant reduction of shaft synapses is not compensated by significant increases in spine density. This might reflect inactivation since previous studies for the passive avoidance training have shown that 24h post

training increases in synapse density and deoxyglucose occur mainly in the IMM of the left hemisphere (Stewart et al., 1984; Rose and Csillag, 1985; Patel and Stewart, 1988). In the StM, however, both hemispheres are affected (Stewart et al., 1987; Hunter and Stewart, 1993; Lowndes and Stewart, 1994), as probably occurs in hippocampus. In this case we could also assume that this reduction may be due to apoptosis or stress as mentioned above.

On the other hand, stress apart from reducing neurogenesis (Gould and Tanapat, 1999) has been shown to cause axon degeneration by Ca^{2+} excitotoxicity which can lead to dendritic swelling and microtubular breakdown (Choi, 1995; Rothstein et al., 1996). Therefore, it could be suggested that stress could cause synaptic density changes and remodelling through apoptosis or necrosis of the cell.

A very interesting finding is that the control group shows enhanced shaft synaptic density in the right hemisphere 24 h post training. In particular, the data indicate that the dorsal part of the right hemisphere shows higher synaptic density in relation to the left hemisphere. Furthermore, the dorsal hippocampus of the left hemisphere shows also reduced shaft density in comparison with the ventral part. The dorsal part of the right hemisphere of the control group shows high synaptic activation, since also at 6h post training it showed higher spine density in relation to the MeA trained group and the ventral part of the control group. Thus this increase 6h after training could indicate a burst of synaptic remodelling that disappears 24 h after training possibly due to synaptic re-organization. These results provide support to previous studies which have shown that the information processing begins at the dorsal hippocampus of the right hemisphere, then being transferred to the ipsi and contralateral ventral part of the hippocampus to end up in the left dorsal hippocampus (Hough et al., 2002), where probably due to the late arrival of information, synaptogenesis begins later.

In summary, there is hemispheric asymmetry in the hippocampus that is probably area dependent. In other words, the dorsal hippocampus of the right hemisphere shows increased synaptogenesis in relation to the left 24 h post training in the control group as well as to the ventral hippocampus of the right hemisphere 6h post training, whilst it appears to have higher synaptic density in the shorter term experiments (6h) in comparison with long term (24h). In addition, the left dorsal Hp 24h after training has a smaller shaft synaptic density than the ventral part. The water and MeA trained group 6h post training demonstrate higher axospinous synapse density in the left ventral hippocampus in relation to the right. Furthermore, in the water trained group it appears that the axospinous synapse density is increased at 24h post training in the right ventral hippocampus, the MeA trained group, does not follow the same pattern of changes. Additionally, the water trained group has higher shaft density in the left ventral hippocampus in comparison with the right hemisphere 6h after training, but again shaft density significantly increases in the right ventral hippocampus 24h after training. It is noteworthy that 6h post training the MeA trained group has reduced spine density in relation to controls, but 24 h after training it shows reduced shaft density in relation to the untrained group. After 6 of training the MeA trained group shows increased symmetric shaft density in relation to controls, but this disappears at 24 h post training.

It is therefore clear that all hippocampal areas are affected by passive avoidance training, the right dorsal and the left ventral more than the rest. Changes occur both at 6 and 24h post training, implying a constant alteration of synaptic plasticity and remodelling. Unlike the results from IMM, no apparent increases were found in the trained groups in relation to controls or to each other. The only exception was the symmetric shaft density of the MeA trained group at 6h, which ceases to exist at 24h post training. Although it is a very interesting finding, its explanation is unclear. It suggests that an inhibitory mechanism must be activated, probably affecting shaft and spine density in this group.

Another issue that arises is the reduction of shaft synapses in right dorsal Hp in the MeA trained group 24 h post training. Is it due to cell death or degeneration? Apoptosis may be a regulatory mechanism which influences synaptic transmission and efficacy. Older neurones may be dying in order to be replaced by new, still immature, without processes at the time point of examination. There is also the possibility of synaptic remodelling and neuronal degeneration due to alterations in the levels of corticosterone (Sandi and Rose, 1997) during PAL. More experimental testing e.g. neurotrophic factors and their expression need to be conducted to elucidate this finding. At 6 h post training the hippocampus shows reduced spine density in comparison to that in controls. This phenomenon may occur due to late onset of synaptogenesis in this area in the MeA trained group or limited dendritic arborisation. If a combination of both of these process were to occur it might be suggested that 24h after training, there is neuronal loss (shaft density reduction), but at the same time, spines may divide, as a result of learning and therefore the total number of synapses in this group does not change in respect to the untrained group. Consequently, the birth of new spines may be counterbalancing synaptic loss due to neuronal death or degeneration.

CHAPTER 4

CELL PROLIFERATION

IN THE

CHICK BRAIN

24 HOURS AND 9 DAYS

AFTER PASSIVE AVOIDANCE

LEARNING

4.1 INTRODUCTION

Cell proliferation persists in the adult avian brain (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990a, b; Alvarez-Buylla et al., 1994; Ling et al., 1997; Patel et al., 1997) in the brain of teleosts (Zikopoulos et al., 2001) as well as in the mammalian brain (Altman and Das, 1965; Kaplan and Hinds, 1977; Gould et al., 1999c; van Praag et al., 1999; Gage, 2002). The production of newborn neurones was first identified in the rat brain by Altman and Das, but their study was largely ignored until Nottebohm (1989) and Alvarez Buylla (1990), showed evidence of adult neurogenesis in the High Vocal Center (HVC), a brain area associated with singing in the song bird.

In the rat brain, neurogenesis has been documented in the subventricular zone (SVZ) (Morshead and van der Kooy, 1992; Lois and Alvarez-Buylla, 1993) and the dentate gyrus (Altman and Das, 1965; Bayer, 1982), in the borders of the hilus with the granule cell layer (Kuhn et al., 1996) and olfactory bulb, although in the latter newborn cells rather migrate long distance from the lateral ventricular zone (Lois and Alvarez-Buylla, 1993; Luskin, 1993; Lois and Alvarez-Buylla, 1994). Recent studies have also identified neurogenesis in Ammon's horn in the mouse (Rietze et al., 2000) and neocortex (Magavi et al., 2000; Rietze et al., 2000), although other researchers argue against neocortical neurogenesis (Kornack and Rakic, 2001; Rakic, 2002b).

Newborn neurones in the dentate gyrus which survive demonstrate that they become part of the neural circuitry by receiving synaptic input on the cell bodies, which are surrounded by synaptic vesicles, and dendrites (Kaplan and Bell, 1984) and by extending axons to the mossy fibre pathway that ends up in CA3 (Stanfield and Trice, 1988; Markakis and Gage, 1999). Furthermore, recent studies have shown that newborn neurones exhibit action potentials and passive membrane properties similar to those of the mature neurones, indicating that they will mature to become functional mature neurones (van Praag et al., 2002).

Additionally, mature neurones are polarised, non-mitotic, with an axon and several dendrites and have the ability to release neurotransmitters at their synapses.

Neurogenesis can be positively or negatively affected by different factors. Excitotoxic and mechanical lesions cause an increase in neuronal progenitor proliferation (Gould and Tanapat, 1997), as well as deactivation of NMDA receptors (Gould et al., 1994; Cameron et al., 1995). On the other hand, activation of NMDA receptors decreases neurogenesis (Cameron et al., 1995). These authors have concluded that NMDA receptors may be regulating the equilibrium of granule cells in the dentate gyrus during learning and inactivity periods. Temporal lobe seizures caused by excitatory amino acids has been also demonstrated to increase neurogenesis (Parent et al., 1997), as well as stroke (Bernabeu and Sharp, 2000; Arvidsson et al., 2002; Sharp et al., 2002).

Neurogenesis can be also increased by i) ephrins and erythropoietin (Conover et al., 2000), ii) neurogenin, directly as a transcriptional factor or indirectly by inhibiting astrogenesis (Sun et al., 2001), iii) retinoic acid (Takahashi et al., 1999), iv) antidepressant treatment including electroconvulsive treatment (Jacobs et al., 2000; Madsen et al., 2000; Malberg et al., 2000; Duman et al., 2001) and adrenalectomy (Cameron and Gould, 1994; Montaron et al., 1999) In particular, suppression of glucocorticoids by adrenalectomy enhances neurogenesis by division of immature cells (Gould et al., 1992; Cameron and Gould, 1996), which can return to normal levels by restoration of diurnal and nocturnal levels of corticosterone (Rodriguez et al., 1998)), v) serotonin (Gould, 1999), vi) BDNF (Benraiss et al., 2001; Lee et al., 2002b) and growth factors (Gensburger et al., 1987; Bovolenta et al., 1996; Frade et al., 1996; Cameron et al., 1998a). Growth factors play a mitogenic role by increasing the number of cells undergoing the cell cycle, but also enhance the survival of dividing precursors that would undergo cellular death under normal circumstances (Drago et al., 1991; Mytilineou et al., 1992; Nakagami et al., 1997). Basic Fibroblast Growth Factor

(bFGF) has been demonstrated to stimulate DNA synthesis in neurogenetic populations in the hilus of the dentate gyrus and the SVZ (Wagner et al., 1999). bFGF has been suggested to participate in hippocampal neuro- and gliogenesis during the perinatal period, since manipulations of bFGF in adult animals does not affect neuronal cell production (Kuhn et al., 1997; Wagner et al., 1999). Epidermal Growth Factor (EGF) and Insulin-like Growth Factor (IGF-1) on the other hand, increase mitosis and granule neuron number in the adult rat hippocampus (Kuhn et al., 1997; O'Kusky et al., 2000) as does Fibroblast Growth Factor-2 (FGF-2), whilst BDNF and NT-3 promote neuronal differentiation (Ghosh and Greenberg, 1995; Vicario-Abejon et al., 1995).

Neurogenesis can be also increased by estrogens (Vicario-Abejon et al., 1995), enriched environment (Vicario-Abejon et al., 1995; Nilsson et al., 1999), exercise (van Praag et al., 1999), trace eyeblink conditioning and water maze training (Gould et al., 1999b). However, not all hippocampus dependent tasks require production of new granule cells and spatial navigation in particular is a task that does not involve neurogenesis (Shors et al., 2002). LTP also increases the number of newly formed granule cells, indicating a direct relationship between learning and neurogenesis (Derrick et al., 2000).

Neurogenesis can be reduced under certain conditions. Dopamine agonists (Teuchert-Noodt et al., 2000), NMDA receptor activation (Cameron et al., 1995; Cameron et al., 1998b), and factors such as stress (Gould et al., 1992) (Lemaire et al., 2000), exposure to predator odours (Tanapat et al., 2001) and ageing (Kuhn et al., 1996; Cameron and McKay, 1999; Montaron et al., 1999) all ease reduced neurogenesis. Estradiol administration in female meadow voles initially enhances cell proliferation, but 48 hours later a reduction has been identified, indicating that non breeding females show higher neurogenesis. However, reproductive active females show increased cell survival (Galea and McEwen, 1999; Ormerod and Galea, 2001).

Pregnancy increases the levels of glucocorticoids (Magiakou et al., 1996) resulting in decreased neurogenesis (Cameron and Gould, 1994). Maternal deprivation has also been shown to reduce neurogenesis (King et al., 2004; Mirescu et al., 2004). Studies in the rat hippocampus have demonstrated enhanced neurogenesis after focal ischaemia, but only after NMDA receptors have been activated; blockage of NMDA receptors resulted in suppression of neurogenesis (Arvidsson et al., 2001). It has been suggested that stress reduces neurogenesis through activation of adrenal steroids and NMDA receptors in rats (Gould et al., 1992; Cameron et al., 1998b), adult shrews (Gould et al., 1997) and monkeys (Gould et al., 1998). Stress does not affect the survival of newly formed neurones, but rather alters the survival of mature neurones (Cameron and Gould, 1996; Gould et al., 1999b).

Dehydroepiandrosterone (DHEA) has been demonstrated to antagonise corticosterone and enhance cell proliferation and neuronal differentiation (Karishma and Herbert, 2002). Corticosteroid removal increases apoptosis of mature neurones (Sloviter et al., 1993a; Cameron and Gould, 1996). Administration of dexamethasone reduced neurogenesis but also LTP in CA1 and the dentate gyrus (Yu et al., 2003). These authors support the idea that since there is no neurogenesis in CA1, synaptic plasticity may not be related to neurogenesis. Recent studies have also shown that psychosocial stress reduces new born cells and neurogenesis (Thomas et al., 2003), unlike chronic mild stress which does not alter cell proliferation and survival of granule cells (Kim et al., 2003).

Ageing reduces hippocampal neurogenesis probably through high levels of corticosterone that can kill or damage dividing cells (Anderson et al., 2002; Harman et al., 2003) although recent studies contradict this hypothesis (Heine et al., 2004). Aged rats show decreased hippocampal volume (Heine et al., 2004), possibly due to reduction in the population of granule cells. At the same time, mature neurones have higher action potentials than younger granule cells, indicating that hyperactivity due to lack of young cells might be

causing damage to CA3 and downregulation in CA1 (Cameron and McKay, 1999). Other studies imply that the decrease of neurogenesis in aged rats may be caused by attenuation of proliferative activity of precursor cells which are located in the subgranular layer, inhibition of migration or increased apoptosis. It has been demonstrated that in aged rats the population of dividing cells is diminished and that reduction of neurogenesis affects only granule cell precursors (Kuhn et al., 1996).

Nonetheless, the effects of ageing can be altered if the animals are housed in an enriched environment, experience social interaction and physical activity (Kempermann et al., 1998). Additionally, after water maze training, aged animals that have performed better during the task illustrate higher numbers of BrdU positive cells, indicating also that proper performance for a task may require a baseline of neurogenesis (Drapeau et al., 2003a).

As mentioned earlier, neurogenesis persists in adulthood in the avian brain. Goldman and Nottebohm (1983) have reported a production rate of new neurones between 0.9% and 2% per day in the HVC of adult canaries. Other studies in the avian hippocampus have shown a similar production rate of between 2.3% to 3.9% (Patel et al., 1997). Neurogenesis was thought to be a developmental process, until demonstration that it was affected by estrogens (Burek et al., 1994, 1995), by singing (Nottebohm, 1989; Nordeen and Nordeen, 1990; Alvarez-Buylla et al., 1992; Alvarez-Buylla and Kirn, 1997; Brainard and Doupe, 2002), by spatial learning (Patel et al., 1997; Lee et al., 1998a),by photoperiod (Krebs et al., 1995), by seasonal changes accompanied with learning (Barnea and Nottebohm, 1994; Smulders et al., 2000), and by passive avoidance learning (Dermon et al., 2002).

Cells are born in the ventricular zone (VZ) of the lateral ventricle and migrate with the help of radial glia; the richer an area is in radial glia, the faster neurones migrate to their target areas (Alvarez-Buylla and Nottebohm, 1988). It has been suggested that radial glia fibres decrease significantly 2-3 mm away from the lateral ventricle (Alvarez-Buylla et al., 1988a).

Cells elongate as they migrate into the telencephalon and differentiate when they reach their final destination (Alvarez-Buylla and Nottebohm, 1988). The more cells migrate outside to the telencephalon, less labelled cells are present in the ventricular zone (Alvarez-Buylla and Nottebohm, 1988). Radial glia are important, because they guide and accelerate migration during the first postnatal week. Furthermore, gliogenesis in birds takes place before neurogenesis (Nottebohm, 1985). 'Hot spots' (places on the ventricular zone were newborn cells are mainly gathered) have been observed in the avian brain which are located in the VZ and undergo cell division resulting in the production of new neurones (Alvarez-Buylla et al., 1990). As in the mammalian brain, again it has been suggested that glial cells may be generating neurones (Alvarez-Buylla et al., 1990).

Additionally, neurogenesis decreases with age (Alvarez-Buylla et al., 1994; Wang et al., 2002). As newborn cells migrate outside the VZ and branching occurs, the parenchyma grows and finally it supersedes the VZ resulting in a minuscule VZ in adult birds (Alvarez-Buylla, 1990a). Many newborn cells die during migration or as they begin to differentiate, because more cells are actually born in the brain than needed. Another possibility is that old cells die in order to create space for the new cells to incorporate into a functional circuit (Alvarez-Buylla and Nottebohm, 1988).

Recent studies (Dermon et al., 2002) have shown that one-trial passive avoidance learning enhances cell proliferation in the mesopallium intermediomediale (IMM), striatum mediale (StM) and tuberculum olfactorium (TuO) 24h and 9 days post BrdU injection. Neurogenesis has been identified also in hippocampus after food storing processes (Clayton and Krebs, 1994), after spatial learning (Patel et al., 1997) and can be affected by seasonal changes (Barnea and Nottebohm, 1994). However, the effects of passive avoidance training on cell proliferation in hippocampus and other areas of the limbic system have yet to be examined.

Studies from our group, extensively described in Chapter 3, have demonstrated synaptic remodelling 6 and 24 hours after passive avoidance training in the chick dorsal and ventral hippocampus. Therefore, in the present study it was decided to study the effects of PAL on structures of the limbic system 24h and 9 days after BrdU injection. This was a follow up to the study of Dermon and collaborators (2002) since the limbic association area is related to emotions and memory storage (Kluver and Bucy, 1997). Additionally, Richard and Davies (2000) have suggested a strong link between limbic structures participating in PAL (e.g. mesopallium, arcopallium) and BO.

4.2 RESULTS

4.2.1 CELL PROLIFERATION

Cell proliferation was determined in the ventral and dorsal hippocampus (vHP and dHP), area parahippocampalis (APH) intermediate and dorsal arcopallium (AI and AD), nucleus taeniae of the amygdala (TnA) and olfactory bulb (BO) after passive avoidance learning (PAL) at day P2 post hatching, and P9. The locations of these regions are shown in the schematic drawing of the coronal sections in Figure 4.1. An example of BrdU labelling in the dorsal and ventral Hp of P2 animals is shown in Figure 4.2.

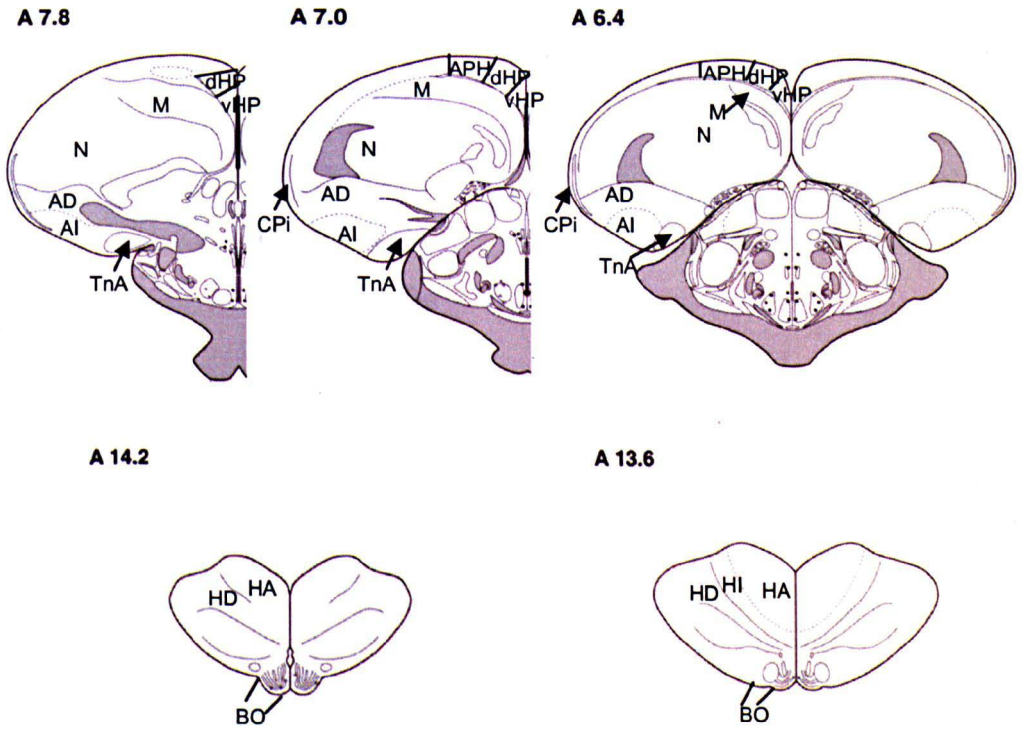


Fig. 4.1. Coronal sections of the chick brain representing some of the different levels where BrdU labelled cells were counted. (A-C) Location of the ventral and dorsal hippocampus (vHp and dHp), area parahippocampalis (APH), nucleus taeniae of amygdala (TnA), intermediate and dorsal arcopallium (AI and AD respectively). (D, E) Location of the bulbus olfactorius in the chick brain. M: mesopallium N: nidopllium, CPi: Cortex piriformis, HA: Hyperpallium apicale, HD: Hyperstriatum densocellulare, HI: Hyperpallium intercalatum

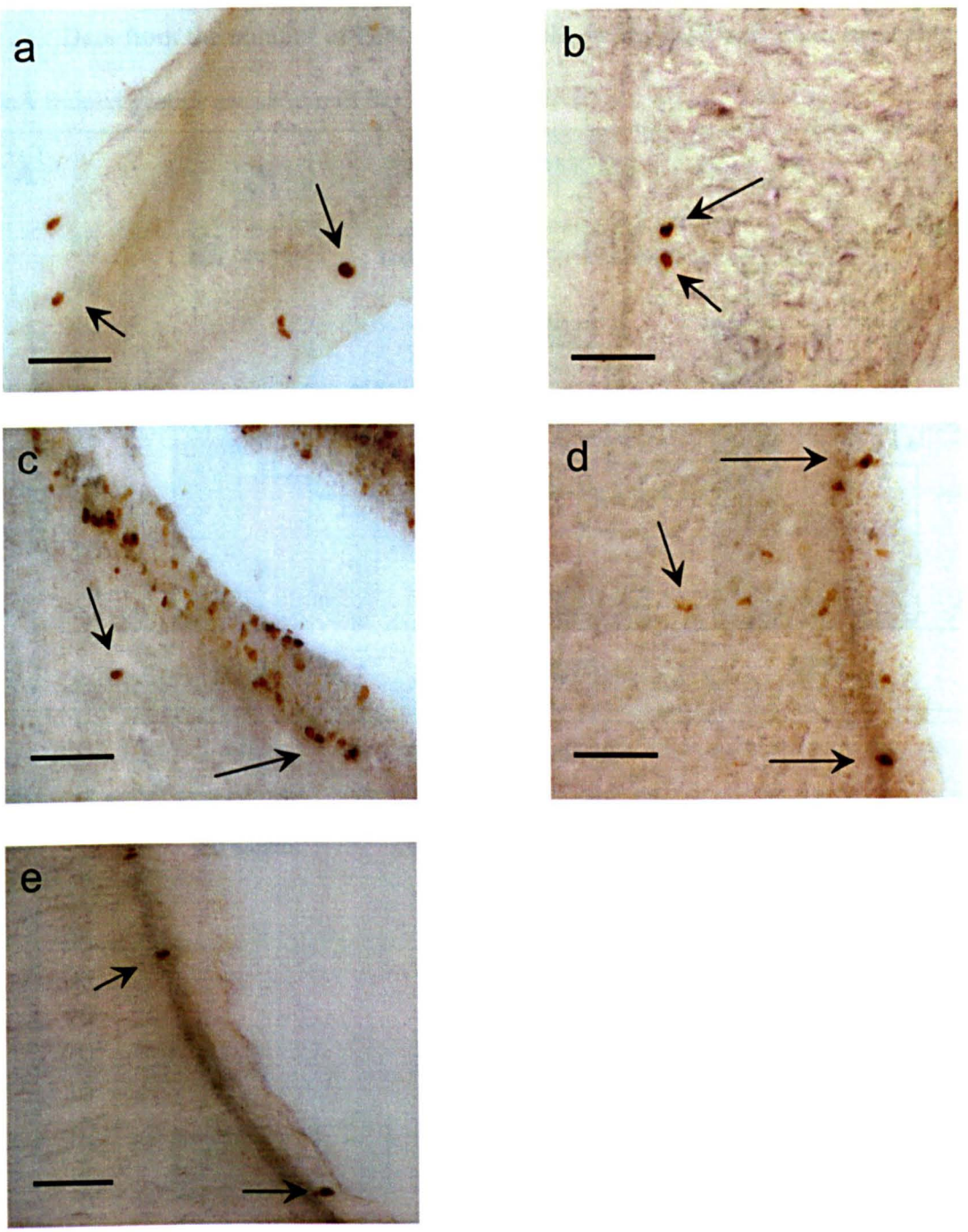


Fig. 4.2. Ventral hippocampus of a control (a) and MeA trained (b) 2 day old chicks. Ventricular zone of the dorsal Hp of a control (c) and a MeA trained animal (d). It is clear that the control animals at P2 have more BrdU labelled cells (arrows) in relation to MeA trained animals (e) dorsal Hp of P9 control animal. There is an obvious reduction in BrdU labelled cells in relation to (c). Scale bars=50 μm.

4.2.1.1 Hippocampus (Hp)

Data from the number of BrdU labelled cells in Hp at P2 and P9 in control, water and MeA trained groups are shown in Fig. 4.3 A and B.

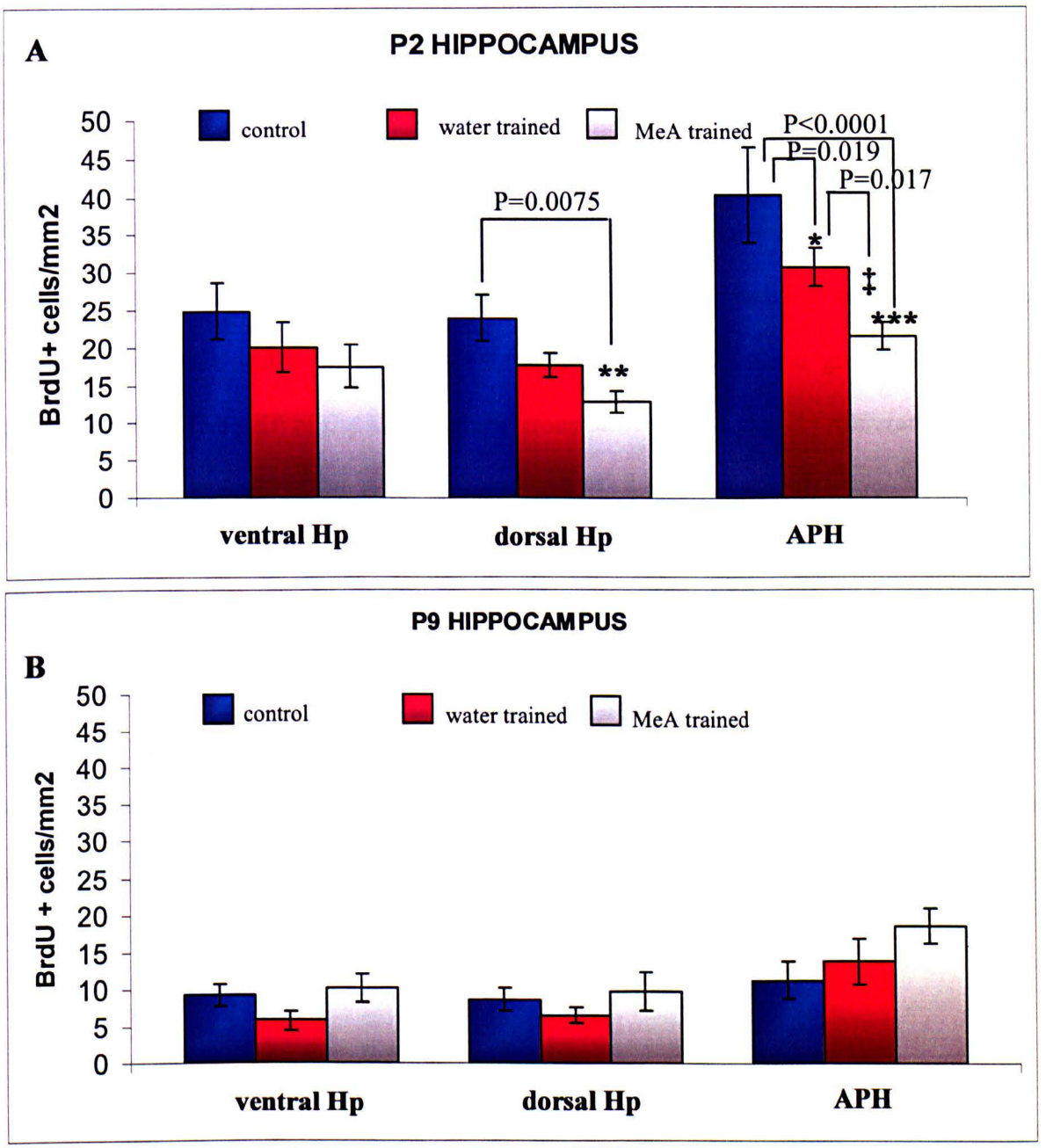


Fig. 4.3. Quantitative analysis for the number of BrdU labelled cells/mm2 at P2 and P9. A. The dorsal hippocampus of the control animals (n=6) shows more BrdU+ cells compared to the MeA trained (n=6) group (**)(P=0.0075). In the APH of the water (n=7) and the MeA trained group there are fewer BrdU positive cells in comparison to controls (P=0.019 (*) and P<0.0001 (***) respectively). The MeA trained group shows further reduction in cell proliferation in relation to the water trained group (‡) (P=0.017). No differences occur between the groups at 9 (control n=5, water n=5, MeA n=5) days post training. All areas of the control and water trained animals show significant reduction in cell proliferation 9 days post training. Columns are means of data and vertical bars indicate standard error means (S.E.M)

There are obvious reductions in BrdU labelling in the dorsal Hp and APH of the MeA trained group at P2 and that was confirmed by ANOVA analysis. A three way ANOVA test showed that there are statistical differences concerning the age of the animals ($F_{1,78}$= 85.35, P<0.0001), the training group ($F_{2,78}$= 4.245, P=0.018), the area of the brain-ventral, dorsal Hp, or APH-($F_{2,78}$=17.93, P<0.0001) as well as the interaction between age and training group ($F_{2,78}$=10.7, P<0.0001). Data for labelling levels in the brain regions examined are shown in Figs. 4.3, 4.4, 4.5, 4.6 and tables 4.1 and 4.2. These results indicate that there is a significant reduction in the number of BrdU labelled cells 24 hours post training in the dorsal chick hippocampus (LSD post hoc analysis, P=0.0075) in the MeA trained group in relation to control animals. The vHP of the MeA trained group shows reduced cell proliferation, but it is not significant (P= 0.073). The APH of water and MeA trained animals shows considerably fewer labelled cells in contrast to controls (P=0.019 and P<0.0001 respectively). However, the MeA trained group shows a further reduction in newborn cells in relation to the water trained group (P=0.017) (Fig. 4.3a). At 9 days post training there are no differences between the groups examined (Fig.4.3b). The control and water trained group show reduced labelling in all areas in relation to animals of control and water trained group of P2 (vHP control P=0.0004, vHP water P= 0.012, dHP P=0.0005 and P= 0.01 correspondingly, APH P<0.0001 and P=0.00012).

4.2.1.2 Nucleus taeniae of amygdala (TnA)

Data for BrdU labelling in TnA in control, water and MeA trained birds are shown in Fig. 4.4 A and B. TnA does not appear to be affected by avoidance training. A multiple way ANOVA showed statistical differences only for the factor of age ($F_{1,31}$=18.7, P=0.00015). At P2 there a slight reduction of cells in the MeA trained group, but this is not significant (P=0.2). No differences appear between the groups at P9. However, there is a significant

reduction 9 days after BrdU injection in the control and water trained group in respect to these groups 24 h post training (P=0.001 and P=0.0075 respectively).

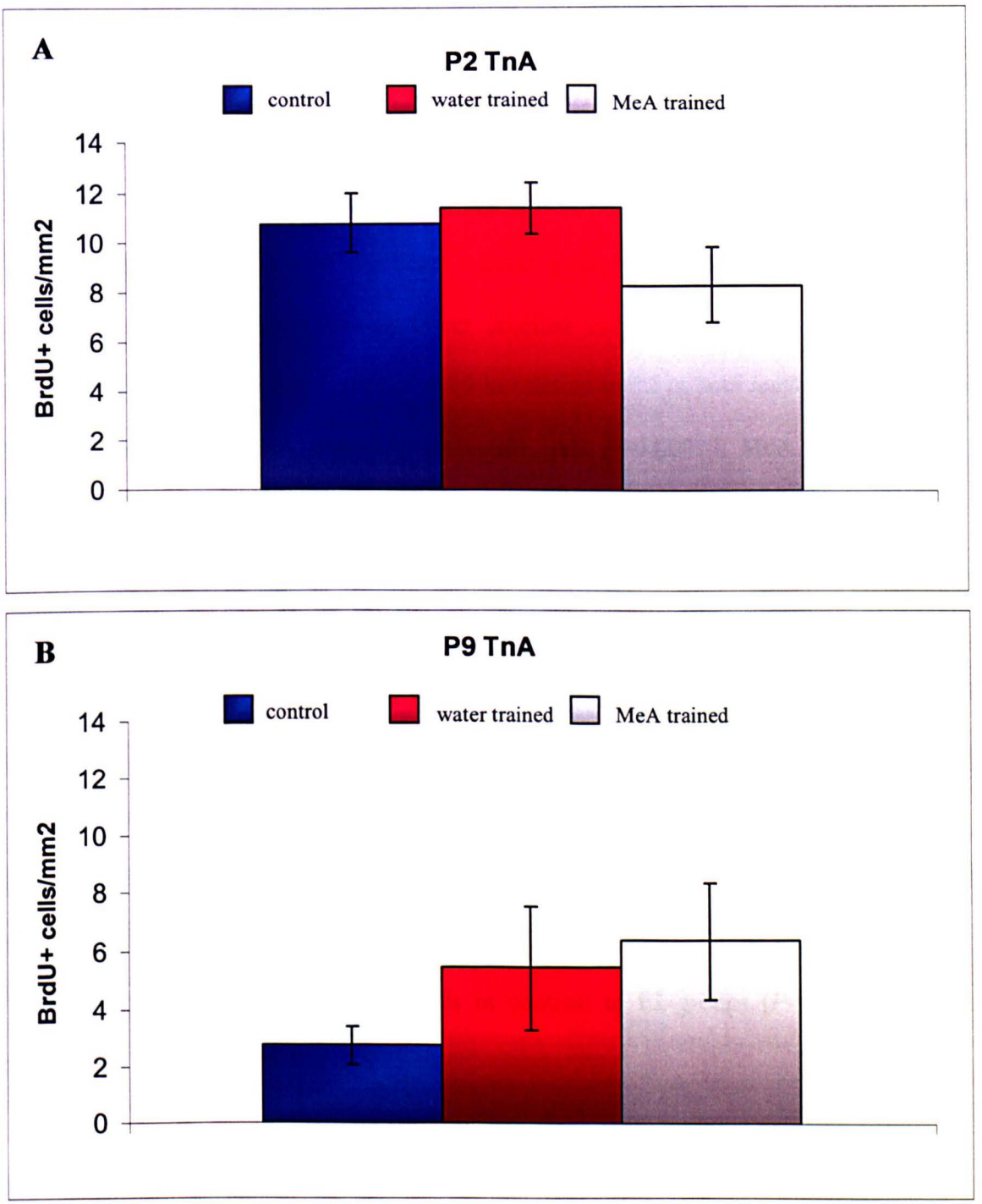


Fig 4.4. Data for the effects of PAL on BrdU labelling in control (n=6), water (n=8) and MeA (n=8) birds. No differences appear between the groups at 24h or 9 days (control n=5, water n=5, MeA n=5) post BrdU injection. 9 days post training there is a significant reduction in the number of BrdU labelled cells in the control and water trained groups in comparison with 24h after training. Columns represent means of data and vertical bars indicate standard error means (S.E.M)

4.2.1.3 Intermediate and dorsal arcopallium (AI and AD)

Data for BrdU labelling in AI and AD in control, water and MeA trained birds at P2 and P9 are shown in Fig. 4.5 A and B. Studies in the arcopallium showed that there are differences between the chicks tested at the different ages ($F_{1,54}$=136.48, P<0.0001), and in the areas examined, intermediate and dorsal arcopallium ($F_{1,54}$=10.74, P=0.0018). In addition, differences occur due to the interaction of age and brain area ($F_{1,54}$=4.75, P=0.034). There is variation between labelling of the MeA trained group in the intermediate and the dorsal arcopallium, with the latter having fewer labelled cells (P= 0.0046). All of the groups demonstrate fewer BrdU labelled cells at P9 in relation to P2 in both nuclei tested (control AI P<0.0001, AD P=0.00046, water AI P<0.0001, AD P=0.00017, MeA AI P<0.0001, AD P=0.0003).

4.2.1.4 Olfactory bulb (BO)

Data for BrdU labelling in BO in control, water and MeA trained birds at P2 and P9 are shown in Fig. 4.6 A and B. Three way ANOVA in the BO shows statistical differences between age ($F_{1,24}$= 16.12, P=0.00051) and training group ($F_{2,24}$=10.33, P=0.00058). The MeA trained group shows more newborn cells at P2 and P9 in relation to controls (P=0.0087 and P=0.013) and the water trained group (P=0.015 and P=0.009). At P9 only the water and the MeA trained group show fewer cells in contrast to P2 groups (P=0.015 and P=0.024 respectively).

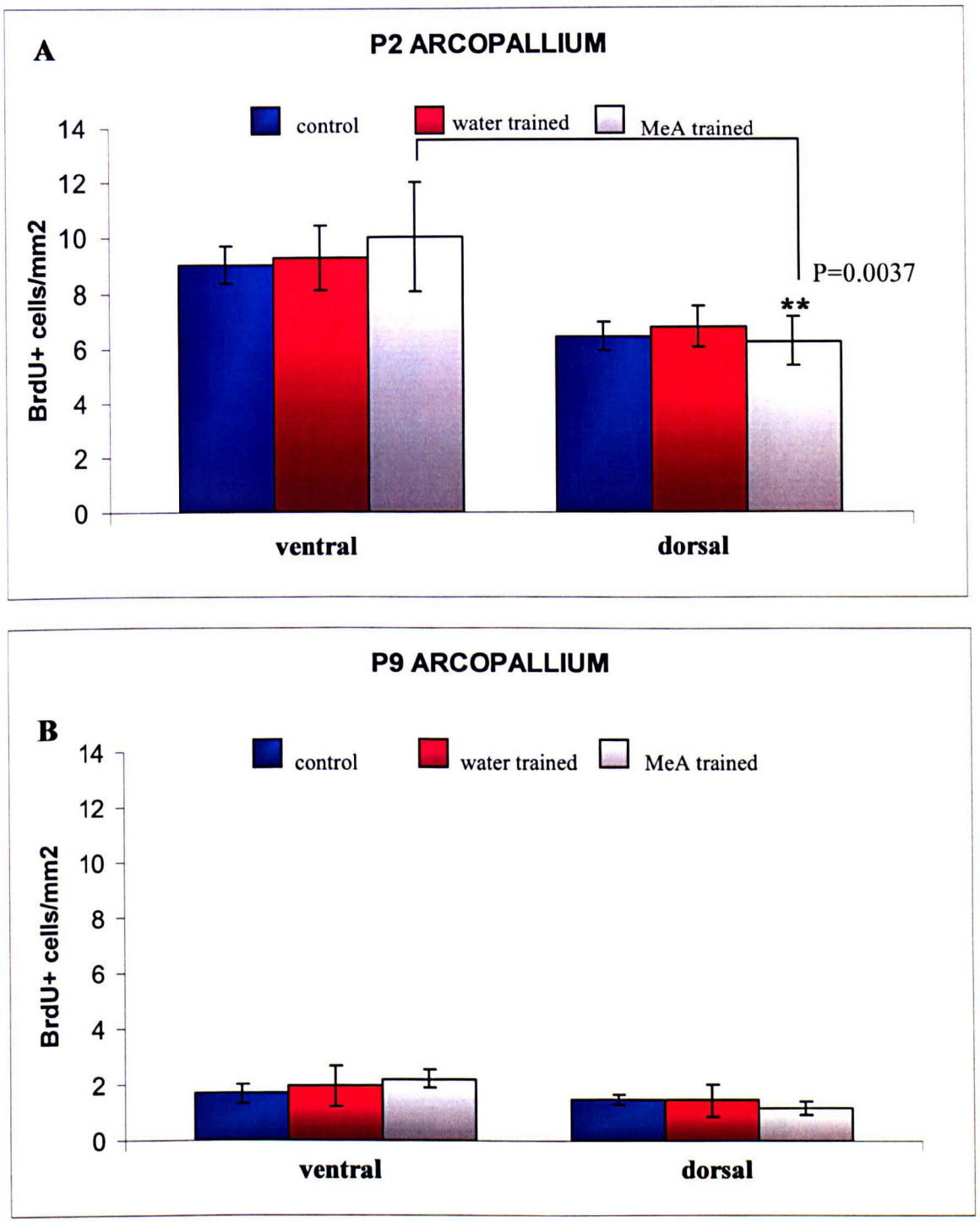


Fig. 4.5. Means of data for BrdU labelled cells in the intermediate and dorsal arcopallium at P2 (control n=6, water n=6, MeA n=6) and P9 (control n=5, water n=5, MeA n=5). In graph A the MeA trained group shows significantly more BrdU labelled cells in the AI in relation to the AD (P=0.0037). No differences arise between animals sacrificed 9 days post BrdU injection. Again after 9 days, the control and water trained groups show reduced BrdU labelling in relation to 24h post training. Columns represent means of data and vertical bars indicate standard error means (S.E.M)

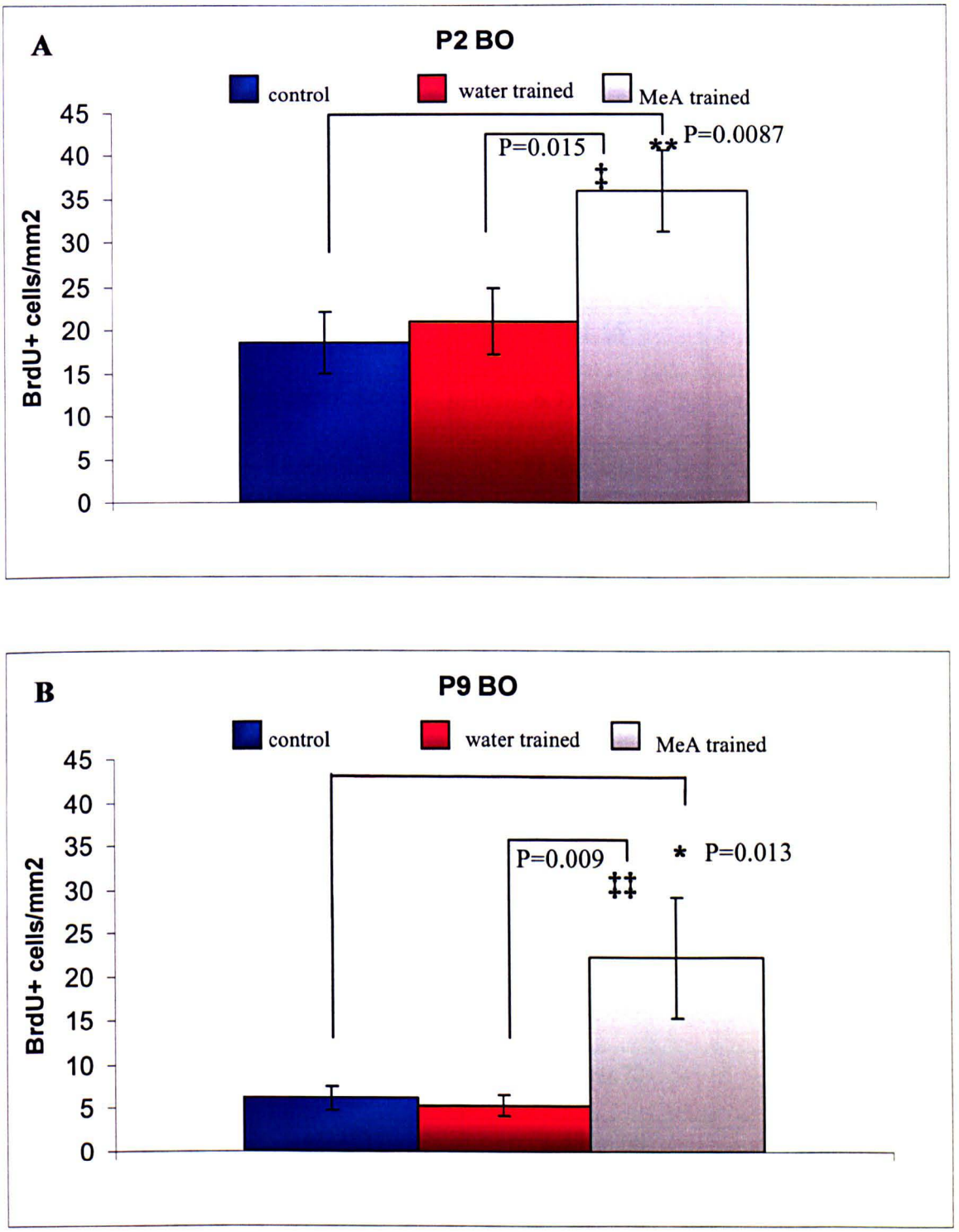


Fig 4.6. Means of data for BrdU labelled cells in the olfactory bulb. The BO of the MeA trained (n=6) animals has more BrdU+ cells in relation to the control (n=6) and water trained (n=6) group 24h (P=0.0087(‡) and P=0.015(**)) and 9 days (controls n=5, water n=5, MeA n=5) post BrdU injection (P=0.013 (*) and P= 0.009 (‡‡)). Nine days post training the water and MeA trained groups show reduced BrdU labelling in relation to 24h. Columns indicate means of data and vertical bars indicate standard error means (S.E.M).

Table 4.1. Summary of cell profile density (BrdU+ cells/mm^2) for each area of interest at P2. The control group refers to developmental control animals.

AREA	Control	Water trained	MeA trained
Ventral Hp	24.85±3.8	20±3.28	17.48±2.84
Dorsal Hp	23.95±3.07	17.57±1.59	12.8(**)±1.32
APH	40.126±6.21	30.7(*)±2.46	21.6(***)±1.79
AI	9.0±0.67	9.3±1.15	10.0±1.97
AD	6.39±0.54	6.79±0.7	*6.27*(**)±0.88
TnA	10.8±10.79	11.4±1.026	8.285±1.52
BO	18.5±3.57	21.0±3.79	36.0(**)±4.75

Means of cell profiles ± S.E.M (control n=6, water n=7, MeA n=6-8). Asterisks (*) indicate differences between groups, italics between subdivisions of the same area

Table 4.2. Summary of profile density (BrdU+ cells/mm2) for each area of interest at P9. The control group refers to developmental control animals.

AREA	Control	Water trained	MeA trained
Ventral Hp	9.25±1.38	5.9±1.3	10.162±1.8
Dorsal Hp	8.63±1.59	6.44±1.075	9.82±2.6
APH	11.2±2.46	13.7±3.12	18.48±2.48
AI	1.68±0.32	1.94±0.72	2.2±0.35
AD	1.45±0.17	1.43±0.58	1.15±0.24
TnA	2.75±0.67	5.43±2.13	6.36±2.03
BO	6.19±1.4	5.36±1.17	22.2(*)±6.89

Means of cell profiles ± S.E.M (control n=5, water n=5, MeA n=5). Asterisk (*) indicates differences between groups. Underlined numbers show significant reduction in comparison with P2.

4.2.1.5 Volume estimation

Data for volume estimation (mm^3) in vHP, dHP, APH, AI, AD, TnA and BO in control, water and MeA trained birds at P2 and P9 are shown in Figs. 4.7 A, B, C, D and E. Three way ANOVA for TnA, BO, AD, AI, Hp showed no differences in volume between the groups. However, in the hippocampus, three way ANOVA showed differences between age ($F_{1,96}$= 61.079, P<0.0001) and the interaction of age and group ($F_{2,96}$=7.45, P=0.00098). Fisher LSD post hoc comparisons demonstrated that there is an increase in volume at P9 in the ventral Hp (43% increase) and APH (55% increase) of control birds in relation to P2 (P=0.017 and P=0.028 respectively). Additionally, all of the areas of the water trained group showed significant volumes increases at P9 in comparison with P2 (vHp 53% increase P<0.0001, dHp 84% increase P<0.0001, APH 108% increase P=0.0027).

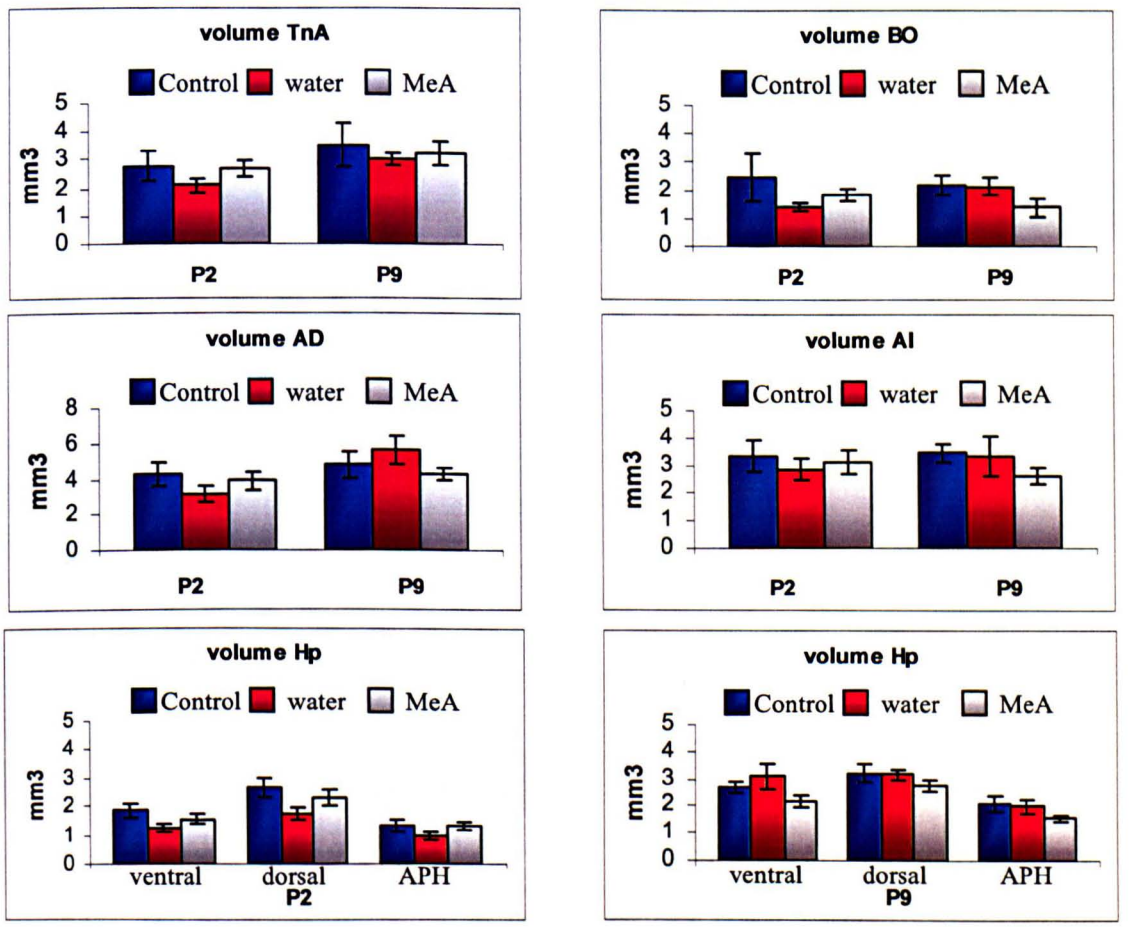


Fig. 4.7. Data representing the volume of TnA, BO, AD, AI and Hp at P2 and P9 in control, water and MeA trained birds. Columns indicate means of data ±standard error means (S.E.M).

4.2.2 DOUBLE LABELLING IMMUNOFLUORESCENCE

Double immunolabelling at 24 hours and 9 days post BrdU injection showed colocalisation of NeuN and BrdU (Fig. 4.8.a-c), and for GFAP and BrdU (Fig.4.9. a-c). Blue stained cells (Alexa Fluor 647) are newborn cells labelled with BrdU, whilst in Fig 4.8 green labelled cells are mature neurones labelled with NeuN, which is a nuclear neuronal marker (Mullen et al., 1992). In Fig 4.9 and 4.10 green fibres are glial cells labelled with glial fibrillary acidic protein (GFAP), which labels mature astrocytes (Eng et al., 2000).

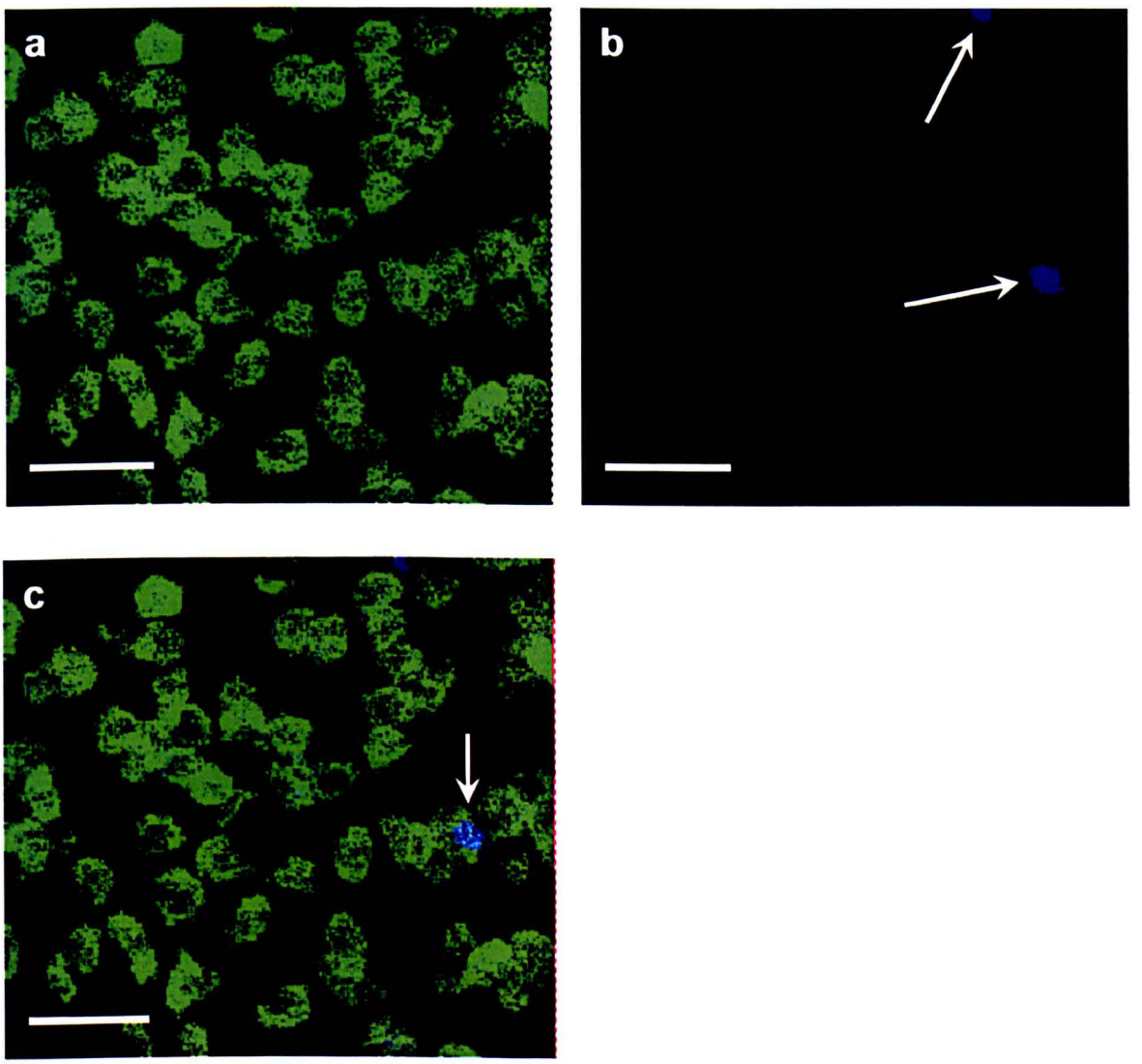


Fig. 4.8. Confocal images of BrdU labelled cells (blue, arrows) and NeuN positive cells (green). A double labelled cell is pointed with an arrow for BrdU and NeuN 24h post BrdU injection (c). The BrdU positive cell on the top of image b is not double labelled for BrdU and NeuN. Scale bars=50μm

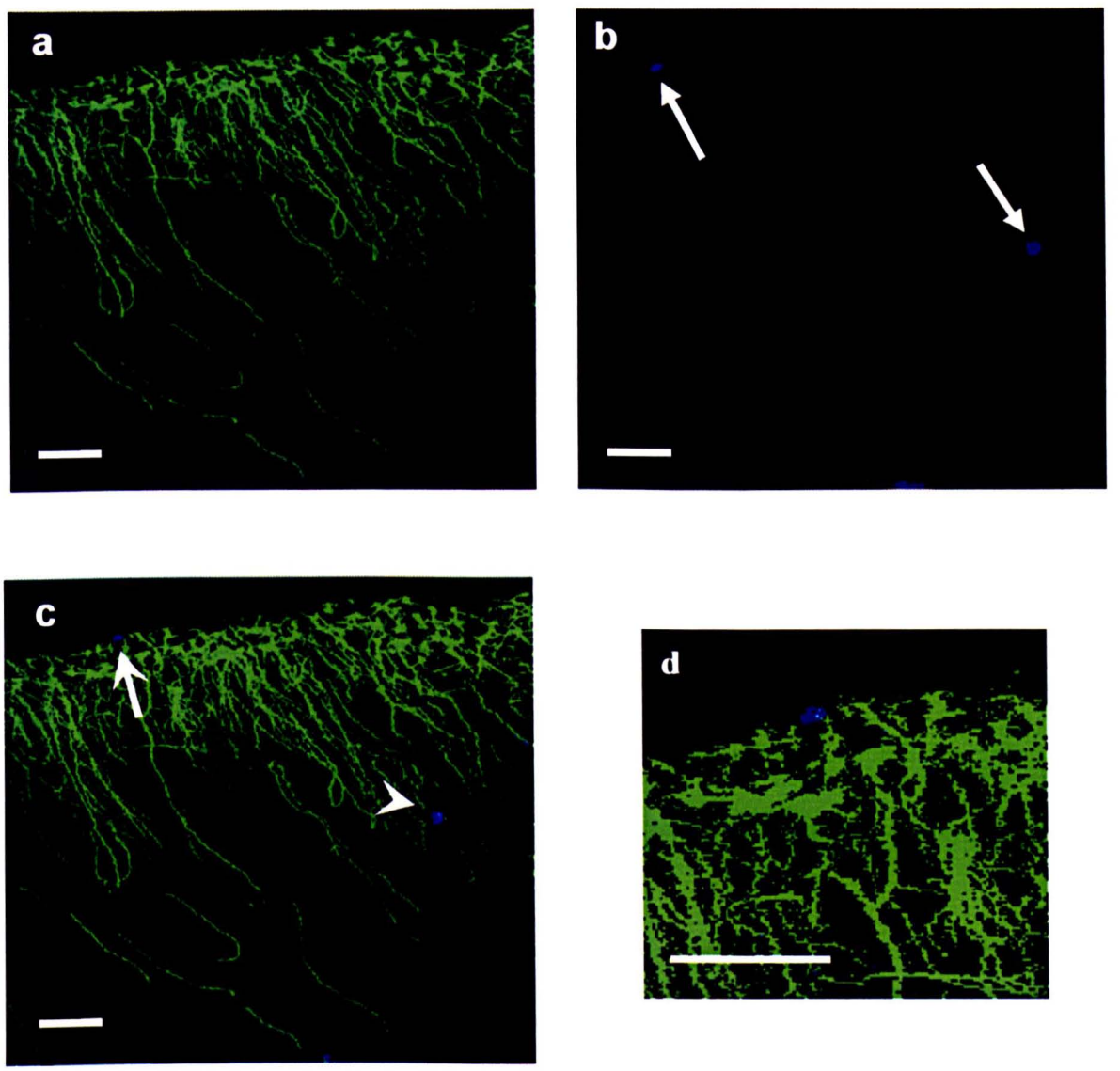


Fig. 4.9. Confocal images of BrdU labelled cells (blue, arrows) and GFAP (green). Representation of double labelled cell for BrdU and GFAP at the dHp close to the pial zone 24 hours after BrdU injection (c, arrow). A cell that migrates to the inner parts of the brain is also visible (not double labelled, arrowhead). Image d shows the BrdU labelled cell of image c which is pointed with the arrow in high power (x2.2 times) where the fibres that derive from it can be clearly seen. Scale bars= 50μm

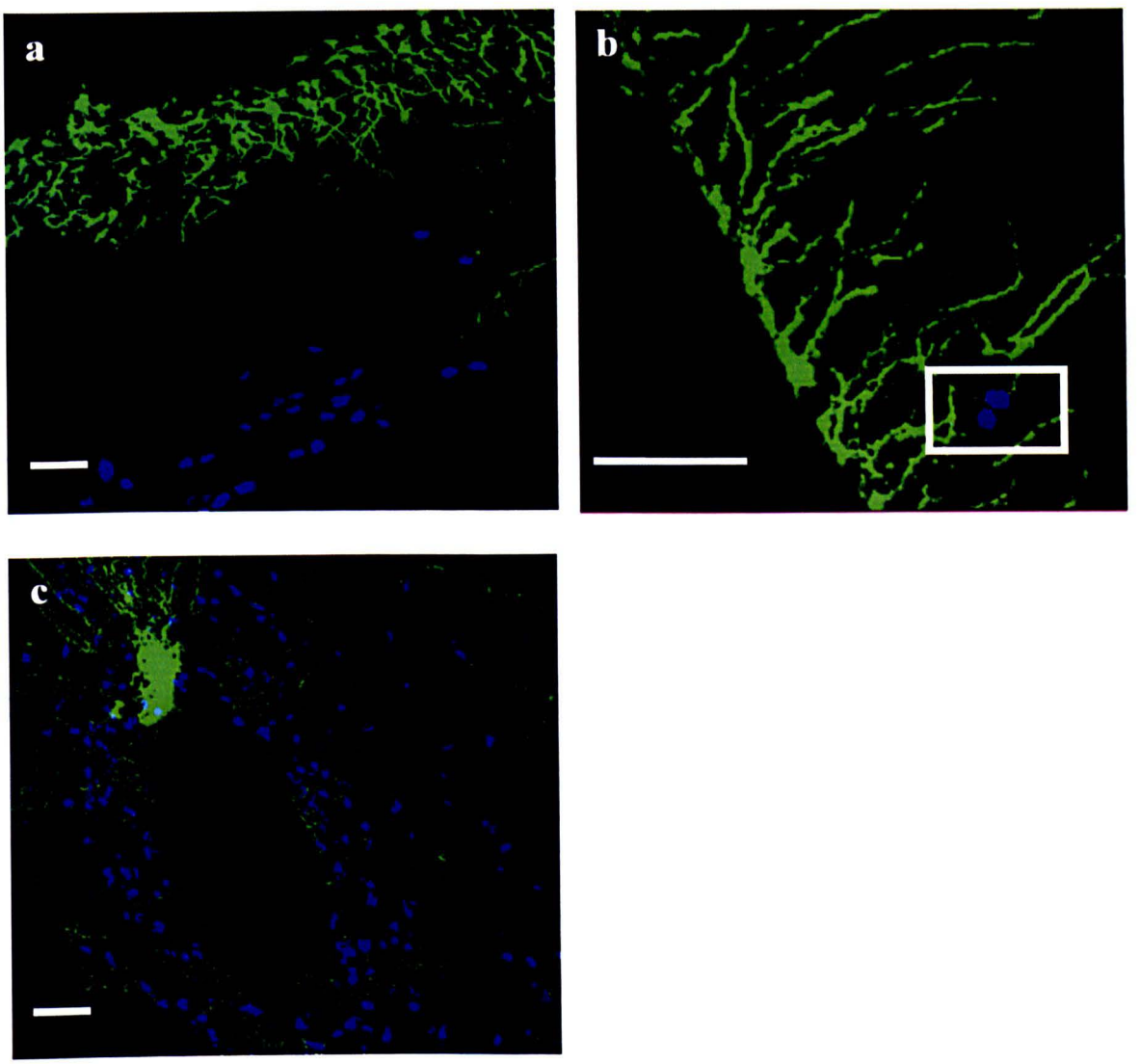


Fig. 4.10. Confocal images of BrdU labelled cells (blue) and GFAP (green). (a)Ventral hippocampus where it is apparent that none of the BrdU positive cells on the ventricular zone are double labelled for GFAP. It is also clear that the ventricular zone does not have an intense glial fibrillary network as occurs close to the pial zone. (b) Two newborn cells (see white square) which may be migrating to other areas of the brain, most likely following the glial fibres. (c) Representation of the olfactory bulb of a 9 days old chick. It can be seen that the majority of cells are not double labelled for BrdU and GFAP. Glial fibres are concentrated on the top of the ventricular zone from where probably cells migrate to other brain nuclei. Scale bars=50 μm

Four way ANOVA revealed statistical differences for the parameter cell type (neurones or glial cells) ($F_{1,83}$=52.2, P<0.0001). The percentages of double labelled cells of either neuronal or glial type do not change significantly between short term and long survival studies. Interestingly we found differences in the percentages of NeuN/BrdU positive cells in the vHP and dHP between the control and the MeA trained group. In the vHP the MeA trained group shows almost double the number of cells labelled for NeuN/BrdU, in comparison to controls (45% increase, P=0.03) (Fig. 4.11a). In contrast, the dHP of the MeA trained group shows fewer NeuN/BrdU cells in comparison to controls (41% reduction, P=0.04) (Fig. 4.11a). Additionally, control animals show more NeuN/BrdU positive cells in the dHP in relation to vHP (53.7% increase, P=0.013) (Fig. 4.11a). At P2, there are more neuronal than glial cells in the dHP of water trained and the vHP of MeA trained animals (67% increase, P= 0.049 and 96% increase, P=0.0028 respectively). At 9 days post training the vHP of all groups shows significant differences in the percentage of neuronal to glial cells (controls 109% increased NeuN positive cells, P=0.025, water trained 164% increase, P=0.012, MeA trained 121% increase, P=0.0036). Only the water and MeA trained groups demonstrate more neuronal cells in the dHP 9 days post training (79.5% more NeuN labelled cells, P=0.018, 65% increase in NeuN+ cells, P=0.04 respectively) with respect to glial cells. In arcopallium (intermediate and dorsal), nucleus taeniae of the amygdala and olfactory bulb (Fig. 4.10 c) the majority of newborn cells are of neuronal type (labelling with NeuN), since only a small percentage are double labelled for GFAP and BrdU (data not shown).

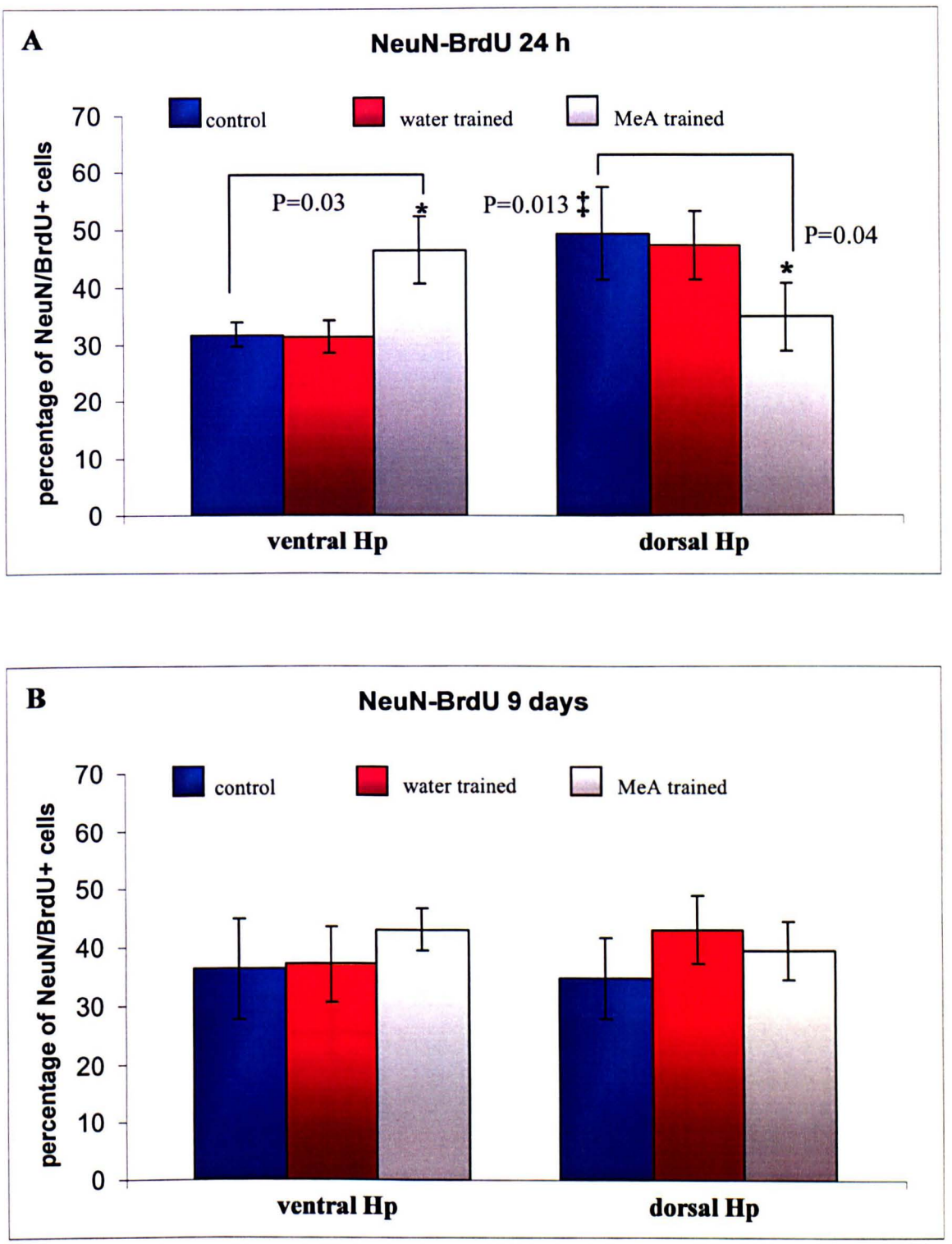


Fig. 4.11. Means of data showing the percentage of double labelled cells for NeuN and BrdU (control n=7, water n=7, MeA n=6) at P2 and P9 (control n=5, water n=5, MeA n=5). A. In the ventral hippocampus the MeA trained group shows more double labelled cells for BrdU/NeuN in relation to controls (P=0.03). The dorsal hippocampus shows reduced percentage of NeuN/BrdU + cells in the MeA trained group in relation to controls (P=0.04). ‡ indicates larger percentage of NeuN/BrdU + cells in the dorsal in relation to ventral Hp. Columns indicate means of data and vertical bars indicate standard error means (S.E.M).

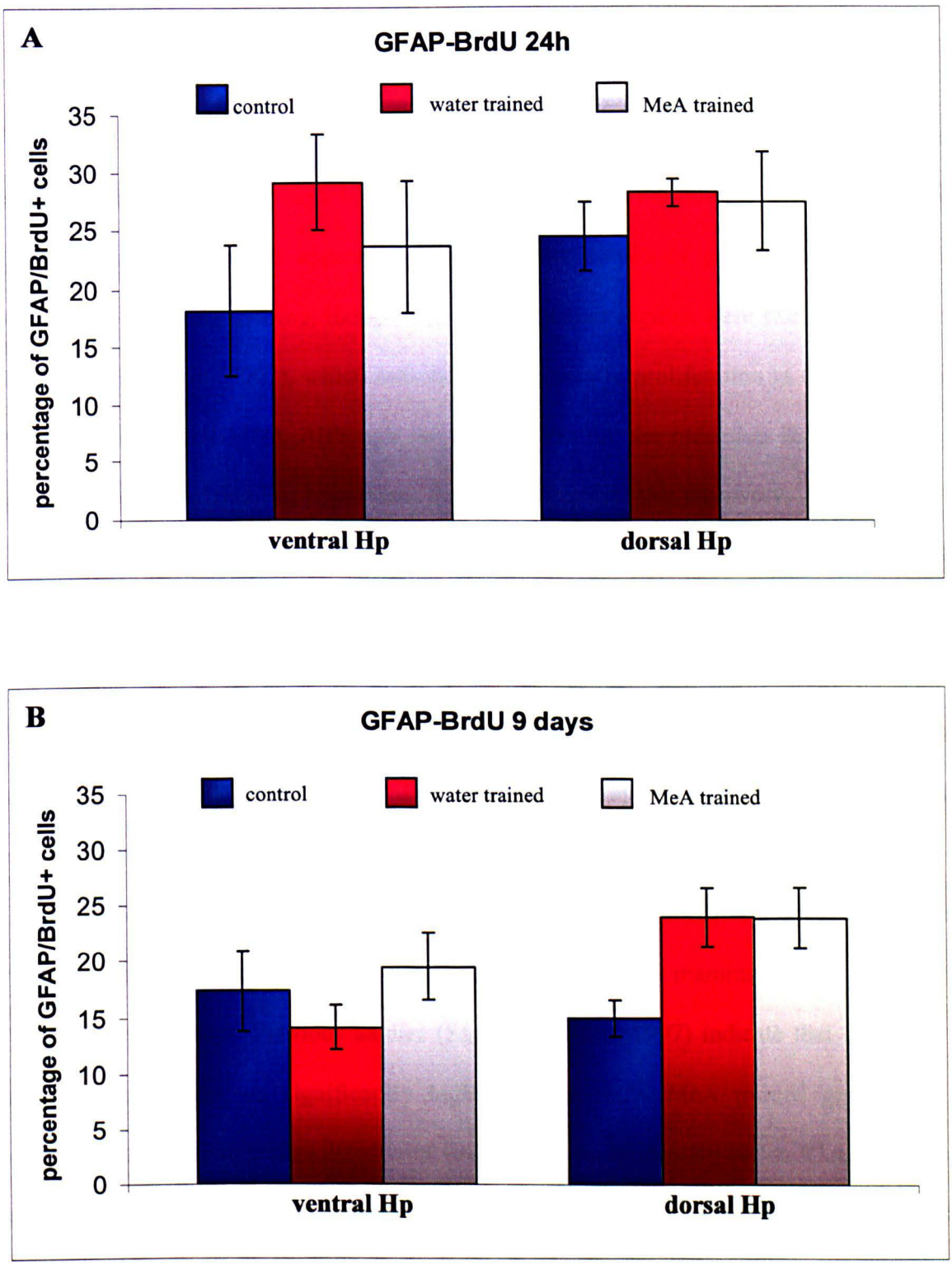


Fig. 4.12. Means of data for the percentage of GFAP/BrdU labelled cells in the dorsal and ventral hippocampus (control n=6, water n=6, MeA n=7) at P2 and P9. No significant differences occur in the percentage of glial cells between the 3 chick groups 24h or 9 days post BrdU injection. Columns represent means of data and vertical bars indicate standard error means (S.E.M).

4.3 DISCUSSION

These data indicate a reduction in cell proliferation 24 hours post BrdU injection in the dorsal hippocampus of the MeA trained animals in relation to controls, whilst no changes were observed in hippocampal volume between the three groups. This finding on cell proliferation was unexpected, because it appears to contradict the results of previous studies on cell proliferation following training, though different behaviour regions were examined, as in the case of Dermon et al. (2002), which indicated an increase in proliferation in the intermediate medial mesopallium (IMM). Although the explanation for the reduction in proliferation is unclear, there are two possible hypotheses that could elucidate this discovery.

Firstly, one may assume that cells undergo a higher rate of apoptosis in the MeA trained animals in comparison with control birds in the regions examined. However, apoptotic studies 24h and 9 days after BrdU administration did not reveal any differences in the number of apoptotic cells although it is possible that apoptosis occurs over a different time scales than those which have been examined here (data not shown). Apoptotic death may show a transient peak soon after training and therefore is not present 24h later.

A second explanation may be that passive avoidance training is a stressful condition which particularly affects cells in the hippocampus as occurs in mammals after similar types of aversive experiences. Previous studies (Sandi and Rose, 1997) indicate that the levels of plasma corticosterone are significantly higher in the 100% MeA trained group than in controls. Corticosterone is associated with stress in the rat hippocampus (de Kloet, 2000) and the effects of this stress may be expressed in terms of a reduced proliferative rate (Gould et al., 1998; Gould and Tanapat, 1999), especially in the dentate gyrus which is equivalent to the dorsal hippocampus of chicks. Furthermore, serotonergic fibres have been identified in the dorsomedial hippocampus (Metzger et al., 2002), which are known to be involved in stress responses (Gruss and Braun, 1997). Here, the double labelling studies with BrdU and NeuN

(nuclear neuronal marker) showed reduced NeuN positive cells in the dorsal hippocampus with respect to control animals. This finding may suggest that stress in the chick brain reduces neurogenesis, whilst it does not affect the number of glial cells (GFAP studies). In contrast, in the ventral hippocampus there is an increase in neurogenesis in the MeA trained group. It is apparent that the two subdivisions of the hippocampus react differently to PAL. This finding was not unexpected, since many studies have shown that in chicks the ventral hippocampus, in relation to the dorsal part, shows different cytoarchitecture (Karten and Hodos, 1967), electrophysiological properties (Siegel et al., 2002) and connectivity (Casini et al., 1986; Szekely and Krebs, 1996; Szekely, 1999; Hough et al., 2002; Kahn et al., 2003).

Furthermore, in mammals many studies have described the diverse roles of the dorsal and the ventral hippocampus; in particular, lesions of the dorsal hippocampus have resulted in impairments in learning the water maze training (Moser et al., 1993; Olsen et al., 1994), spatial tasks (Sinnamon et al., 1978; Moser and Moser, 1998) and fear conditioning (Maren et al., 1997), whilst biochemical alterations after water maze training have been found in the dorsal but not the ventral hippocampus of rats (Blum et al., 1999). In rats, the ventral hippocampus has been implicated in emotional and social actions (Moser and Moser, 1998). It should be mentioned, however, that the ventral and dorsal Hp differ in mammals and birds since in rats the ventral Hp refers to posterior parts of the brain unlike the case in the avian brain.

At 9 days post training in chicks the MeA trained group shows no further reduction in BrdU labelling in contrast to control and water trained animals. This might suggest that there are two main effects. Cell proliferation is reduced immediately after training in the MeA trained group, possibly due to the action of corticosterone. On the other hand, after the effects of stress have passed, cell death may be prevented by learning whilst cell survival may be enhanced as happens after learning in rats (Kempermann et al., 1998; Gould et al., 2000;

Leuner et al., 2004). Moreover, these newborn cells which mostly become neurones, migrate with the help of radial glia (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla, 1990b) and reach their final destination where they differentiate and stop dividing (Alvarez-Buylla and Nottebohm, 1988).

It is interesting to mention, though, that the control and water trained groups show significantly increased hippocampal volume at P9 in comparison with P2, unlike the MeA trained group, which only demonstrates 38% volume increase in the ventral Hp between 24h and 9 days post training, however statistical analysis showed that this increase is not significant, indicating possibly an effect of stress on hippocampal volume.

In the APH there is a significant reduction in cell proliferation in the water trained group compared to controls at P2. One explanation may be that the presentation of a novel object (bead) causes alterations in cell proliferation. Connectivity studies have demonstrated that the APH receives afferents from the HI/HD (Shimizu et al., 1995) which are major parts of the visual Wulst in the avian brain (Pasternak and Hodos, 1977; Watanabe, 2003). These projections may render APH sensitive to visual cues, causing synaptic modifications after visual experiences. Although reduction in cell proliferation occurs in the water trained group, double labelled studies for neuronal markers could shed some light on the events taking place in APH during the passive avoidance training. The fact that in the water trained group there are significantly more BrdU labelled cells in contrast to MeA trained animals might further support the idea that methylanthranilate can possibly invoke stress responses in some regions of the chick brain.

It is worth mentioning that after 9 days, the MeA trained group does not show significant reduction in cell proliferation in the APH. One explanation for this finding could be that cell survival is promoted by PAL after the possible effects of stress have passed or that cell proliferation is induced after the levels of corticosterone have gone back to basal levels.

However, we cannot exclude the possibility that both are simultaneously happening with the stronger effect having more influence on cell proliferation. Another hypothesis could be that the MeA trained group does not show reduced newborn cell density, because the volume of APH does not increase in P9 animals in contrast to the other groups.

Cell proliferation is increased in the AI (intermediate arcopallium, Reiner et al, 2004, previously called ventral intermediate archistriatum) of the MeA trained group in relation to the AD (dorsal arcopallium). AI has been suggested to show homology to the mammalian amygdala (Zeier and Karten, 1971). Since AI is sensitive to fear, it would also likely be affected by stress and respond to fearful conditions, by influencing avoidance behaviour which is translated into a refusal to peck the dry bead after PAL, because of the previous unpleasant experience. Lesions of the intermediate arcopallium have been shown to cause memory impairments for passive avoidance training (Lowndes and Davies, 1994) and filial imprinting (Lowndes et al., 1994). Although these studies have not distinguished between dorsal and ventral parts of the intermediate arcopallium, other data have suggested that the limbic part is the intermediate arcopallium, previously termed as ventral intermediate archistriatum, whilst the "dorsal intermediate archistriatum (dorsal arcopallium) is involved in somatosensory system regulation (Zeier and Karten, 1971; Davies et al., 1997).

The amygdala in mammals is activated by high stress and learning (Akirav et al., 2001), so there may be similar effects in the avian brain. The effects of passive avoidance training are expressed differently in the arcopallium and the hippocampus as occurs in mammals (Akirav et al., 2001). One possibility for the present result may be related to differential responses to stress and fear following the avoidance training experience. The AI, additionally, has been proposed to be part of a circuitry that connects the mesopallium intermediomediale and the striatum mediale and therefore it is strongly affected by PAL (Csillag, 1999), probably provoking avoidance behaviour.

An interesting point is that the AI receives afferents from the dorsal nidopallium (Nd) (Wild et al., 1993; Metzger et al., 1998), an area which has been shown to receive inputs from field L (Bonke et al., 1979) and nucleus ovoidalis (Wild et al., 1993), which are auditory areas, indicating possibly that the distress calls that chicks emit during PAL could further affect the AI. This might even affect control animals that hear the voices since all of the groups are at the same room, provoking activation of AI. In this case, cell proliferation may be also affected in the AI of control animals in a lesser extend, though this would be difficult to quantify without much further experiments. The AD in contrast is not affected by PAL, probably because it does not belong to the limbic system and for that reason it shows no differences among the groups, whilst it demonstrates reduced BrdU labelling in contrast to AI. Interestingly, though, serotonergic fibres were found in the AD and TnA (Metzger et al., 2002). Serotonin has been suggested to participate in stressful conditions such as social separation and 5HT+ fibres have been found in mesopallium after auditory filial imprinting (Gruss and Braun, 1997). Although the presence of serotonin in AD and TnA may not agree with the hypothesis that AD is part of the somatosensory system, and serotonin inhibition (Stephenson and Andrew, 1994) is associated with memory impairments in passive avoidance training in chicks, previous studies have not directly associated the AD with passive avoidance training. Rather they focus on intermediate arcopallial lesions (Lowndes and Davies, 1994) in general or the involvement of AI in a circuitry that mediates the passage of information from the IMM to StM (Csillag, 1999). Therefore, since serotonin is also involved in aggression and anxiety behaviour (Lucki, 1998) its presence in AD and TnA may imply social and emotional features, which would agree with studies in TnA and its involvement in male sexual anxiety in the presence of females and mating (Thompson et al., 1998).

Future double labelling studies could clarify if there are any disparities in neurogenesis between MeA trained and control groups in the AI. Although no variations in cell proliferation

are apparent, there might be alterations in the number of proliferative cells that develop into neurones. No volume changes were observed in AI and AD either between the groups or the diverse time points examined.

No differences in volume or newborn cell density are shown in the nucleus taeniae between the different groups. Although recent studies in the medial nucleus of the amygdala (homologue to the nucleus taeniae of the amygdala) of adult rats demonstrate a reduction in PSA-NCAM immunohistochemistry after chronic stress in the rat (Cordero-Campaña et al., 2004), here the reduction in cell proliferation is not significant. This might be explained by the fact that one day old chicks are not sexually mature, so they may display different activation of the nucleus taeniae of amygdala (TnA, Reiner et al., 2004).

Another possibility is that TnA may not be affected by PAL; studies have shown that it is involved in male sexual behaviour (Thompson et al., 1998; Absil et al., 2002), but there are no studies to indicate its participation in PAL. However, TnA receives afferents from the dorsolateral hippocampus (Casini et al., 1986; Szekely and Krebs, 1996; Atoji et al., 2002), and the olfactory bulb (Reiner and Karten, 1985), whilst it sends efferents to the APH (Cheng et al., 1999), the dorsal hippocampus (Atoji et al., 2002) and the StM and IMM (Cheng et al., 1999), areas that have been shown to be directly implicated in PAL.

It is noteworthy that at 9 days post training the MeA trained group is the only one that does not show significant reduction in cell labelling in relation to 24h after BrdU injection. This finding may indicate that TnA shows a slower response to PAL in respect to the other areas so far examined. Thus, in this nucleus there may be cell proliferation between 24h and 9 days, or cell survival may be enhanced after PAL.

No studies have been conducted in the chick brain to test cell proliferation or neurogenesis in the olfactory bulb after PAL. Although the olfactory bulb in rats has proven to be one of the areas where neurogenesis persists in adulthood (Kaplan and Hinds, 1977; Bayer,

1983; Luskin, 1993; Suhonen et al., 1996; Winner et al., 2002) no evidence exist for postnatal neurogenesis in the adult chick. Our studies indicate that neurogenesis continues in the olfactory bulb of chicks after they hatch. Double labelling experiments demonstrated that the majority of newborn cells become neurones, since only a very small percentage of BrdU labelled cells showed double labelling for GFAP and BrdU.

The BO of MeA trained animals especially shows significantly elevated cell proliferation with regard to controls and water trained animals. It is known that chicks react to smell (Wenzel and Sieck, 1972; McKeegan, 2002; McKeegan et al., 2002) so an increase in cell proliferation was not unexpected. An interesting point for behavioural studies is that chicks can smell methylanthranilate (MeA) and react aversively to it (Marples and Roper, 1997). It has been suggested that MeA may play a role in memory consolidation (Burne and Rogers, 1997). Other bitter but odourless substances (e.g. denatonium benzoate, quinine, Bourne et al., 1991; Marples and Roper, 1997) do not seem to be strongly aversive stimuli for chicks and have a weaker ability as aversive memory stimulants in PAL, suggesting a strong between limbic structures participating in PAL (e.g. mesopallium, arcopallium) and BO (Richard and Davies, 2000).

The BO shows connectivity with the hippocampus, especially the APH via the piriform cortex (Reiner and Karten, 1985; Bingman et al., 1994), TnA (Reiner and Karten, 1985) and IMM and StM (Rieke and Wenzel, 1978), all these areas potentially involved in avoidance learning. Furthermore, gene expression studies for vasoactive intestinal polypeptide (VIP) have revealed that the BO, along with the hippocampal complex, the piriform cortex, the mesopallium, hyperpallium densocellulare, hyperpallium intercalatum and medial and intermediate arcopallium demonstrated high levels of VIP mRNA, suggesting that they could be part of the visceral system, indicating possibly further connections between these areas (Kuenzel et al., 1997).

At 9 days post BrdU injection all of the areas of the control and water trained groups show reduced cell proliferation in comparison with animals sacrificed 24 hours after BrdU injection. The likelihood that this is due to migration of cells has yet to be proven. Another possibility is that BrdU is diluted due to continued cell divisions until the cells become postmitotic. Studies have shown (Dayer et al., 2003) that BrdU is diluted between 24h and 4 days post BrdU injection. Finally, newborn cells could undergo apoptosis as a process of plasticity in order for the brain to keep balance in incorporation of new cells and total neuronal density.

Interestingly, the only area where there are differences in cell proliferation 9 days after BrdU injection between the groups is the olfactory bulb (BO). In this nucleus, the MeA trained group continues to show increased BrdU labelling even 9 days post training, but this dissimilarity may be due to migration of cells from other highly proliferative areas such as the ventricular zone of StM, StM or tuberculum olfactorium (TuO) (Dermon et al., 2002), the latter having olfactory functions. Another explanation could be that the olfactory receptor neurones possess the ability to regenerate throughout adult life (Barber, 1982). Even so, there is a significant reduction in the number of BrdU labelled cells between 24h and 9 days in this group. It is interesting that the BO volume does not change between 24h and 9 days post training.

It is noteworthy that unlike the rat dentate gyrus where newborn cells require three weeks to mature (Cameron et al., 1993; Kuhn et al., 1996), in the chick Hp we found double labelled cells for NeuN and BrdU 24 hours after bromodeoxyuridine injection. The percentage of new neurones did not appear to alter between short and long term survival times of newborn cells. One explanation may be that the cells can express the proteins of mature neurones at an early stage after genesis although they are not fully mature. On the other hand, surprisingly, none of the cells that resided on the ventricular zone co-expressed NeuN and

BrdU, even 9 days after birth. These cells may be immature neurones since there were not labelled for GFAP. Thus 2 days old chicks have a less developed glial fibre network in comparison to 10 days old animals, which has been also shown from other studies (Kalman et al., 1998). In the hippocampus, labelling for GFAP is mainly localised at P1 in the pial surface and less intensely in the ventricular zone, where the fibres are shorter and less closely packed. However, the chick brain expresses GFAP (labels astrocytes) from the first day post hatching (Kalman et al., 1998), indicating rapid maturity of the chick brain from the early stages of life.

Further studies are necessary in order to clarify precisely the events which occur in the chick hippocampus after passive avoidance learning. Studies concerning corticotrophin-releasing factor (CRF), which is involved in stress and anxiety responses, have shown an extensive network of fibres containing CRF in the hippocampus, the arcopallium, the nucleus taeniae of the amygdala and other areas (Richard et al., 2004). If the chick hippocampus is similarly affected by stress, as in mammals, then the chick model may be additionally exploited to study stress and neurodegeneration.

CHAPTER 5

CORTISOL IN THE CHICK BRAIN AND THE EFFECTS OF PASSIVE AVOIDANCE LEARNING

5.1 INTRODUCTION

Stress is a condition that can cause morphological changes to the brain (Fuchs et al., 2001) and may also incite pathological conditions such as memory impairment (Liu et al., 2003; Sauro et al., 2003; Shors and Leuner, 2003). Excess glucocorticoids can cause synaptic remodelling in pyramidal neurones of CA3 in the rat hippocampus (Woolley et al., 1990), restraint stress can decrease branching and dendritic length (Watanabe et al., 1992; Magarinos and McEwen, 1995; Sandi et al., 2003), effects similar to psychosocial stress (Magarinos et al., 1996). In contrast, restraint stress has been demonstrated to increase branching in the amygdala, indicating the fear causing character of the training (Vyas et al., 2002). Recent studies have also shown reduced dendritic branching in CA1 and dentate gyrus (Sousa et al., 2000).

Neurogenesis can be affected by high levels of corticosterone (Gould and Tanapat, 1999; Harman et al., 2003; King et al., 2004). Corticosterone injections during the first postnatal week caused deprivation of granule cell precursors in the dentate gyrus of the rat (Gould et al., 1991b). Other studies have shown that adrenal hormones can reduce cell proliferation in the dentate gyrus (Gould et al., 1992). Adrenalectomy, on the other hand, has been shown to increase neurogenesis (Gould et al., 1992; Cameron and Gould, 1994) indicating the likelihood that adrenal steroids create a natural boundary for cell death and neurogenesis or gliogenesis. Neuronal loss is determined also by the type of the stressor, for instance, acute stress such as the odour of a predator inhibits neurogenesis (Tanapat et al., 1998), albeit acute restraint stress does not suppress neurogenesis (Pham et al., 2003). These authors have demonstrated that repeated restraint stress although reducing neurogenesis, also causes a concomitant increase in the expression of polysialic acid neural cell adhesion molecule (PSA-NCAM), which agrees with recent light and electron microscopy studies (Cordero et al., 2003).

Although moderate levels of corticosterone has been demonstrated to enhance memory formation (Sandi et al., 1997), high levels of the above mentioned hormone can impair memory facilitation and learning (Diamond et al., 1999). Stress can affect learning by a U form of corticosterone concentrations; very low or extremely high levels of CORT (Anderson, 1976) do not improve memory. In the chick brain corticosteroid receptors blockage causes amnesia to one day old chicks (Sandi and Rose, 1994b), whilst corticosterone enhances memory formation for the weal passive avoidance task (Sandi and Rose, 1994a), implying that a moderate amount of stress is necessary for memory formation (Sandi and Rose, 1997). These authors have demonstrated that high levels of corticosterone did not help the animals remember the training task and have also shown an increase in the levels of plasma corticosterone 5 min post passive avoidance training (Sandi and Rose, 1997).

Corticosterone has been shown to be the dominant stress steroid present in the chick brain (Nagra et al., 1960; Urist and Deutsch, 1960; Kalliecharan and Hall, 1976). Quantitative studies in the chick brain have demonstrated that at 2 day old chicks except from corticosterone, other steroids circulate also in the plasma, and cortisol comes second in concentration after corticosterone (8.75ng/ml and 17.20ng/ml). Adrenal steroids including progesterone and cortisone are also present, but in lower concentrations (Kalliecharan and Hall, 1974). Other studies have shown that the levels of circulating cortisol are much lower in the chick brain; in particular it has been suggested that the levels of cortisol in the plasma decline after hatching, in the 17^{th} day of embryonic life being 1.7ng/ml, reaching 0.8 and 0.3ng/ml by 3^{rd} and 7^{th} day post hatching to disappear at 14 day old chicks (Nakamura et al., 1978). Nonetheless, cortisol is present in young chicks and indicates the existence of 17a-hydroxylase which is necessary for cortisol formation, unlike rats and mice which lack the enzyme (McEwen et al., 1976). It is interesting to mention that cortisol is the dominant steroid in the human brain (Underwood and Williams, 1972; West et al., 1973).

Data here from cell proliferation, neurogenesis and synapse formation studies indicate a reduction in the MeA trained group in relation to controls, especially in the dorsal hippocampus. However, data from apoptotic studies (chapter 5) did not resolve the question about the reason for cell loss present in the MeA trained group. Passive avoidance learning has been claimed to be a stressful experience due to the elevation of plasma corticosterone in the chick brain (Sandi and Rose, 1997). Based on the experiments of the latter authors, the levels of cortisol were examined in the present in chick brain tissue 5 and 20 minutes after passive avoidance training. Studies focused on the hippocampus, the striatum mediale, an area strongly associated with learning (Stewart and Rusakov, 1995; Csillag, 1999; Dermon et al., 2002) and arcopallium.

The purpose of this study was to investigate the effects of PAL on cortisol levels that may demonstrate if PAL is a stressful paradigm which could explain the synapse density and cell proliferation reduction observed in the hippocampus.

5.2 RESULTS

5.2.1 Five minutes after training

Data for ng of cortisol/g of brain area are presented in Fig 6.1 and table 6.1. Three way ANOVA indicated significantly statistical differences for the area ($F_{2,14}$=5.3, P=0.019) of study, the minutes after training ($F_{1,14}$=145.94, P<0.0001), the training group ($F_{2,14}$=33.87, P<0.0001) and the interaction between area and group ($F_{4,14}$=23.14, P<0.0001), between minutes after training and group ($F_{2,14}$=20.82, P<0.0001) and between area, minutes and training group ($F_{4,14}$=30.43, P<0.0001).

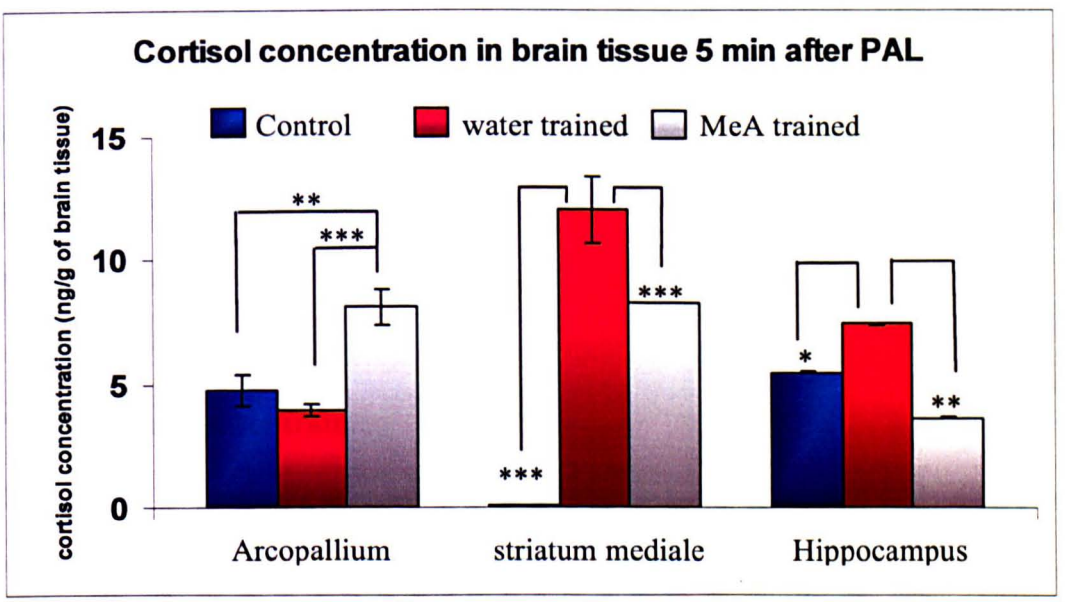


Fig. 5.1. Means of data showing the concentration of cortisol in ng per g of brain tissue (ng/g) in the arcopallium, the striatum mediale and the hippocampus of control, water trained and MeA trained group 5 (developmental control n=12, water trained n=14, MeA trained n=14) minutes after PAL. Five minutes after PAL in the water trained group the striatum mediale and the hippocampus shows higher concentration in relation to control and MeA trained birds. In the arcopallium the control and water trained groups show lower levels of cortisol in comparison with controls. Columns represent means of data and vertical bars indicate standard error means (S.E.M).

In the arcopallium, the MeA trained group shows increased cortisol concentration in relation to control and water trained birds. In particular, the MeA trained animals demonstrate a 72% (P= 0.00037) and 107% (P<0.0001) increase in comparison with the control and water trained group respectively. The arcopallium of the control group shows increased cortisol

levels (47 times higher) in relation to the striatum mediale (P<0.0001). In contrast, the water trained group demonstrates higher levels of cortisol in the striatum mediale (207% increase, P<0.0001) and Hp (89% increase, P=0.0015) in comparison with the arcopallium. The MeA trained group demonstrates higher levels of cortisol in the arcopallium in relation to Hp (124% increase, P=0.00018). In the striatum mediale, the control group shows 55 times lower levels of cortisol in comparison with the Hp (P<0.0001). The water trained group shows significantly higher levels of cortisol in relation to control (120 times higher, P<0.0001) and MeA trained birds (46% increase, P=0.00074) in the striatum mediale, whilst the MeA trained group is 82 times higher than the control group (P<0.0001). The water trained and the MeA trained groups of the striatum mediale show 62% and 127% increased levels of cortisol in relation to Hp (P=0.00014, P=0.0005 respectively). Finally, in the hippocampus, the water trained group shows a 35% and 105% increase in comparison with the control (P=0.048) and MeA trained (P=0.00075) groups.

5.2.2 Twenty minutes after training

Data for ng of cortisol/g of brain area are presented in Fig 6.2 and table 6.2. Twenty minutes after PAL, the arcopallium shows reduction in cortisol concentration in all groups in relation to results conducted 5 minutes post training. The control group shows a 64% decrease at 20 minutes in comparison with 5 minutes (P<0.0009), the water trained group shows 42% reduction (P=0.039), whilst the MeA trained group shows the most considerable reduction (67%, P<0.0001) in relation to the MeA trained group sacrificed 5 minutes after training. The levels of cortisol in the MeA trained group in the arcopallium 20 minutes after training are lower (39% decrease, P=0.035) in comparison with the Hp. In the striatum mediale data show a contrasting pattern; the control group shows 26 times increase 20 minutes after training in relation to the results from 5 minutes (P=0.0044), whilst the water and the MeA trained group

show a 79% (P<0.0001) and 53% (P=0.0002) decrease respectively. Although the MeA trained group in the striatum mediale shows a 46% and 49.5% increase in relation to the control and water trained groups, these differences are not significant (P=0.1 and P=0.12 respectively). In the hippocampus, the control group shows reduction in relation to five minutes training (59% decrease, P=0.0028). Most importantly, the MeA trained group shows increased cortisol levels in comparison with the control (91% increase, P=0.013) and water trained (85.5% increase, P=0.016) birds.

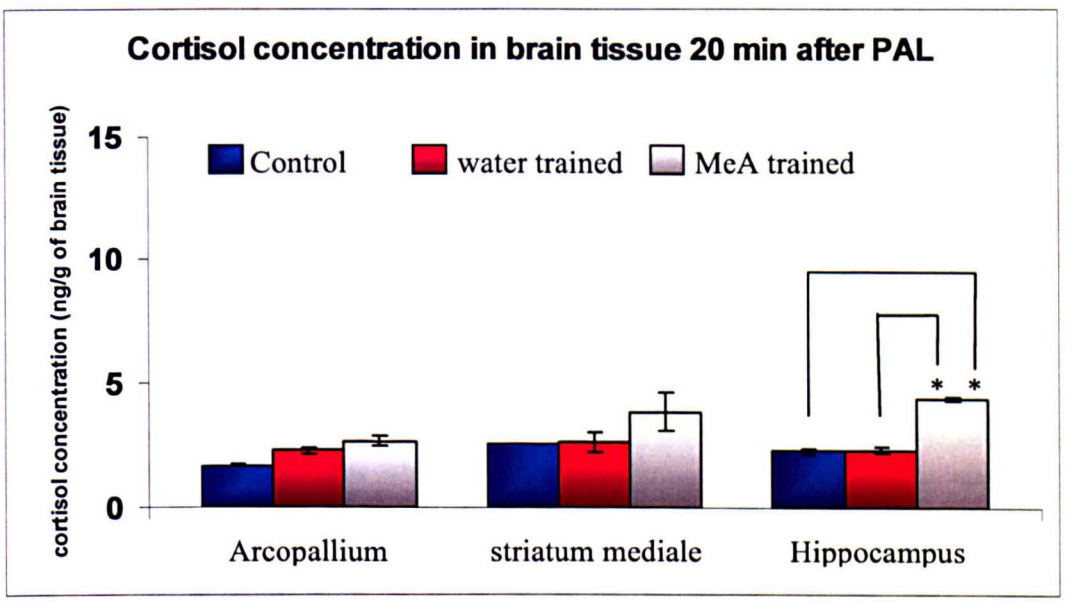


Fig. 5.2. Group mean values showing the concentration of cortisol in ng per g of brain tissue (ng/g) in the arcopallium, the striatum mediale and the hippocampus of control, water trained and MeA trained group 20 minutes (developmental control n=12, water trained n=14, MeA trained n=14) Twenty minutes after training, only in the hippocampus the MeA trained group shows significantly higher cortisol levels in relation to control and water trained groups. Columns indicate means of data and vertical bars indicate standard error means (S.E.M).

Table 5.1 Data from cortisol radioimmunoassay demonstrating the levels of cortisol in brain tissue (ng/g) ± S.E.M **5 minutes** after passive avoidance learning. Control animals are developmental controls

	CONTROL	WATER TRAINED	MeA TRAINED
Arcopallium	4.73±0.65(**)	3.93±0.27(***)	8.12±0.73
Striatum mediale	0.105±0.001(***)	12.05±1.35	8.23±0.008(**)
Hippocampus	5.49±0.02(**)	7.43±0.03	3.63±0.05(**)

Asterisks (*) indicate significant differences between the groups in the area of study. Underlined numbers demonstrate differences between the different areas in the same training group.

Table 5.2 Data from cortisol radioimmunoassay demonstrating the levels of cortisol in brain tissue (ng/g) ± S.E.M **20 minutes** after passive avoidance learning. Control animals are developmental controls

	CONTROL	WATER TRAINED	MeA TRAINED
Arcopallium	1.7±0.05	2.28±0.12	2.64±0.17
Striatum mediale	2.56±0.01	2.63±0.41	3.84±0.77
Hippocampus	2.27±0.12(*)	2.34±0.13(*)	4.34±0.09

Asterisks (*) indicate significant differences between the groups in the area of study.
Underlined numbers demonstrate differences between the different areas in the same training group

5.3 DISCUSSION

The data presented in this chapter indicate that after passive avoidance training there is an increase in the levels of cortisol in the arcopallium, the striatum mediale and hippocampus, all areas being closely influenced by PAL (Sandi et al., 1992; Hunter and Stewart, 1993; Lowndes and Davies, 1994; Csillag, 1999; Dermon et al., 2002). Cortisol has been suggested to be the main adrenal steroid in the primate brain (Uno et al., 1994; Magarinos et al., 1996; Karssen et al., 2001), whilst it has been shown to be one of the major steroids in the chick brain during embryonic and early postnatal life (Kalliecharan and Hall, 1974, 1976; Nakamura et al., 1978). In primates, it has been exhibited that high levels of cortisol are excreted after stressful conditions and in subjects suffering from depressive diseases (Swigar et al., 1979; Lupien et al., 1999; Ng et al., 2003; Pico-Alfonso et al., 2004; Ritsner et al., 2004).

Stress has been shown to have a severe impact on synaptic plasticity (Magarinos and McEwen, 1995; Sandi et al., 2001; Sandi et al., 2003), whilst it has been also demonstrated to reduce neurogenesis (Lemaire et al., 2000; Pham et al., 2003). However, although high levels of stress impair memory (Bodnoff et al., 1995; Sousa et al., 2000), moderate amounts of stress are needed during learning procedures to enhance acquisition of the task (Sandi and Rose, 1994a; Sandi et al., 1995; Loscertales et al., 1997; Sandi et al., 1997).

Earlier studies have demonstrated that PAL can be a stressful experience; in particular, Sandi and Rose (1997) have shown that the levels of circulating plasma corticosterone are higher in the MeA trained group in relation to controls 5 minutes after passive avoidance training, implying the possibility of a stress-related memory impairment in the MeA trained group after methylanthranilate administration (usually ~30% of the MeA trained birds cannot remember the task in comparison with the water trained group which show lower percentages of inability to recall the task).

Stress has been closely associated with the limbic system (Vermes and Telegdy, 1977; Sapolsky et al., 1985a; Cook, 2002; Sapolsky, 2003). In mammals, the limbic association area is related to emotions and memory storage (Sapolsky et al., 1985a).

In monkeys, it has been suggested that the limbic system is also closely associated to imprinting (Mishkin, 1982), a memory task that has been extensively used for studies in the chick brain (Horn et al., 1985; Horn, 1998). Lesions in the arcopallium has been demonstrated to impair the acquisition of imprinting (Lowndes et al., 1994), whilst the arcopallium has been suggested to be equivalent to the mammalian amygdala (Zeier and Karten, 1971; Davies et al., 1997), which implies that it is affected by stress and emotions as in mammals (LeDoux et al., 1990b; LeDoux, 1995) indicating a strong link between functions of the mammalian and the avian limbic system. The nucleus taeniae of the amygdala is an area which plays an important role in male sexual behaviour (Thompson et al., 1998) and is homologous to the medial amygdala, an area that has been also associated with stress responses (Cordero et al., 2004). Finally, the limbic system is involved in learning, stress (Sapolsky, 2003) and movement strategy, functions that both in mammalian and avian species are regulated by the hippocampus (Moser et al., 1993; Olsen et al., 1994; Gould et al., 1999b; Sandi et al., 2001; Shiflett et al., 2004; Suge and McCabe, 2004).

In the present studies, changes in the levels of cortisol were observed both 5 and 20 minutes post training. The arcopallium, which is homologous to the mammalian amygdala (Davies et al., 1997), would be expected to react to stress in a similar way as in rats (Dayas et al., 1999; Akirav et al., 2001; Vyas et al., 2002). Five minutes after PAL, the levels of cortisol are elevated in the MeA trained group in relation to control and water trained birds. However, this effect is not present 20 minutes post training. The striatum mediale on the other hand, shows a gross increase of cortisol in the water trained group in relation to control and MeA trained group. The control group particularly shows miniscule levels of cortisol 5 minutes

after training, and twenty minutes after training, these effects do not exist anymore. It is noteworthy that both areas show a reduction in cortisol levels in all the chick groups 20 minutes after PAL. The striatum mediale of the MeA trained group shows a 59% reduction 20 minutes after PAL in relation to levels at 5 minutes, in comparison to the water trained group which shows a 79% decrease. As a result, 20 minutes after training, the MeA trained group shows higher cortisol levels in the striatum mediale in relation to the water trained birds. In contrast, in the arcopallium, where 20 minutes after training the MeA trained group shows a 67% reduction, the water trained group shows only a 42% decrease. It is interesting to note that the levels of cortisol 20 minutes after training in these two groups resemble the levels of the control group, indicating possibly a return of cortisol to the basal levels in the arcopallium of these groups.

It is noteworthy that the only area which shows an increase in cortisol levels after 20 minutes is the hippocampus and specifically the MeA trained group. The control and water trained group show a 59% and 68% reduction 20 minutes after training in relation to the levels at 5 minutes, whilst the MeA trained birds demonstrate a 8.4% increase in the levels of cortisol in the hippocampus. Consequently, 20 minutes after training the MeA trained group exhibits 91% and 85.5% increased cortisol levels in comparison with the control and water trained birds respectively.

This raises a question: does this finding indicate a later onset of changes in the hippocampus of the MeA trained group, or does it imply a longer lasting effect in this area? In Chapter four, it was demonstrated that the dorsal hippocampus of the MeA trained group shows reduced cell proliferation and neurogenesis in relation to control and water trained group. Could these two events be connected? Possibly, since it is known that stress reduces neurogenesis (Gould et al., 1997; Lemaire et al., 2000). If the effects of cortisol in the chick hippocampus last longer, then it is possible that reduction in cell proliferation would occur,

especially in the dorsal part, which is homologous to the dentate gyrus (Erichsen et al., 1991; Krebs et al., 1991; Siegel et al., 2002), as happens in mammals (Pham et al., 2003). Furthermore, studies in the ventral hippocampus of rats have shown that it is not as heavily affected by learning and stress as does the dorsal part (Moser and Moser, 1998; Akirav et al., 2001). Therefore, it might be suggested that the chick ventral hippocampus shows a milder reaction to stress resulting in minor cell proliferation reduction in comparison with the ventral part.

Although the arcopallium of the MeA trained group and the striatum mediale of the water trained groups and hippocampus show high levels of cortisol five minutes after passive avoidance training, cell proliferation is not reduced in these areas, in any group 24h post BrdU injection, not as Dermon et al. (2002) have shown, are there any increases in BrdU labelled cells in these areas. There is the possibility that at longer time points (e.g. 20 minutes or even longer) cortisol changes are required to induce cell proliferation reduction. For this reason, it may be that the hippocampus is the only area demonstrating numerical cell density reduction 24h after training in the MeA training group. Another explanation could be that the arcopallium and the striatum mediale are affected by elevated levels of cortisol in a diverse way and therefore do not demonstrate the typical characteristics of stress such as reduced BrdU labelling. Double labelling studies in the arcopallium could enlighten the present data by demonstration of dual labelled cells for BrdU and NeuN, an indication of neurogenesis: although cell proliferation may not appear affected, neurogenesis may be.

The increases in cortisol concentration in the water trained group could indicate that the actual presentation of the bead causes a stressful reaction on discrete brain regions. However, this assumption cannot be fully supported with the data presented in this thesis. Further cortisol studies at earlier time points might be able to enlighten the true facts happening in the chick brain after water training. It may also be possible that the water trained group shows an

earlier response to the training in relation to the MeA training group. Studies ten or fifteen minutes after PAL could demonstrate if the MeA trained group shows significantly higher cortisol levels in the striatum mediale or the arcopallium.

It would also be interesting to check the time availability of higher cortisol levels in the hippocampus of the MeA trained group. Are twenty minutes of high cortisol levels enough to enhance cell birth inhibition? Possibly the effects of cortisol even for only twenty minutes may have negative effects on the cell cycle and as a consequence on cell proliferation.

To sum up, cortisol levels are elevated in a number of the brain areas studied, all parts of the training experience, indicating probably a stressful reaction to passive avoidance training. Nonetheless, only the hippocampus shows cortisol elevations twenty minutes after training, whilst at this time point the arcopallium and the striatum mediale demonstrate a significant fall of cortisol levels. It could therefore be assumed that the hippocampus may be the only area studied so far that is most strongly affected by stress, since both synapse formation and cell proliferation studies have demonstrated reduction in synapse and cell genesis respectively in the MeA trained group, in contrast to the striatum mediale (Hunter and Stewart, 1993; Dermon et al., 2002) and intermediate medial mesopallium (Patel and Stewart, 1988). If this hypothesis is proven, then the chick model could be used for future studies regarding neurodegenerative diseases caused by stress.

CHAPTER 6

DISCUSSION

AND

FUTURE STUDIES

6.1 The main findings of research in this thesis can be summarized as follows:

- 5 minutes after PAL: increased levels of cortisol in arcopallium of the MeA trained group in relation to control and water trained birds
- 20 minutes after PAL: increased levels of cortisol only in the Hp of the MeA trained birds in relation to control and water trained groups.
- 6 hours after training: numerical synaptic density reduction of axospinous synapses in the dorsal Hp of the right hemisphere
- 24 hours after training: numerical synaptic density reduction of axodendritic synapses in the dorsal Hp of the right hemisphere
- 24 hours post BrdU injection: cell proliferation and neurogenesis reduction in the MeA trained group in comparison with control birds in the dorsal Hp
- 24 hours and 9 days after PAL: increased cell proliferation in the olfactory bulb of the MeA trained group in relation to control and water trained animals
- 9 days after training: no increase of hippocampal volume of the MeA trained animals in comparison with 24h after training, in contrast to control and water trained groups

6.2 STRESS?

PAL appears to be a stressful experience based on data from Sandi and Rose (1997), and from the data presented here after measurements of cortisol in brain tissue. Stress has been suggested to reduce cell proliferation (Gould et al., 1991b; Gould et al., 1992; Gould et al., 1998) and increase cell death (Reagan and McEwen, 1997; Lucassen et al., 2001; Lee et al., 2002a; Minana et al., 2002). It is unclear whether stress attenuates the cell cycle and therefore

if cell proliferation is diminished. It has been suggested that stress induces apoptosis which in turn does not cause an inflammatory response due to rapid phagocytosis from macrophages (Wyllie et al., 1980). If newborn cells die as a result of stress immediately after their birth and are phagocytosed, then BrdU will not be detected. However, Roy and Sapolsky (2003), claimed that stress causes necrosis rather than apoptosis. Our apoptosis studies did not show significant differences between MeA and control groups (data not shown). If the hypothesis of Roy and Sapolsky is accepted, then apoptotic death should not be as considerable as necrotic. We have not tested necrosis, so we could not tell as certain if our results agree with this idea. Nevertheless, it is interesting to mention that other studies have shown that glucocorticoids do not lead to DNA cleavage (Masters et al., 1989), indicating that high levels of glucocorticoids could reduce cell number either by suppressing the survival of cells or by cell death without the characteristic signs of apoptosis.

Six and twenty four hours after PAL the synapse density was reduced in the dorsal Hp. In particular, the axospinal density of the MeA trained group demonstrated a reduction 6h after training in relation to control groups, which disappears at 24h after PAL. On the other hand, the shaft density of the MeA trained group appears reduced at 24h in respect to controls. Preiously, studies have demonstrated that stress causes apical dendrite atrophy in CA3 in rats (Magarinos and McEwen, 1995; Magarinos et al., 1996) and branching elimination (Woolley et al., 1990). Here, stress may be having similar effects, such as dendritic branching eradication and suppression of arborization with consequential synaptic changes.

Many studies have shown that the brain is a very plastic region and synaptic remodelling is a very rapid process; Toni et al. (2001) have shown that as soon as half an hour after LTP induction there is an increase of perforated synapses, indicating new synapse formation through splitting (Toni et al., 1999). Furthermore, studies in the chick brain have demonstrated a 77% increase of spine synapses in the right IMM 1h post training (Doubell and

Stewart, 1993). Thus, we can assume that 6h is a sufficient time scale to allow synaptic remodelling. However here there was a decline in synapse number in the MeA trained group, contrasting with previous results obtained from IMM and StM which show a spectacular multiplication of synapses and synaptic elements (Doubell and Stewart, 1993; Hunter and Stewart, 1993; Stewart and Rusakov, 1995). If PAL is a stressful experience, it would be expected that it should possibly affect other areas that have been shown to be affected by PAL, which in turn should demonstrate a decrease of synaptic density. However, this is not the case; our results from the cortisol studies in Hp, StM and arcopallium demonstrated that only the hippocampus shows significantly elevated levels of cortisol in the MeA trained group in comparison with the control and water trained group. There were no differences between the groups in StM and arcopallium.

The intermediate arcopallium has been suggested to be a limbic area (Zeier and Karten, 1971; Davies et al., 1997) and therefore probably affected by stress, since the arcopallium has been proposed to be homologous to the mammalian amygdala (Cohen, 1975; Dafters, 1975; Puelles et al., 2000).

However, because during dissection the whole area of the arcopallium was removed (including dorsal and posterior), differences in cortisol concentrations may have been counterbalanced between limbic (intermediate) and non limbic (dorsal) arcopallium. Nevertheless, cell proliferation studies did not show any differences between the diverse groups in the intermediate arcopallium suggesting that PAL may not be a sufficiently stressful experience to activate the arcopallium; in rats high stress activates in a diverse mode the amygdala and hippocampus, the former showing activation only after high stress accompanied by learning (Akirav et al., 2001).

Additionally, studies have shown that corticosterone is essential for PAL acquisition (Sandi and Rose, 1994a; Sandi et al., 1995) and water maze training (Sandi et al., 1997). So, if

the levels of corticosterone, and similarly of cortisol, are elevated due to stress, then learning is facilitated and the hippocampus experiences the effects of stress by exhibiting reduced cell proliferation and synapse genesis as it happens in rats and primates (Gould et al., 1991b; Magarinos and McEwen, 1995; Gould et al., 1998; Mirescu et al., 2004).

Nine days post training there are no effects in cell proliferation between the groups possibly because the effects of elevated glucocorticoids have faded away. As Sandi and Rose (1997) have shown, 15 minutes after PAL, corticosterone returns to its basal levels. In tissue, however, the changes occurring are quite different. Cortisol levels show significant arousal in the MeA trained group in relation to controls and water trained 20 minutes after training only in the chick hippocampus. Additionally, only the above mentioned group shows higher levels of cortisol 20 min after PAL when compared to 5 min. Although the explanation for this finding is unclear, it may indicate that adrenal steroids are present in the brain tissue for longer than in plasma. Another explanation could be that elevated glucocorticoids first pass into the circulation (blood) and then travel into the hippocampus, pointing possibly to a later response in the brain in comparison with plasma, meaning that since cortisol needs more time to invade the brain, the time points of peak stress steroid concentrations in the brain may appear later in relation to the blood.

The arcopallium and the striatum mediale on the other hand show elevated levels of cortisol in the MeA and water trained group respectively 5 minutes after training, however these effects are not present twenty minutes after training implying a shorter term exposure to cortisol. Furthermore, the water trained group has demonstrated higher levels of cortisol 5 minutes after training in the chick hippocampus. Although an answer to these data cannot be specified, it is important to mention that in none of these chick groups, in the areas studied, is there increased cell proliferation 24h after BrdU injection. On the contrary, the striatum mediale shows increased levels of cell proliferation in the MeA trained group 9 days post

training (Dermon et al., 2002), whilst data presented in Chapter four indicate a slight reduction in the water trained group in the ventral and dorsal Hp 24 after PAL in relation to controls, whilst the MeA trained group in the intermediate arcopallium shows no differences in comparison with control birds. It may, therefore, be presumed that significant reductions in cell proliferation exist mainly after long term action of cortisol.

In conjunction with the results from the volume estimation, it might be suggested that the persistence of cortisol in the MeA trained group 20 minutes after training could be responsible for the lack of volume increase in this group 9 days post training. In tree shrews for example, it has been shown that psychological stress causes a reduction in hippocampal volume (Ohl et al., 2000), whilst in rats chronic restraint stress induces a 5% volume reduction in the granule cell layer (Pham et al., 2003).

6.3 FUTURE STUDIES

The studies described in this thesis indicated that one trial passive avoidance learning induces synaptic and neuronal plasticity alterations in discrete areas of the chick brain. Additionally, apoptotic and cortisol studies were conducted in order to investigate the reductions in synapse and cell genesis observed in the chick hippocampus. Although the latter studies suggest that PAL may be a stressful experience for the hippocampus, further studies need to be performed to elucidate this finding by examining cell proliferation, synapse density levels and tissue cortisol levels at different times after training.

6.3.1 Synaptogenesis studies in the chick dorsal and intermediate arcopallium

Electron microscopy studies could reveal if there is synaptic reorganization in the arcopallium of the chick brain. It has been suggested that the arcopallium is affected by PAL in chicks, whilst studies in rats have shown that the amygdala, is involved in stress and

learning. Differences between the distinct groups as well as between the areas (AI and AD) or the hemispheres could enlighten the events occurring in the chick brain after PAL.

6.3.2 Neurogenesis studies in the arcopallium

Double labelling studies may help investigate possible differences in neurogenesis in the two functionally diverse areas of the arcopallium. The data in chapter four showed an increase in BrdU labelling in the AI of the MeA trained group in comparison with the AD 24 hours after training. Is neurogenesis also increased in the AI? Are there any differences in the percentage of double labelled cells for BrdU and NeuN between control, water trained and MeA trained birds? These questions could be possibly answered by confocal microscope imaging.

6.3.3 Cell proliferation in the Hp

Cell proliferation studies demonstrated a reduction in BrdU labelled cells in the dorsal Hp. Although synaptogenesis data indicate also a decrease of axodendritic synapses in the dorsal Hp, the two results could be better matched if cell proliferation had been studied separately in each hemisphere. No studies so far have examined cell proliferation for hemispheric asymmetries, it would be interesting to know if cell birth shows 'hemispheric preference' as it occurs during synapse formation. Furthermore, since a decrease in axospinous synapses was found 6 hours after training, cell proliferation studies at this time point could exhibit possible links between synaptic and neuronal plasticity.

6.3.4 Neurogenesis studies in the nucleus taeniae and olfactory bulb

Although double labelling studies have shown that the majority of newborn cells in both areas become neurones, a quantitative analysis could clarify the rate of neurogenesis and

gliogenesis in these two brain regions. Furthermore, it has been suggested that TnA is a sex related area implying possibly sexual dimorphism in cell proliferation between male and female birds.

6.3.5 Apoptotic studies

Earlier studies have shown that stress affects cell survival and death. Apoptotic studies one to two hours after PAL could illuminate the effects of PAL in cell survival and cell death. Another interesting time point for future studies is twenty minutes after PAL, since radioimmunoassay studies showed increased cortisol levels in the MeA trained group of the chick Hp. Furthermore, the AI, AD, BO and TnA would be important to be tested for differences in cell death which could then give a clearer explanation of the events taking place after PAL. Finally, TUNEL studies 5 minutes after PAL could elucidate any possible alterations in cell proliferation in arcopallium dorsale and intermediale.

6.3.6 Cortisol studies 10, 30, 45 minutes and 1 hour after PAL

Cortisol levels are decreased 20 minutes in comparison with 5 minutes after passive avoidance training in arcopallium and striatum mediale. However, in the Hp, the levels of MeA trained group show no changes between 5 and 20 minutes. Additionally, 20 minutes after PAL, the MeA trained group demonstrated increased levels of cortisol in relation to control and water trained birds. Is this effect long-lasting? And if yes, how long does it last? Different time point studies twenty minutes post training would clarify the duration of the cortisol effect in the chick hippocampus. If the effects appear to be long lasting, then the hypothesis that PAL is a stressful experience will be able to be strongly supported. Furthermore, studies 10 minutes after PAL could possibly demonstrate if cortisol shows a

similar decline to that observed after 20 minutes, which would possibly clarify the time course of cortisol availablility.

CONCLUSION

Although the questions remaining to be answered are many, the studies conducted for this thesis showed a different aspect of PAL and its effects firstly in the chick hippocampus and secondly in other areas of the limbic system. If future studies prove that PAL induces stress-related alterations, then the chick model could be possibly further used for the study of neurodegenerative diseases.

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