Synaptic Plasticity in the Chick Lobus Parolfactorius During Development and Following Memory Formation

Thesis

SYNAPTIC PLASTICITY IN THE CHICK LOBUS PAROLFACTORIUS DURING DEVELOPMENT AND FOLLOWING MEMORY FORMATION

Alistair Hunter BSc Hons (Anat)

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789 East Eisenhower Parkway
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DECLARATION

I hereby declare that this thesis is the work of the candidate. It describes the outline of experiments which were conducted solely by the candidate. It has not been accepted in any previous application for this, or any other degree. All verbatim extracts have been highlighted by quotation marks, and the sources of all information obtained from other studies are specifically acknowledged. Some of the data obtained from experimental studies conducted in respect of this thesis, have been published previously in learned journals, and as abstracts in conference proceedings.

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From the dreams on the barbed wire at Flanders and Bilston Glen,
From the Clydeside that rusts from the tears of its broken men,
For the realisation that all we've been left behind,
Is to stand like our fathers before us in the firing line.

(Derek William Dick 1987)

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ABBREVIATIONS USED

ANOVA	Analysis of variance
ASh	Asymmetric shaft synapses
ASp	Asymmetric spine synapses
GABA	γ amino butyric acid
$\bar{H}_{syn}$	Mean synaptic projected height
IMHV	intermediate and medial hyperstriatum ventrale
ITM	Intermediate-term memory
LPO	Lobus parolfactorius
LTD	Synaptic long-term depression
LTM	Long-term memory
LTP	Synaptic long-term potentiation
M-trained	Chicks trained to avoid a bead coated with methyl anthraniiate
N_{syn}	Synaptic number
Nv_{syn}	Synaptic density per μm^{-3}
PAL	Passive avoidance learning
PSD	Post-synaptic density
Sa_{syn}	Synaptic surface area
SSh	Symmetric shaft synapses
SSp	Symmetric spine synapses
STM	Short-term memory
$\bar{V}v_{bouton}$	Mean volume density of the synaptic boutons
$\bar{V}v_{shaft}$	Mean volume density of the dendritic shafts
$\bar{V}v_{spine}$	Mean volume density of the dendritic spines

ABSTRACT

The lobus parolfactorius (LPO) is an avian basal forebrain nucleus. The morphology of synapses within the LPO has been shown to be altered 24 hours following training of chicks (*Gallus domesticus*), using an aversive taste (methyl anthranilate) as the training stimulus (Stewart *et al* Brain Res. (1987) **426**:69-81). It is not known when synaptogenesis takes place in this nucleus during normal development, and what other factors may influence it. Lesion of the LPO has been shown to cause amnesia for the passively learnt task, only if made post-training (Gilbert *et al* in press). This suggests that the LPO is involved in storage of memory for the task, and not in its acquisition. The present study aimed to investigate the time course over which changes in synaptic morphology take place in the day-old chick LPO, and to clarify the onset of synaptogenesis during normal development.

Chicks were reared to the ages of either 16 days *in ovo*, 1-, 9-, or 22-days post-hatch, when they received anaesthesia intra-peritoneally (Sagatai, 6mg/chick). They were then killed by cardiac perfusion, with a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer. Each brain was extracted from its cranium, and the LPO was subsequently processed for electron microscopy. Unbiased stereological methods were used to make estimates of synaptic density (Nv_{syn}), on micrographs taken of a systematic random sample of electron microscopic fields. An estimation was also made of the mean projected synaptic height ($\bar{H}_{syn}$) within the EM section. Results indicated that the 1-day old chick has a lower complement of synapses compared with the 9-day old chick. There is a substantial increase in synaptic density between these ages, although there is a hemispheric asymmetry in this

increase, with the left hemisphere exceeding the right by 62%. Synaptic height does not change significantly between 16 days *in ovo* and 1 day post-hatch, but increases slightly on or before 9 days post-hatch, after which it remains stable.

The time-course study involved the training of day-old chicks, using the aversive taste of a bead coated with methyl anthranilate (M-trained), as the training stimulus. Control chicks were trained using water-coated beads. M-trained chicks avoided pecking a similar, but dry bead, 30 minutes later, whereas control chicks re-pecked. At 1, 6 12, 24 or 48 hours after training, chicks were killed by cardiac perfusion and the LPOs obtained as described above. Using unbiased stereological methods, estimates of Nv_{syn}, $\bar{H}_{syn}$, synaptic contact surface area (Sa_{syn}), mean dendritic shaft volume ($\bar{V}v_{shaft}$), mean dendritic spine volume ($\bar{V}v_{spine}$) and mean synaptic bouton volume ($\bar{V}v_{bouton}$) were made. A significantly larger mean Nv_{syn} (approx. 30%) was seen in the left hemisphere of M-trained chicks 24 hours after training, compared with control chicks. A difference of approximately 10% was seen in this hemisphere 48 hours post-training. M-trained chicks also had a greater mean Nv_{syn} (approx. 18%) in the right hemisphere at 48 hours. The estimators of synaptic size showed an increase predominantly in the left hemisphere of M-trained chicks. The analysis of $\bar{V}v_{bouton}$ was inconclusive, although no significant differences were found between control and M-trained chicks. $\bar{V}v_{spine}$ was significantly increased in the left hemisphere 48 hours following training, but no differences were found in the estimates of $\bar{V}v_{shaft}$ between the two groups. These results show that memory formation results in a number of lasting synaptic and dendritic morphological changes in the LPO, and that some of these changes are lateralized to the left hemisphere. The results are consistent with the hypothesis that synaptic plasticity occurs following long-term memory formation.

CHAPTER 1 : INTRODUCTION

1.1 : THE IMPORTANCE OF MEMORY

a) Types of memory: Some definitions

One of our most valued possessions is memory. We treasure experiences, and store a hoard of detail, some trivial, some profound. Memory can be broadly defined as the retention of knowledge, or experience. The acquisition of such memories is the process of 'learning'. if the memory is to be of benefit to the individual, there has to be ready access to the memory 'store'. That is, there has to be 'recall' of the memory, which may or may not lead to a change in the behaviour of the individual. It is via the modulation of behaviour that researchers are able to deduce that memory has occurred. A lack of behavioural change however, cannot be concluded to be from a deficit of memory.

The simplest form of learning is when an organism's responsiveness to a stimulation either increases (sensitization) or decreases (habituation). A more complex form of learning is that of association, or conditioned reflexes. This type of learning (classical conditioning) involves the association of a conditioned and an unconditioned stimulus. Establishing a conditioned reflex increases the probability that an initiaiiy ineffective, or neutral, stimulus will elicit a response that is normally given as a reflex to some other stimulus.

A second type of associative learning, is that of operant learning (Thorndike 1911). Operant learning involves the association of a reward or punishment with a particular behaviour. For example, if food is presented to a rat whenever a lever is pressed, the frequency of lever pressing will subsequently increase as a result of operant learning. This is a form of 'active reinforcement learning',

where reward is given for a particular behaviour. Many different paradigms of learning have arisen from this basic conditioning task. One may equally reward the lack of lever pressing, in which case one would describe the condition as 'passive reinforcement learning'. Another such paradigm is active avoidance learning, where the animal must elicit a given behaviour to avoid punishment. Similarly, passive avoidance learning, is where the animal can choose simply not to do something, that it might otherwise be tempted to do. Such a type of learning is the one chosen for investigation in this thesis.

The taste-aversion paradigm employed here to investigate the processes of memory formation in the chick, has been used in modified form in a much wider role. In the treatment of chronic alcoholism, the taste of beer or spirits has been paired with nausea, by giving the subject a powerful emetic. Aversion to alcohol results from this after repeated trials. The same principle is used to treat those suffering from obesity, by association of illness with the intake of certain foods.

There may be more than one type of memory. Recent clinical findings have shown that patients with an inability to recall recently learned events, can display recall for learned motor skills (Squire and Zoia-Morgan 1988). These two forms of memory have been termed 'declarative', where information can be brought into conscious recall, and 'procedural', where learned skills can be demonstrated sub-consciously (Squire and Zola-Morgan 1988). This latter type of memory may also be used to describe simple classical conditioning.

b) Amnesia

The fear of forgetting is common to most of us. Memory is so fundamental to human existence, that a person without the ability to remember, seems almost

non-human, and is likely to have great difficulty in coping with normal society. Unfortunately, this is the case for numerous patients suffering from Alzheimer's disease. This disease primarily affecting the aged population, results in a progressive loss of memory. There are currently over half a million sufferers in the UK. Loss of memory has also been seen in individuals involved in accidents with resultant serious head injury, or in cases of more calculated loss of brain tissue through surgery. At the opposite pole, there are some people who have an apparently limitless memory, a so-called 'iconic memory'. Such people can memorize pages from a book within minutes, and recite them back faultlessly. They appear to have what some may describe as a 'photographic memory', as though a visual image was simply being 'read' from their mind. Cases such as these provide a stimulus to the researcher. It is of great significance that one should try to understand the mechanisms involved in memory loss, by first gaining an insight into the process of memory acquisition and storage.

1.2 : A SHORT HISTORY OF MEMORY RESEARCH

In 1894 it was suggested by Ramon y Cajai that learning might be the result of prolonged changes in the effectiveness of connections between nerve cells, and that these changes may serve as the basis of memory (Ramon y Cajal 1894). This idea is still prevalent today and forms the main thrust of this thesis. This idea was developed further by the Polish psychologist Konorski in 1948, when he presented a paper describing changes within nerve cells as a consequence of sensory stimuli. These changes were two-fold; firstly, an invariant and transient excitability change, and secondly an enduring plastic change (Konorski 1948).

In a book entitled "The Organisation of Behaviour" published in 1949, Donald O. Hebb put forward the idea that 'ceil assemblies' may be organized within

nervous tissue, to form a reverberating closed system. It was postulated that this may act to cause structural changes associated with learning, after the initial stimulation has ceased (Hebb 1949). Hebb also described a mechanism whereby a growth of the PSD accompanied synaptic activity. It was suggested that this may reduce synaptic resistance and therefore increase synaptic efficacy (Hebb 1949). Hebb's theories have played an important role in subsequent studies of changes in brain morphology, as a consequence of learning and memory.

A popular hypothesis in the 1960's was that memory was coded by molecules synthesized by neurones. The molecular properties, shape or composite atomic sequence, could somehow code for a memory. It had been shown that long-term memory formation could be blocked by inhibition of protein synthesis (for review, see Deutsch 1969). This led to the idea that proteins might be responsible for information storage (Davis and Squire 1984). An equally plausible suggestion was that RNA may form the code, since RNA synthesis precedes protein synthesis (Hyden and Egyhazi 1962, Zemp *et al* 1966). This led to the 'transfer of learning' idea; that if memory was so encoded, it should be possible to train a given animal in a novel task, isolate the coded molecule that represents the acquired information, and transfer it to a naive animal. The latter, having been un-trained in the particular task, should respond in a manner akin to the trained animal (McConnell 1962). Many such studies were conducted in the 1960's and early 1970's (for review, see Fjerdingstad 1971), but a report by Dyal (1971) showed that of 133 published papers on this subject, 130 were negative or inconclusive. By the late 1970's, the publishing of papers attempting to demonstrate the direct transfer of information molecules, had ceased. The theory is now rightly viewed with some scepticism (Squire 1987).

Today, research on the cellular basis of learning and memory focuses more upon specific events in particular *loci* within the brain. Rather than memory being dictated by a diffuse molecule, memory is thought to be a consequence of cellular changes of the neuronal elements of the brain, and changes of circuitry within given brain regions. Thus, localization of memory has become an important issue, and this is discussed in detail in Chapter 2. Among the many cellular changes, the modulation of synaptic parameters is of particular interest here, and these are dealt with in Chapter 4, Section 3.

1.3 : MODELS USED TO STUDY MEMORY PROCESSES

Models are required to reduce the intrinsic difficulties in studying elaborate neural systems. Their purpose is to reduce the variables involved in the analysis, which are primarily environmental factors ('experience'). Memory is the result of an interaction between an individual and the environment, although it is sometimes guided by specific preferences (Horn 1985b). The drawback of many learning models is that the data obtained from them may not apply to the more complex learning paradigms. However, at a cellular level, models as diverse as habituation in *Aplysia*, avoidance learning in chicks and hippocampal long-term potentiation (LTP), provide data that show many similarities. Such data will be discussed later in this thesis.

a) Invertebrate models

Neuroscientists are confronted with enormous difficulties when attempting to decipher the complexities of the mammalian brain. Changes in behaviour that result from learning and memory, arise through a complex weave of interacting neuronal processes. In an attempt to simplify the experimental approach to studying the underlying trends of memory formation, many neuroscientists have

opted to study the foundation of such behavioural changes in invertebrates.

Intensively studied invertebrate models are *Hermissenda* (Aikon 1987) and *Aplysia californica* (for a review see Kandel and Schwartz 1982). Studies by Kandel and his co-workers in *Aplysia* provided the first cellular theories of habituation and sensitization. In *Aplysia*, the gill syphon withdrawal reflex is paired with a weak tactile stimulus. Repeated touch produces a weaker withdrawal reflex (habituation), however, paired with a strong electric shock to the tail, a subsequent touch unpaired with shock can elicit an increased withdrawal response (sensitization) (Carew *et al* 1981). Since the neural circuitry of the siphon withdrawal reflex is well known (24 sensory neurons and 13 motor neurons), the neurophysiological responses of the neurons have been characterised. Sensory neurons undergo an increased excitatory post-synaptic potential following conditioning (Hawkins *et al* 1983).

Another invertebrate model is that of food suppression in *Limax maximus*, the giant garden slug (Gelperin 1975, Sahley et al 1986). The procedure involves the pairing of food with carbon dioxide poisoning (Gelperin 1975) or with quinidine, a bitter tasting substance (Sahley *et al* 1986). This type of operant learning has many similarities to the chick passive avoidance learning in the present investigation, where a long-lasting memory is achieved usually after a single trial. A detailed analysis of the cellular changes as a result of this learning however, has not as yet been elicited.

b) Vertebrate models

Invertebrate models of learning and memory may be representative only of simple learning tasks, and not the type of learning encountered by more evolved

animals in complex environments. As a result, many neuroscientists have attempted to gain insight into learning, by using animals capable of responding to either Pavlovian or instrumental conditioning. This type of learning is in direct contrast to that exhibited by the invertebrate models.

c) Electrophysiological models

Much research in this field has focussed on LTP, which has been considered as a possible mechanism of memory formation. LTP can be identified however, in only a few selected regions of the mammalian brain, particularly the hippocampus (Teyler and Aiger 1978). Studies of the electrical activity in the hippocampus, show a stable and enduring increase in the responsiveness (magnitude of the excitatory post-synaptic potential or population spikes) of post-synaptic cells after a brief tetanic afferent stimulation (Anderson and Lomo 1966). This phenomenon has subsequently been reported for a number of brain regions, and in a number of different species (for review, see Swanson *et al* 1982). Since the cellular mechanisms persist for a relatively long-time (hours to weeks), it is thought that LTP may be involved in information storage in the brain (Swanson *et al* 1982). Recently, it has been shown that long-term potentiation causes morphological changes in addition to the detectable physiological changes (Desmond and Levy 1986, Desmond and Levy 1988, Desmond and Levy 1990, Schuster *et al* 1985, Morris and Baker 1984, Wenzel *et al* 1985). These studies are considered in detail in Chapter 4, Section 3. A decrease in efficacy has been reported to occur after sustained synaptic use (Stevens 1989). This phenomenon is known as long-term depression (LTD). Such a mechanism has been shown to be involved in memory processes in the cerebellum (Ito 1987).

Studies of LTP or LTD have primarily been concerned with the mechanisms of pharmaco-kinetics of the synaptic junction, and whether the process involves pre-synaptic (Bliss 1990), or post-synaptic (Baranyi and Szente 1987) alterations. These aspects of synaptic involvement in memory processes are outside the scope of this thesis.

d) Avian models

Imprinting : The young chick must recognize its mother to whom it will go for warmth and protection. The chick instinctively attaches itself to the first moving object after hatching, and is said to be 'imprinted' upon that object. Normally this is the mother, but the natural mother may be replaced by a wholly un-natural object. In the absence of 'mother', the chick will approach a wide range of objects, particularly if they are moving. The visual characteristics of the chosen object are quickly learned, and if the 'imprinted' object moves, the chick will follow. The chick will preferentially move towards an imprinted object, and may actively avoid an unfamiliar object. There is a critical time period, usually lasting no more than a few hours, when the chick will approach any novel object. Although imprinting on a visual stimulus is the most easily recognized form of early learning in the chick, preferences for both auditory and olfactory stimuli can also be demonstrated (Horn 1985a).

Passive avoidance learning : Passive avoidance learning (PAL) in the chick was first described by Cherkin and Lee-Teng in 1965. It is passive because it involves a negative or inhibited response in reply to a given choice. It is quickly learned (often after a single trial), and the memory is stable for a substantial time after training (Gold 1986). Such trials often involve some form of punishment as part of the training programme. Because of this, there has been

some controversy over the usefulness of such studies, since the effects of stress, arousal and shock are likely to confound the results (Sahgal 1984). It is hence important to isolate the effects of memory from other concomitants of the learning experience. In an analysis of ^{3}H-fucose incorporation into chick forebrain following PAL, Rose and Harding (1984) showed that increases were found in chicks trained to avoid the aversive substance, methyl anthranilate (MeA), but not in control chicks (water trained). Another group of chicks trained with MeA (M-trained), but immediately made amnestic by application of a sub-convulsive trans-cranial shock, showed no elevated ^{3}H-fucose incorporation. A further group of M-trained chicks received delayed shock (after 10 minutes). These chicks were not amnestic, and showed elevated levels of ^{3}H-fucose incorporation. Hence it was shown that its increased incorporation into chick forebrain was due entirely to the memory, and not due to some other facet of the experimental procedure. In addition, shock alone was shown to have no effect on the measures of ^{3}H-fucose (Rose and Harding 1984). It is clear however, that memory is likely to be enhanced in conditions of stress and arousal. This is undoubtedly under hormonal control (Gold 1984). Since stress is directly involved in memory enhancement, it is not altogether advisable to have a model of memory which eliminates stress. However, this is only so, if the component of stress alone, without subsequent memory formation, is not involved in alteration of the relevant brain structures.

The present studies have used a one-trial passive avoidance learning paradigm, with the day-oid chick (*Gallus domesticus*) as the experimental animal. The young chick shows preferences for learning. When newly hatched, it has to learn many new things, and experiences a variety of novel situations. Some of these experiences are important to its survival. It also has to feed itself (since

the domestic chick is born precociousiy). There is an instinctive pecking behaviour which is elicited toward any object resembling food. Of course, not all small round objects are food, and it must learn which of those are to its benefit, and which are possible dangers (poisons). The learning paradigm used in this study takes advantage of this drive for learning, since in this situation, there might be a more positive anatomical change than in non-specific learning. The chick is allowed to peck at a bright chrome bead which has been coated with a bitter tasting substance, such as methyl anthraniiate. Chicks that have pecked the bead show a characteristic 'disgust' response. This is displayed by the onset of distress calls, head shaking, rapid swallowing and by the chick wiping its beak on the floor of the pen. 80% of chicks will avoid a second encounter with a similar but dry bead, even if tested several hours after the initial experience (Rose 1986). Such a dramatic alteration of the chick's behaviour, and the permanency for the recall, makes such a trial a useful one in which to test the biology of memory processes.

CHAPTER 2 : LOCALIZATION OF MEMORY IN THE CHICK

There are two possibilities for the location of memory. Firstly it may be represented diffusely throughout the brain, or secondly in discrete localized regions of the brain. The historical perspective of the debate on the localization of memory, is discussed in detail by Larry Squire in his recent book (Squire 1987), and will not be entered into here. There follows however, a discussion of some of the evidence of regionalized localization of memory processes, with particular relevance to the chick brain.

2.1 : LESION STUDIES

Lesion of selected regions of the brain may allow the experimenter to gain an insight into the functions of that region. However, the technique is fraught with difficulties in interpretation, because an altered behaviour following a lesion may be due to factors other than the loss of function of the region itself (Olton 1986). Firstly, there is the possibility that the lesion may damage all or part of a neural pathway which runs through the lesioned region. Secondly, the region may only be part of a complex chain of centres involved in a given function, and the lesioned part of this chain need not play an important role (e.g. an integration centre) in the behavioural deficit. Any break in the link however, will bring about a complete loss of function. Thirdly, there may be technical difficulties associated with achieving complete destruction of the target area, without affecting surrounding regions.

Various studies using mammalian species, and some human clinical findings, have shown that the medial temporal lobe (in particular, the hippocampus) (Milner 1970), and parts of the diencephalon (particularly the thalamus)

(Winocur 1984), are concerned with the iocaiization of memory. The avian brain however, has some fundamental differences to the mammalian brain in terms of its organization and function, and therefore direct comparisons with mammalian counterparts are not entirely valid. This Chapter will therefore be restricted to a discussion of memory localization in avian species, particularly in the chick. The reader should refer to Winocur's review article for details of localization of memory in the mammalian brain (Winocur 1984).

The earliest accounts of lesions of the chick brain coupled with analysis of consequent learning and memory deficits, was performed by Evelyn Lee-Teng of the Californian institute of Technology, in 1969, using one-trial passive avoidance learning. The lesions were crude, involving aspiration of the brain surface, either totally, dorso-laterally or dorso-medially. The conclusions drawn from this study could be only as specific as the limitations of the experimental design permitted:- "... the dorsal forebrain was critically involved in both acquisition and retention of the one-trial learning.". It was conceded that the results *may* allow the interpretation that the lateral portion of the forebrain was more important than the medial portion (Lee-Teng and Sherman 1969).

The importance of the lateral forebrain area was again suggested following lesions to this region, after passive avoidance learning (Benowitz 1972). Benowitz found that in addition to a complete loss of retention for the task, there was also a complete loss of ability to re-learn the task. Other workers have provided evidence for an involvement of the lateral cerebral area in avoidance learning tasks in the chick (Saizen and Parker 1975), and in imprinting (Salzen *et al* 1975a, Salzen *et al* 1975b, Saizen *et al* 1978).

Less severe deficits were seen in groups of chicks receiving extensive hyperstriatal lesions and frontal forebrain lesions (Benowitz 1972), although these groups were impaired significantly from Controls. In this same study, lesions of the dorso-medial hyperstriatum impaired acquisition of memory only. This compares favourably with recent data of hyperstriatal lesions and memory acquisition (Patterson *et al* 1990a).

There are some areas of the avian brain which have been shown to be intimately involved with 'specialized' memories, such as Area X and its involvement with song-learning (Sohrabji *et al* 1990), and the hippocampus with food-storing (Sherry *et al* 1989, Krebs *et al* 1989). Neither of these types of memories play a role in the behaviour of the chick, and so are not discussed further.

Many of the early experimenters made lesions of the dorsal telencephalon, which included all or part of the hyperstriatum (Lee-Teng and Sherman 1969, Benowitz 1972, Benowitz and Lee-Teng 1973, Saizen *et al* 1978). It was subsequently shown that a bilateral lesion of a restricted part of the medial hyperstriatum ventrale (intermediate part (IMHV)) causes an impairment in both the acquisition (McCabe *et al* 1981) and the retention (McCabe *et al* 1982) of an imprinting preference. It was thought that the IMHV was specifically involved in the imprinting behaviour, since lesioned birds did not seem to be impaired on an operant learning task (Johnson and Horn 1985). However, lesion of the hyperstriatum has been shown to disrupt classical conditioning in the pigeon (Reilly 1987, MacPhail and Reilly 1987, Reilly 1988). Memory impairment also followed a passive avoidance learning paradigm in chicks receiving bilateral lesions to the IMHV (Davies *et al* 1988). It has

been suggested that the effects of lesions on the IMHV may be due to a reduced ability of the bird to learn or recognize the visual characteristics of the imprinting stimulus (Bolhuis *et al* 1989) or of the training stimulus, such as the colour of the bead, in passive avoidance learning trials (Davies *et al* 1988). However, visual recognition is unlikely to be the sole function of the IMHV, since a recent study has shown that memory for the avoidance task is only disrupted if the lesion of the IMHV is made pre-training. Lesions made 1- or 6-hours after training, did not significantly affect the chick's ability to avoid the training stimulus (Patterson *et al* 1990a). It has further been shown using uni-lateral lesions of the IMHV, that only the left IMHV is necessary for the acquisition of the avoidance learning behaviour (Patterson *et al* 1990a). It should be noted that one study (Saizen *et al* in preparation), has failed to find any deficit in memory for the avoidance task following IMHV lesions, using a similar learning paradigm to previous studies cited above.

in an early study, frontal lesions of the chick forebrain were found to impair retrieval of pre-surgically acquired information, while not affecting storage of information acquired post-surgicaliy (Benowitz 1972). This region of the forebrain included most, if not ail, of the LPO. This indicates that frontal brain region is necessary for memory storage, but not its acquisition. A more recent series of experiments, which involved more specific lesions to the LPO, showed that pre-training lesions of the LPO do not affect subsequent avoidance learning, whereas post-training lesions do (Gilbert *et al* in press). These authors have further shown that uni-lateral lesions to the LPO are without effect, suggesting that either left or right LPO on its own is sufficient to maintain the memory trace. The timing of the effects of lesions suggests that the LPO is involved in the storage of memory, and not its acquisition (Gilbert *et al* in press).

To date there have only been two other studies involving lesions to the LPO whilst testing memory performance. Wesp and Goodman (1978) used pigeons to investigate the effect of lesions on an operant conditioning task. They reported that although there was no significant impairment to general feeding and drinking activity following lesions, there was a suppression of performance. In a study on heart rate conditioning in the pigeon, Cohen and Goff (1978) could not find any significant reduction in the response levels of lesioned birds, compared with controls.

2.2 : STIMULATION STUDIES

Electrical stimulation of cortical and sub-cortical structures, is used to disrupt the neural activity in localized regions of the brain. If current levels are kept low enough, the disruption of neural activity which may be consequent upon learning prior to memory formation, may be reversible. This has obvious advantages over lesion studies to investigate localization of memory. This technique has principally been used to study memory formation in mammalian species, including stimulation of the amygdala, caudate nucleus, and hippocampus (For review, see Berman 1986). Electrical stimulation may also be used to enhance memory formation in particular nuclei eg. dorsal hippocampus and lateral hypothalamus (Berman 1986). The results of experiments on the effects of electrical stimulation are difficult to interpret however, since the alterations in observed performance may be due to factors other than memory formation, such as increased or decreased attentiveness, motivational states, and sensory and motor disturbances (Cannon and Salzen 1971). There have only been two studies of the effects of localized electrical stimulation in the avian species. McCollum and Goodman (1974) reported that electrical stimulation of the lateral cerebral area of the pigeon brain, resulted in amnesia of a passive avoidance learning task.

McCabe *et al* (1979) showed that electrical stimulation of the IMHV at 1.5 or 4.5 trains per second, could influence the chicks preference when it was given a choice of two flashing stimuli, operating at either 1.5 or 4.5 flashes per second.

2.3 : ^{14}C-2-DEOXYGLUCOSE METHODS

The search for regional changes in brain activity following learning can be achieved by examining the utilization of glucose. The assumption underlying such studies, is that the formation of memories must inevitably be associated with the use of energy. A common method for studying such consumption of energy, is labelling with 2-deoxyglucose, an analogue of glucose (Sokoloff *et al* 1977). The uptake of 2-deoxyglucose is similar to that of glucose, but once phosphorylated is unable to be further metabolized. Accumulation of phosphorylated 2-deoxyglucose in the cells, can be detected by using a radioactive label such as ^{14}C. The technique has previously been used to map neural pathways involved in memory storage in the rat (Destrade *et al* 1985), and following auditory imprinting in the chick (Wallhäusser and Scheich 1987).

Autoradiographic analysis of accumulated ^{14}C-2-deoxyglucose-6-phosphate showed that three regions of the chick brain were particularly active for at least 30 minutes after a passive avoidance learning task (Kossut and Rose 1984). These were the *hyperstriatum ventrale-posterior*, LPO, and *paleostriatum augmentatum*. Rapid assay for the radioactivity confirmed the involvement of two of these sites (*hyperstriatum ventrale-posterior* and LPO), and demonstrated that most of the activity was confined to the left hemisphere (Rose and Csillag 1985). The *medial hyperstriatum ventrale* had also been been shown to have an elevated level of ^{14}C-2-deoxyglucose-6-phosphate after imprinting (Kohsaka *et al* 1979).

CHAPTER 3 : AVIAN NEUROANATOMY

3.1 : THE PALEOSTRIATAL COMPLEX

The lobus parolfactorius is one of the nuclei of an important group of basal forebrain structures, collectively known as the paleostriatal complex. This complex has three major subdivisions; the *paleostriatum augmentatum*, *paleostriatum primitivum*, and *nucleus intrapeduncularis*. Medial to the *paleostriatum augmentatum*, lies the LPO. For descriptive purposes early workers considered the LPO as part of the *paleostriatum augmentatum*, but it has since been shown to be entirely separable from the *paleostriatum augmentatum*, both in terms of its function and by its cellular mass (Karten and Dubbeldam 1973).

For many years the avian paleostriatal complex has been considered homologous to the mammalian basal ganglia (Karten and Dubbeldam 1973). The *paleostriatum primitivum* was suggested to be comparable to the mammalian *globus pallidus*, whereas the *paleostriatum augmentatum* was thought to be similar to the mammalian *caudate nucleus-putamen* (Ariens-Kappers *et al* 1936). More recently, evidence has accumulated to show that the avian *paleostriatum primitivum* is comparable to the mammalian *globus pallidus* (Karten and Dubbeldam 1973, Brauth *et al* 1978). In contrast, it has been suggested that the entire avian *paleostriatum augmentatum-lobus parolfactorius-nucleus accumbens* region is comparable to the mammalian striatal complex (caudate nucleus, putamen, and nucleus accumbens) but not on a one-to-one basis (Kitt and Brauth 1981, Reiner *et al* 1983). The paleostriatal complex is important in that it may control motor function, and also control spatial orientation and attention (Brauth *et al* 1978).

3.2 : THE LOBUS PAROLFACTORIUS

Stereotaxic atlases describe the chick brain such that a line between the ear bars and the beak bar is at an angle of 25.5° with the horizontal axis of the frame (Salzen and Williamson, unpublished, Youngren and Phillips 1978). Using an anterior posterior zero in the centre of the ear bars, with the lateral zero in the median plane, the co-ordinates of the LPO in the 3-day old chick are calculated as Anterior 4.5 mm, Lateral 1.0 mm and a depth of 4.75 mm. It is hence a basal forebrain nucleus (Fig.3.1a,b). It is adjacent to the ventricular surface on its medio-basal surface, and has the *neostriatum* dorsally. The *nucleus basalis* lies antero-laterally, whilst the lateral relation is the lateral forebrain bundle. Also lateral, though slightly more caudally, lies the *paleostriatum augmentatum*, and *paleostriatum primitivum*. The LPO extends caudally as far as the appearance of the *nucleus accumbens*, and the medial divergence of the *paleostriatum augmentatum*.

a) Afferent projections

Using retrograde transport of horseradish peroxidase to label the afferent input into the chick LPO, Boxer and Csillag (1986) report that labelled cells were found in the following regions; *nucleus superficialis parvocellularis*, *area ventralis tegmentalis of Tsai*, *nucleus tegmenti pedunculo-pontinus*, and the *archistriatum* (Fig.3.1c,d). Injections of agglutinin-conjugated horseradish peroxidase into the dorsal thalamus of the pigeon (mainly *nucleus subhabenularis lateralis*), produced heavy labelling throughout the ipsilateral LPO, and sparse labelling throughout the contra-lateral LPO (Wild 1987).

There may be a functional sub-division of the LPO, since its lateral portion

has been reported to receive inputs from the *neostriatum*, namely the *neostriatum frontale, pars intermedia-dorsalis*, and *neostriatum frontale, pars medialis* (Dubbeldam and Visser 1987). Note however, that these results were obtained from investigations of the brain of the mallard (*Anas Platyrhynchos L.*). The lateral portion also receives a small input from the *hyperstriatum ventrale* (Dubbeldam and Visser 1987), although the precise origin of the afferents is unknown, since retrograde tract tracing was not performed. These latter results are of particular interest, since both the *lateral neostriatum* and *hyperstriatum ventrale* of chicks and pigeons have known involvement in memory processes (see Chapter 2).

There is a projection to the LPO from the *locus coeruleus* and *subcoeruleus dorsalis* in the midbrain of the pigeon (Kitt and Brauth 1986a). It is interesting to note that a similar projection exists to the *hyperstriatum ventrale* and *paleostriatum augmentatum* (Kitt and Brauth 1986a), the latter nuclei of which has also been implicated in the formation of memory in the chick (see Chapter 2). The connections of the LPO are almost entirely ipsilateral (Kitt and Brauth 1981, Kitt and Brauth 1986b, Boxer and Csillag 1986). There is however a contribution to the LPO on one side, from various structures on the other side of the brain, via the anterior commissure (Zeier and Karten 1973). In the pigeon, the fibres to the LPO cross in the anterior branch of the commissure, the *pars bulbaris* (Zeier and Karten 1973).

b) Efferent projections

The output of the LPO is two-fold. Firstly, a tract of rostrally directed axons, which course through the substance of the LPO, and secondly, a caudally-directed tract which passes ventral to the inferior margin of the LPO, and forms the

largest part of the medial forebrain bundle (Karten and Dubbeldam 1973). Terminations include the *nucleus superficialis parvocellularis*, *area ventralis tegmentalis of Tsai* (Kitt and Brauth 1981, Kitt and Brauth 1986b), and the *nucleus tegmenti pedunculo-pontinus* (Kitt and Brauth 1981, Kitt and Brauth 1986b).

Other efferents terminate in the *ventral paleostriatum* (Kitt and Brauth 1981, Kitt and Brauth 1986b), although there is some suggestion that these efferents may only arise from the lateral part of the LPO (Dubbeldam and Visser 1987). There are also reports of efferents to the *hypothalamus* (Benowitz 1980), particularly its rostro-lateral portion (Kitt and Brauth 1981), arriving via the medial forebrain bundle.

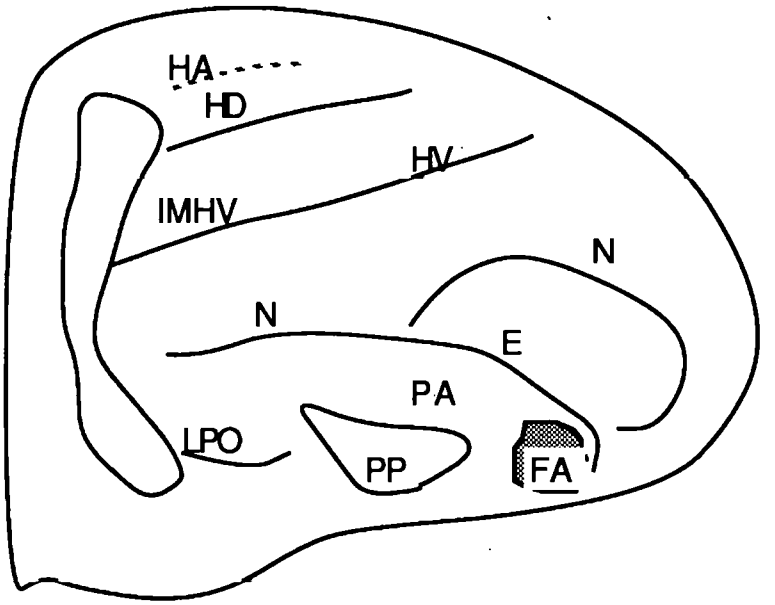


Fig.3.1a : Left hemisphere coronal section (A9.25). E-ectostriatum, FA-tractus fronto-archistriatalis, HA-hyperstriatum accessorium, HD-hyperstriatum dorsale, HV-hyperstriatum ventrale, IMHV-intermediate and medial hyperstriatum ventrale, LPO-lobus parolfactorius, N-neostriatum, PA-paleostriatum augmentatum, PP-paleostriatum primitivum

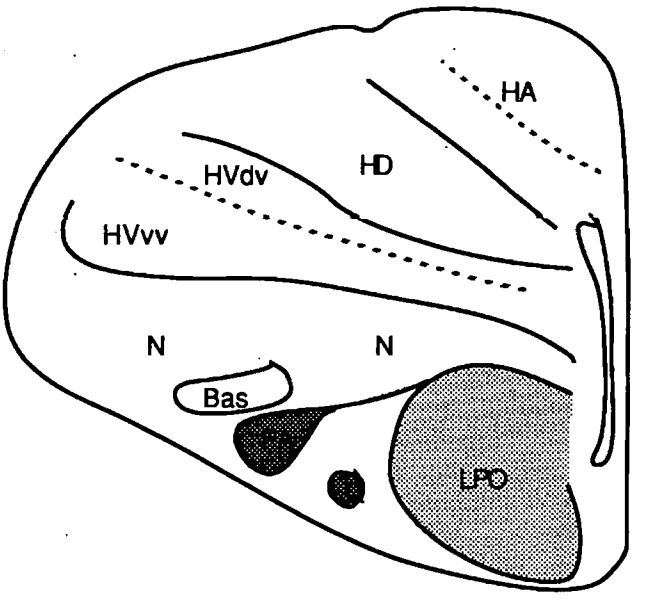


Fig.3.1b : Right hemisphere coronal section (A11.50). Bas-nucleus basalis, FA-tractus fronto-archistriatalis, HA-hyperstriatum accessorium, HD-hyperstriatum dorsale, HVvv-hyperstriatum ventrale ventro-ventrale, HVdv-hyperstriatum ventrale dorso-ventrale, LPO-lobus parolfactorius, N-neostriatum, PA-paleostriatum augmentatum, Q-tractus quintofrontalis

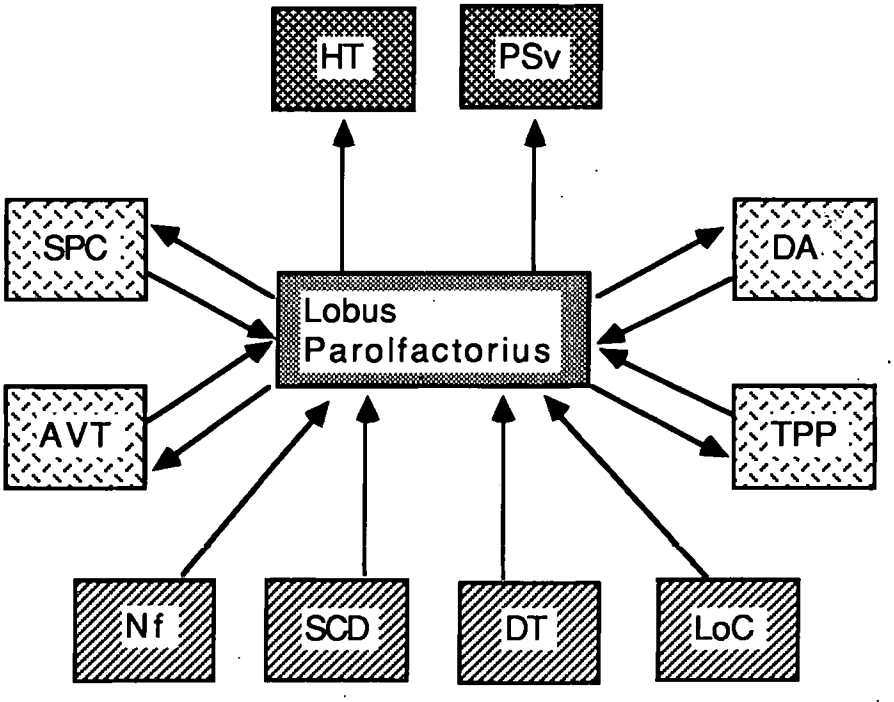


Fig.3.1c : Summary of LPO connections: AVT - Area ventralis of Tsai, DA - dorsal archistriatum, DT - dorsal thalamus, HT - hypothalamus, LoC - locus coeruleus, Nf - Neostriatum frontale, PSv - ventral paleostriatum, SCD - subcoeruleus dorsalis, SPC - superficialis parvocellularis, TPP - nucleus tegmenti pedunculo-pontinus.

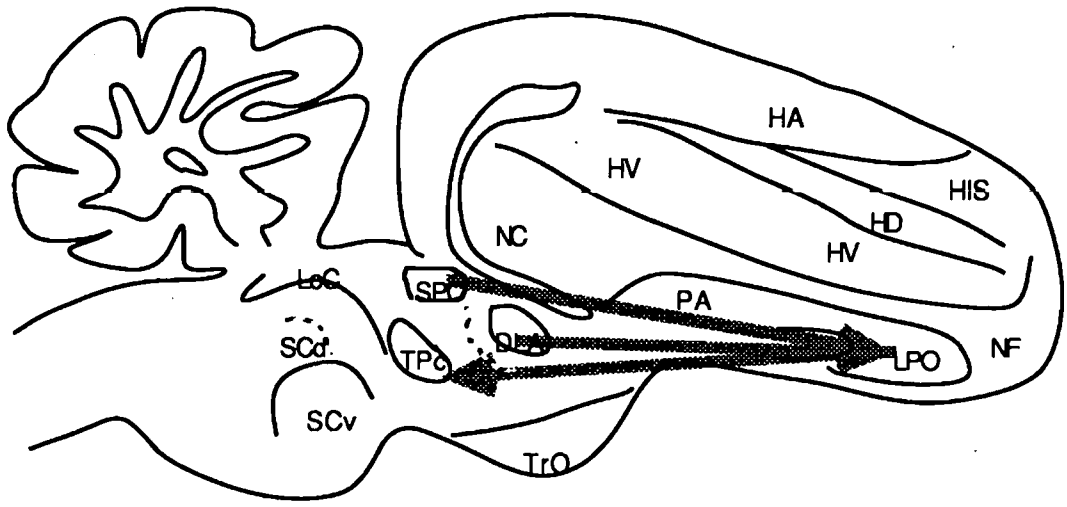


Fig.3.1d : Parasagittal section of chick brain (L2.00). DLA-N.dorsolateralis ant.thalami, HA-hyperstriatum accessorium, HD-hyperstriatum dorsalis, HIS-hyperstriatum intercalatus superior, HV-hyperstriatum ventrale, LoC-locus coeruleus, LPO-lobus parolfactorius, PA-paleostriatum augmentatum, SC-N.sub-coeruleus, SPC-N.superficialis parvocellularis, TPc-N.tegmenti pedunculo-pontinus.

c) Cell types and possible functions

The neuronal population of the chick LPO is generated between day 6 and 9 of embryonic life (Tsai *et al* 1981a). The LPO is thus one of the last regions to undergo cellular proliferation in the avian telencephalon (Tsai *et al* 1981a). The mature neurons are mostly small (12-20 μm) bipolar cells (Karten and Dubbeldam 1973, Kitt and Brauth 1981). Tömböl (1988) has described in detail, four types of projection neurons from the LPO, two of which are aspinous, and three local circuit neurons.

There is a wide variety of neurotransmitters contained within the cells of the LPO, but there is a predominance of cholinergic (both muscarinic and nicotinic) fibres (Horn 1985b, Karten and Dubbeldam 1973, Richfield *et al* 1987, Dietl *et al* 1988a). It is also rich in dopamine (both D1 and D2 receptors have been identified) (Dietl and Palacios 1988). On the basis of the small size of the cells, and the predominance of cholinergic and dopaminergic neurotransmitters, a

comparison to the mammalian *caudate-putamen* has been made (Karten and Dubbeldam 1973, Reiner et al 1984), since these descriptions would fit the known features of these basal ganglia.

There are moderate amounts of γ-amino butyric acid (GABA) (Dietl *et al* 1988b) and serotonergic fibres (Sako *et al* 1986). Enkephalin-like immunoreactive substance was also found in the LPO (Reiner *et al* 1984), and large amounts of substance P (Reiner *et al* 1983). The substance P containing neurons travel via the medial forebrain bundle to project upon catecholaminergic cells in the midbrain.

The functions of the LPO are unknown, although the idea that it may be a homologue of the mammalian basal ganglia suggests that it may be involved in regulation of motor output. The chick LPO was shown to be immunoreactive to corticoliberin, a neuropeptide that causes the release of adreno-corticotrophic hormone from the anterior pituitary (Kuenzel and Blaehser 1989). It has therefore been suggested that the LPO has a neuroendocrine function, involved in the integration of emotional behaviours (Kuenzel and Blaehser 1989). This was also suggested by Wesp and Goodman (1978), in their discussion of the effects of lesions to the LPO in the pigeon. These studies could have important consequences for the interpretation of data in the present thesis, since stress of the experimental procedure may well cause an excitation of the LPO.

3.3 : THE GUSTATORY SYSTEM

The chick has approximately 300 taste buds, located in the tongue, upper beak epithelium, floor of the anterior lower beak, and the mandibular portion posterior to the tongue (Ganchrow and Ganchrow 1985). Afferent fibres proceed caudally via the facial and glossopharyngeal nerves to the solitary nucleus.

Another brain structure that has been associated with the sense of taste is the *stratum cellulare externum*. Lesions of this region result in hyposensitivity to chemical solutions applied to the buccal cavity (Gentle 1975). It has been shown that the parabrachial nucleus of the dorsolateral pons projects to the *stratum cellulare externum*, as well as to the *paraventricular nucleus* (Wild *et al* 1987). The *paraventricular nucleus* in turn, projects to the *nucleus tractus solitarius*, hence the *stratum cellulare externum* is indirectly connected to the main gustatory system. The lobus parolfactorius has no known direct or indirect involvement in the sensation of taste.

Chicks are indifferent to solutions containing common sugars, but can detect the presence of sodium chloride (Pick and Kare 1962). They will actively avoid drinking a toxic salt solution. Most chicks will readily drink an acid solution at pH2 without hesitation, and it is therefore thought that their ability to detect 'sour' solutions is markedly impaired (Sturkie 1965). The chick's sensation of a 'bitter' taste are the most markedly different to our own. There are some compounds (eg. sucrose octa-acetate) that are offensive to humans, which the chick will accept readily (Sturkie 1965), whilst others such as *dimethyl anthranilate*, a substance used in the human food industry, is offensive to many species of birds (Kare and Pick 1960). A derivative of this compound (methyl anthranilate) is used as the aversive stimulus in the experimental work of this thesis.

3.4 : THE OLFACTORY SYSTEM

The olfactory projection occurs along the entire rostrocaudal extent of the *dorsolateral telencephalon*. The neural structure identified in this region is the *pyriform cortex* (Reiner and Karten 1985). A second projection was found to

terminate in the *olfactory tubercle*, and a third was shown to enter the *nucleus tæniæ* (Reiner and Karten 1985). The *nucleus tæniæ* is considered to be a component of the avian *archistriatum*, which is thought to be homologous to the amygdala of mammals (Zeier and Karten 1973).

One study of the olfactory connections of the pigeon brain, has conciuded that the LPO is the terminal field of the projection from the olfactory bulb (Reike and Wenzel 1978). This claim is substantiated by use of both electrophysiological and neuroanatomical methods. Electrical stimulation of the olfactory bulb resulted in Type I recordings (direct monosynaptic connection) from *hyperstriatum ventrale*, *lobus parolfactorius*, and *cortex prepiriformis*. Neuroanatomical degeneration studies have confirmed these three locations as terminal sites of the olfactory bulb projection. The projection to the LPO was found to be primarily ipsilateral, although some fibres were found contralateral in the caudal portion of the LPO (Reike and Wenzel 1978). A subsequent study using anterograde radioisotope labelling of projections from the pigeon olfactory bulb fails to confirm these claims, but concedes that '..a certain amount of radioactive amino acids did spread from the injection site to rostral portions of the lobus paroifactorius...' (Reiner and Karten 1985). The authors suggest however, that this may be the consequence of technical inadequacies of the experiment, rather than a true neuroanatomical connection.

It has been suggested that chicks show very little use of the sense of smell under normal circumstances; " Domesticated birds reveal no concern for the odors of their environment. Howéver, the presence of neuroanatomical structures suggests that olfactory information can be transmitted even if it is not behaviourally meaningful." (Sturkie 1965).

CHAPTER 4: PLASTICITY FOLLOWING LEARNING AND MEMORY

This thesis has proposed that memory may be the result of lasting changes in neural networks. Such changes are likely to include a variety of biochemical, morphological and physiological mechanisms. All of these alterations are likely to be intricately interwoven. A change in one of these mechanisms is likely to bring about changes in the others. In particular, a change in the chemistry of the system is likely to be the basis for structural and functional change.

4.1 : BIOCHEMICAL CHANGES

Imprinting studies in the chick have demonstrated an increase in tritiated lysine incorporation into proteins of the forebrain (Bateson *et al* 1969), and also of uracil into RNA (Bateson *et al* 1973, Bateson *et al* 1975, Rose *et al* 1970). Both of these experiments suggest that protein synthesis is involved in the memory process. Inhibition of protein synthesis using drugs such as anisomycin (Mizumori *et al* 1987a), cyclohexamide or emetine (Patterson *et al* 1986), results in amnesia for a given learning task, provided that testing is delayed for several hours after training. This amnesia occurs even if the drug is administered 5 minutes after the initial training period (Gibbs and Lecaneut 1981). It has therefore been concluded that long-term memory is dependent upon protein synthesis, whilst short-term memory is unaffected (Matthies 1982, Gibbs and Ng 1977, Rosenzweig and Bennett 1984a).

There is however, some opposition to this suggestion. There is evidence that various 'treatments', such as vasopressin and oxytocin (Kovacs *et al* 1985, DeWied 1984), can reverse memory impairments or indeed, complete amnesias (Dunn 1980). It is therefore considered that memory may be 'state-dependent';

that is, dependence upon the hormonal state of the individual, during and after a learning task (Izquerdo 1984, Gold 1984). A number of hormones, such as peripheral catecholamines, may have a direct influence on the transmitter complexes in the brain at the time of learning (McGaugh and Liang 1985, Gold 1984). Others, such as opioids, may act in a more indirect way by altering the arousal state of the animal, and therefore influencing adrenergic modulation (Introini-Collison and Baratti 1986).

Recent experiments have demonstrated 'state-dependency' in the chick during a passive avoidance learning task (Bradley and Galal 1987, Bradley and Galal 1988). Anisomycin, injected intra-peritoneally 30 minutes prior to training, was shown to inhibit memory formation after 6 and up to 12 hours. Avoidance was established if the drug was injected 30 mins prior to the testing period, as well as 30 minutes prior to training. The authors suggest that memory is formed independent of the inhibition of protein synthesis, but that 'recall' for the memory is dependent upon a 'humoral state' similar to that in which the memory was formed. A subsequent study has shown however, that the mode of injection (i.e. intra-peritoneally) may have been a major factor in producing the observed results. This is supported by data which shows that intra-peritoneal injection of anisomysin at the concentrations used by Bradley and Galal (0.8mg/chick), causes a deficit of only 8% in incorporation of amino acids into proteins (Patterson *et al* 1989).

In addition to the studies on protein synthesis, attention has been directed to the glycoproteins that form a major component of the post-synaptic densities. A transient elevation of ^{3}H-fucose incorporation into glycoproteins was seen up to 24 hours after passive avoidance training. This activity had returned to control

values after 48 hours (Sukumar *et al* 1980). It was subsequently shown that this elevation takes place primarily within the synaptic membranes (Burgoyne and Rose 1980).

4.2 : PHYSIOLOGICAL CHANGES

It has been shown that in the IMHV of day-old chicks, neurones discharge action potentials spontaneously. In an investigation of neuronal activity in relation to imprinting, chicks were imprinted on a rotating, flashing red box. The rate of firing of these neurones, 1-hour after training, was less in chicks making more frequent approaches to the imprinting stimulus during a 3-hour training period. That is, there was a negative correlation between the neuronal activity and the number of approaches made to the imprinting stimulus (Payne and Horn 1984). Since no such correlation was seen in the *hyperstriatum accessorium*, this finding was not due to some generalized effect, such as motor activity.

With recording electrodes placed in the IMHV of anaesthetised chicks, Mason and Rose (1987) were able to demonstrate an increase, compared with controls, in spontaneous multi-unit 'bursting' activity in chicks trained with methyl anthranilate 1-13 hours previously. 'Bursting' is described as high frequency (400 Hz), large amplitude (200-450 μV) spikes of short duration (15-20 ms). These 'bursts' increased by as much as 320% in the left hemisphere, and 350% in the right hemisphere. The mean number of spikes per burst also increased in M-trained chicks, by 66%. 'Bursting' may represent an efficient method of consolidation of connections between neurons within brain networks, associated with memory formation (Gigg 1991).

Subsequent studies have revealed that the increase in bursting activity in the IMHV is restricted to the time period between 3 and 7 hours post-test (Gigg 1991). With the exception of the time period between 6 and 7 hours post-test, there was no significant difference between the hemispheres with respect to the pattern of bursting activity.

Multi-unit recordings have also been taken from the LPO at times ranging from 1-10 hours post-methyl anthranilate training and test (Gigg 1991). The time-course for an increase in bursting activity in LPO neurons, is much the same as that for the IMHV, with a peak of activity between 4 and 7 hours post-test. By the administration of a sub-convulsive electroshock 5 minutes post-training, Gigg (1991) rendered M-trained chicks amnestic for the aversive taste. These chicks failed to show any significant increase in bursting activity in comparison to water-trained controls. Thus, bursting changes are due to the formation of a memory for the taste, and not some other facet of the experimental procedure, such as the taste or smell of the bead. This lack of change in bursting activity of amnestic chicks had also previously been demonstrated for the chick IMHV (Mason and Rose 1988). These authors had demonstrated that after a delay of 10 minutes, application of a sub-convulsive shock (12 mA, 110 V, 220 ms duration at 50 Hz through hand-held trans-dermal electrodes) results in 40% of chicks becoming amnestic, whilst immediate shock causes 63% of chicks to become amnestic.

4.3 : ANATOMICAL CHANGES

a) Synaptic changes

The idea that the synapse might be the key structure involved in the process of memory formation, is not a new one. Hebb proposed in the 1940's, that the

synapse may be modifiable in such a way that specific nerve connections may be enhanced in response to a given learning situation (Hebb 1949). It is clear that any alteration of neural functioning, will inevitably result in some modification in the responses of the synapses, and probably to an alteration in their structure. Indeed, it is thought that it is precisely this form of altered structure, that may form the basis of a stored 'memory'.

In principle, there are three types of morphological change that might occur in synapses as a consequence of learning. Firstly, it is possible that through a growth in size of the synapses, there would be an increase in the number of receptor sites along the post-synaptic thickenings, and hence an increase in the efficacy of the synapses. Conductance may also change, via growth of the synapse (Herrera *et al* 1985). A second type of change could arise as a consequence of an increase in the number of axons, or axon collaterals. The end result would be an increase in the number of synaptic sites by synaptogenesis. The third type of morphological change which will be considered here, is that of a modification of receptor sensitivity. Some of the evidence for such mechanisms as a consequence of learning and memory, will be reviewed in the following pages.

It is possible that structural changes, particularly those occurring at the synapse, may be a consequence of repetitive activation (Hebb 1949). The changes made in this way, may in themselves result in a facilitation of synaptic transmission. This may be one of the mechanisms that form a basis for the formation of memory. It is important for our model, that the proposed synaptic modifications exist for the adult as well as for the developing animal. There may of course be some differences, since adult forms may exhibit different learning strategies and abilities. Most studies regarding synaptic plasticity have been performed on developing systems, since this is when the greatest changes can be

observed. But are adult neuronal populations capable of plastic adaptability? Vrensen and Nunes-Cardozo (1981) showed that such changes were observable in adult rabbits that had undergone visual discrimination training. There is also evidence that the adult brain has a number of 'potential' or inactive synapses (Wall 1977). If activated, these offer a method of responding to given environmental situations and experiences. In addition, there is the possibility of axonal sprouting, or the formation of polysynaptic connections. Both of these have previously been reported to occur in response to manipulation (lesion) of the adult brain (for review, see Petit 1988).

There is considerable evidence, that a variety of structural changes (e.g. size and shape of processes, vesicle density, cleft width and curvature and paramembranous densities) in existing synapses may occur as a result of different environmental experience (for review, see Greenough and Chang 1985). Since memories are dependent upon experience, it is reasonable to suggest that synaptic change may be involved in the formation or facilitation of memory. Environmental enrichment does not cause any significant increase in synaptic numerical density in rat visual cortex (Bhide 1983), indicating that synaptic changes may be restricted to specific regions of the brain. Indeed, there is good evidence that measurable morphological changes occur in distinct localized regions of the brain, in response to learning experiences (Greenough and Chang 1985).

Synaptic number changes

The number of synapses in the cerebral cortex is highly influenced by experiential factors. For example, animals exposed to enriched environments have a greater number of synapses per neuron than controls (Turner and

Greenough 1985), whilst sensory deprivation causes a decrease in this parameter (Cragg 1975).

Increases in synaptic number occur following the induction of LTP in the *dentate gyrus* of the rat hippocampus (Wenzel *et al* 1985, Desmond and Levy 1983, 1986), and following repetitive activation of rat hippocampal neurons (Petit *et al* 1989). This was also seen in rat hippocampus after a brightness discrimination task (Matthies 1989a, Wenzel *et al* 1980). The latter study showed that trained animals had an increase in synaptic number of 40% in comparison with controls. Synaptogenesis was also seen following the acquisition of a new behaviour of female canaries, after administration of testosterone (DeVoogd *et al* 1985). In *Aplysia*, there is an increase in synaptic number following sensitization (Bailey and Chen 1983, Bailey and Chen 1989a, Bailey and Chen 1989b, Bailey and Chen 1990). These studies suggest that an increase in synaptic number follows a wide variety of situations involving environmental stimulation, and learning.

A decrease in the number of synapses is seen in the *dentate gyrus* of the hippocampus in aged rats, and is associated with a memory deficit (Geinisman *et al* 1986). In *Aplysia*, there is also a synaptic loss following long-term habituation (Bailey and Chen 1983). These studies support the idea that morphological change is closely linked with synaptic use.

A report by Stewart *et al* (1983) did not find any significant increase in synaptic density in the LPO, following passive avoidance learning in the day-old chick. This was also the case for other brain regions of the chick, such as the IMHV (Horn *et al* 1985, Bradley *et al* 1985b, Bradley and Galal 1987),

hyperstriatum accessorium (Bradley and Horn 1979), and *paleostriatum augmentatum* (Stewart *et al* 1983). These studies were conducted using either imprinting, or passive avoidance learning trials. A study of visual training in the rabbit (Vrensen and Nunes-Cardoso 1981), also found no alteration in synaptic number as a consequence of learning. A subsequent study by Stewart *et al* (1987), has however reported a significant increase in the density of synapses in the LPO, in both hemispheres. A comparison of the estimates between the two studies (1983 and 1987) by Stewart *et al*, shows that the baseline estimates for controls differs by a considerable degree (data published in Stewart and King 1984). In the first report the mean estimate was approximately 0.85 μm^{-3} for the left hemisphere, and 0.74 μm^{-3} for the right hemisphere. Subsequent values reported were 0.33 μm^{-3}, and 0.27 μm^{-3} respectively. These studies represent the only published morphological data from this region of the chick brain. All estimates were produced using 'biased' counting techniques (for details, see Chapter 7).

Synaptic size changes

One of the proposed mechanisms of morphological change associated with learning and memory, is that of a change in size of the synapse. As stated previously, an increase in synaptic size would undoubtedly result in an increase in synaptic efficacy (Herrera *et al* 1985). Alterations in synaptic size could affect both the amount of transmitter released (via an alteration in the number of vesicles released), and the extent of post-synaptic receptor activation.

It has been shown that synapses in the visual cortex of rats, show a decrease in cleft width and an increase in PSD thickness, in response to a visual learning task (Vrensen and Nunes-Cardoso 1981). These authors did not find any change

in the length of the PSD. However, an increase in PSD length is seen in the rat hippocampus following the acquisition of a brightness discrimination task (Wenzel *et al* 1980, Matthies 1989b). In *Aplysia*, the PSD size is larger in 'sensitized' animals and decreased in 'habituated' animals (Bailey and Chen 1983). Subsequent studies by Bailey and Chen (1989a, 1989b, 1990) have shown that for the sensitized animals at least, this increase in synaptic size is transient in nature. The mean size of the PSD doubles compared to that of the control animals in the first 48 hours following sensitization, but has reduced in size to equal that of the controls by one-week. This time course does not follow that of the observed behavioural response of 'sensitized' animals (Bailey and Chen 1989b). The authors conclude that an increase in absolute number of active sites (which had been reported previously (Bailey and Chen 1983)), is the main factor controlling the maintenance of the sensitization.

6.5 hours after the onset of imprinting, chicks have been shown to exhibit an increase in the length of synapses on dendritic spines in the left IMHV (Bradley *et al* 1981, Bradley *et al* 1985b). No corresponding increase was found in the right hemisphere of these chicks, suggesting that there may be an asymmetry in function in this region of the chick brain. The IMHV of chicks trained on a passive avoidance task, also show an alteration in post-synaptic density length in the left hemisphere 12 hours following training, but is restricted to the PSDs of symmetric synapses (Bradley and Galal 1987). This increase in PSD size was blocked by injecting 0.8 mg anisomysin (a protein synthesis inhibitor) (Bradley and Galal 1987, 1988).

Another study of synaptic changes in the chick IMHV 24 hours following a passive avoidance task, showed that a hemispheric asymmetry which existed in

control chicks was abolished by training, via a decrease in synaptic length in the right hemisphere (Stewart *et al* 1984). This, and the study by Bradley *et al* (1981), using imprinting as the learning paradigm, appear to be in contradiction. There is however, a considerable time difference between the two studies (chicks were studied 6.5 hours and 24 hours after training respectively). It is interesting to note that the values obtained for synaptic length in the left hemisphere of control chicks, are in close agreement (though direct comparison is impossible due to the classification strategy of Bradley *et al*). The conflicting results may therefore be due, at least in part, to different procedural errors. Of course, it is quite probable that there are different strategies used by the chick for the two different types of learning. These different strategies may account for the involvement of the two hemispheres, reacting in seemingly opposing ways.

Changes in the length of the post-synaptic thickening in the LPO have been reported after a passive avoidance learning task, that are broadly similar to those reported for the IMHV (Stewart *et al* 1984). However, a subsequent study of synaptic structural changes in the LPO following passive avoidance learning, reported no significant differences in synaptic length, among asymmetric synapses of the left and right hemisphere. There was however, a lateralization of length of symmetric synapses, which is greater in the left hemisphere of Control chicks, by 13%. This asymmetry is reversed following training, such that the right hemisphere exceeds the left by 10% (Stewart *et al* 1989). This data however, was based upon few animals, and must therefore remain inconclusive.

Synaptic shape changes

It has been shown previously that memory can be Inhibited by the administration of a protein synthesis inhibitor (Gibbs and Ng 1977, Bradley and

Galal 1987). Production of new synaptic proteins may be associated with the re-organization of the internal cytoskeleton (Fifkova 1987). One might argue that such changes may be associated with an alteration in synaptic shape.

Results of particular relevance to the present study, are those of Stewart *et al* (1987), who showed that there was no alteration in the shape of the post-synaptic thickening in the LPO, following a taste-avoidance training procedure. They categorized synapses as either convex, concave, or flat, relating to the shape of the pre-synaptic component of the membrane. Curvature (K) was assessed by the formula; K=1/r, where r is the radius of equivalent circle. The value of K remained constant following training. Other workers have classified these synaptic shapes as; smile, frown or flat synapses respectively (Petit and Markus 1987). Petit *et al* (1989) studied the morphological effects of repetitive activation of synapses in the rat hippocampus. Their results, although inconclusive, could support the idea that there was a change in synaptic appearance, from frown to smile-shape. The conclusions reached by the authors of this study however, are inconclusive, since their results may be explained by a selective gain or loss of synaptic sub-types. Another report of structural correlates of long-term potentiation in the rat hippocampus, showed a significant increase in 'concave spine' synapses (Desmond and Levy 1983, Desmond and Levy 1986). These presumably correspond to the 'convex' or 'smile' synapses described previously. A loss of concavity of the pre-synaptic membrane in hippocampal synapses, was found following acquisition of a brightness discrimination task in rats (Wenzel *et al* 1977).

One final feature of the plastic synapse is that of 'perforation'; synapses that have or develop 'holes' (Dyson and Jones 1984). There is a recent and

comprehensive review of the role of this remarkable feature of synapses, both during development and as a function of plasticity in the brain, by Calverley and Jones (1990a). Perforated synapses are generally large, and are present in increasing numbers throughout development into adulthood (Dyson and Jones 1984). They are therefore considered to be active and mature synapses (Petit 1988). There have been few studies of the role of perforations or 'complex' synapses in memory formation. One such study has shown an increase in perforations in synapses in the visual cortex of rabbits following visual training. However, one cannot say whether this was due to memory formation or consolidation, or some other aspect of visual function. A loss of perforated synapses in the hippocampus, has been correlated with a loss of spatial memory in aged rats (Geinisman *et al* 1986). This loss was not observed in non-perforated synapses from the same region. This leads one to conclude that perforation of synapses may have an important function in the modification of neural function, particularly in relation to learning and memory.

b) Pre-synaptic changes

Synaptic bouton size

In their investigation of morphological changes in the rat hippocampus following a brightness discrimination task, Wenzel *et al* (1980) found that the mean area of the pre-synaptic bouton decreased significantly in trained animals. The significance of these results is unclear, since it has been shown that a similar reduction occurs in the *ectostriatum* of monocularly deprived birds (Nixdorf 1990a).

Studies of imprinting in young chicks have failed to find any effect of the learning experience upon the size of the pre-synaptic bouton in the IMHV

(Bradley *et al* 1981, Bradley and Galal 1987). Using a taste-avoidance training procedure however, Stewart *et al* (1984) report a significant increase of 23% in the volume of the pre-synaptic bouton in this same region, 24 hours after training, and a 19% increase in the left hemisphere *paleostriatum augmentatum*. These differences however, were not seen in the chick LPO 24 hours after the training procedure (Stewart *et al* 1987).

Synaptic vesicle number

In studies of habituation and sensitization in *Aplysia*, Bailey and Chen (1983, 1989a, 1989b, 1990) report an alteration in synaptic vesicle number which is correlated with the degree of synaptic activation; ie. increased in sensitized animals, and decreased in habituated animals. This change is time-dependant, being present only transiently (up to 48 hours) (Bailey and Chen 1989a). Learning has also been shown to cause a decrease in the number of vesicles associated with the synaptic terminals of rat hippocampal neurons (Wenzel *et al* 1977). This study involved the acquisition of brightness discrimination. It was demonstrated that synapses in the *stratum radiatum* of CA3 neurons had a decreased number, and a decreased size, of vesicles following learning. There was no significant change in vesicle density, either following 8 hours of induction of long-term potentiation in the rat hippocampus (Schuster *et al* 1985), or following passive avoidance learning in the chick IMHV (Bradley and Galal 1987). This latter study is in contradiction to that of Stewart *et al* (1984), who showed that there was an increase in the number of synaptic vesicles (61%) in the left hemisphere IMHV, of chicks trained on a passive avoidance task, although the study of Bradley and Galal was carried out 12 hours post-training, whilst that of Stewart *et al* was 24 hours post-training. Some scepticism has been expressed as to whether synaptic vesicle number can be

reliably obtained using current methods of counting (Gündersen 1986, Fox 1988). However, if the differences are as large as that reported, then one does not require very precise counting methods.

It can be seen from the various reports in the scientific literature summarized above, that there is no consistent pattern with regard to the size of the pre-synaptic elements, or with the number and size of the pre-synaptic vesicles. Although one might assume a probable correlation between the number of vesicles and the degree of activation of a particular terminal. It would appear that this is not always the case (Herrera *et al* 1985).

c) Post-synaptic changes

Given that structural changes have been shown to occur in synapses following memory formation, it is not unreasonable to suppose that there may also be a change in the number or size of the post-synaptic targets for those synapses. It has been shown that exposing weanling rats to enriched environments, causes an increase in dendritic branching (Greenough 1985, Greenough *et al* 1985). A number of research approaches have shown that early experience is associated with an increase in the size of the spine heads, and a shortening of the spine stems (see Coss and Perkel 1985 for review). Conversely, animals deprived of stimulation may display a decrease in dendritic parameters (Petit and Markus 1987, Gray *et al* 1982). Chicks reared in the dark have a significantly reduced spine density in the *hyperstriatum accessorium* (Galal *et al* 1990).

In a recent study, Patel and Stewart (1988) have shown that passive avoidance learning in the day-old chick, causes a significant increase in the density of dendritic spines, in multipolar projecting neurons of the IMHV. These

authors also showed that there was a significant increase in mean spine head diameter, and a significant decrease in mean spine stem length. Chicks receiving a sub-convulsive trans-cranial shock, 5 minutes following training, were rendered amnestic for the avoidance task. They did not show any significant increase in spine density. This provides clear evidence that the dendritic structural changes seen in the trained chicks who subsequently showed a behavioural avoidance to the training stimulus, are due entirely to the learning experience, and not to some concomitant experience, such as the taste of the bead (Patel *et al* 1988).

Recent data suggests that an increase in dendritic spine density following memory formation, is not restricted to the IMHV. Lowndes and Stewart (unpublished) have preliminary evidence of an increase in spine density in the left hemisphere LPO of trained chicks.

Increases in spine number and dendritic branching would increase the amount of transmission between inter-connected cells. Enlargement of the spine head and a reduction of the stem length would alter its cable properties, thus increasing the probability of an electrical potential passing into the dendritic shaft or soma (Shepherd *et al* 1985).

4.4 : NEUROTRANSMITTER MODULATION

The hypothesis stands that learning, and subsequent memory formation, is the result of adaptive changes in synaptic function. A possible candidate for such alteration of function, is the molecule involved in the transfer of signal from one nerve cell to another; the neurotransmitter. An alteration in the receptive function, mode of action, or sensitivity in the post-synaptic receptor for the transmitter may also be involved. An alteration in transmitter release is known

TABLE 4.1 : Summary of changes in synaptic structure in three regions of the chick forebrain, 24 hours following passive avoidance learning.

Forebrain region	Synaptic parameter	Nature of change
Hyperstriatum ventrale (intermediate and medial)	Synaptic density	No change in either hem. following training.
	Post-synaptic thickening length	Length in right hem. of control chicks > left by 12%. Difference abolished on training.
	Mean pre-synaptic bouton volume	Increases in left hem. by 20% on training.
	Mean synaptic vesicle number (per synapse)	Increases by 60% in left hem. of M-trained chicks.
Hyperstriatum ventrale (posterior)	---------- no synaptic changes ----------	
Lobus parolfactorius	Synaptic density	Increases by 20% in both hems. following training.
	Mean post-synaptic thickening length	Length in right hem. is 10% > left; difference is reversed on training.
	Mean synaptic vesicle number (per synapse)	Increases by 60% in the left hem. of trained chicks.
Paleostriatum augmentatum	Mean synaptic vesicle number (per synapse)	Greater by 15% in right hem. of control chicks. Asymmetry disappears on training.
	Mean pre-synaptic bouton volume	Greater by 19% in left hem. following training.

All changes referred to are statistically significant at $p<0.05$ or less, unless otherwise stated. (Adapted by the author, from Stewart 1990)

to be involved in habituation (Horn 1985b), and it can be argued, may also be involved in more complex learning situations. It may be that a reduction in the rate of release of an excitatory neurotransmitter in a given fibre, is brought about by an increase in inhibitory transmitter release onto the cell. Indeed there are many plausible theories regarding complex regulation of neural circuitry via single or multiple control of transmitter release, and it is therefore important to know something of the local circuitry of the LPO, and other anatomical structures reputed to be involved in memory and learning, and of its neurotransmitter content.

There is much evidence for the involvement of a wide range of neurotransmitters in memory formation. A decrease in the number of nicotinic receptors (Aleksidze *et al* 1981), and a transient increase in muscarinic receptors (Rose *et al* 1980) has been found to occur 30 minutes after passive avoidance learning in the chick. This suggests an involvement of cholinergic synapses early in memory formation, perhaps comprising some component of 'short-term memory'. Enhancement of dopaminergic function in four-day old chicks, using the dopamine agonist, apomorphine, caused an increase in key-peck response to an aversive stimulus (McDougall *et al* 1987). Recent studies have shown that the receptors for γ-aminobutyric acid (GABA) in the chick left hemisphere, posterior forebrain roof, increase significantly after a passive avoidance learning task (Bourne and Stewart 1985). In addition, injection of GABA into the chick brain during the first week of life causes a retardation of learning (Hambley and Rogers 1979). Chicks injected with L-proline and L-glutamine, two amino acids which inhibit endogenous glutamate release, demonstrated a retrograde amnesia for a one-trial passive avoidance learning task (Cherkin *et al* 1976). These studies cited above give further evidence of

the involvement of synapses in the process of memory formation and/or storage.

Research into transmitter involvement in memory has largely concentrated on transmitter systems and localized circuits. It should not be forgotten however, that such modifications require profound changes in individual synapses, and neurons. There is considerable evidence that a given neuron is capable of immense plastic change throughout its life-span (Petit 1988). It is even capable of partially, or completely changing its neurotransmitter chemical in response to a given environmental stimulus (Black 1984). Adaptive change must therefore be viewed with regard to both network systems, and to the individual neurons which serve that network.

CHAPTER 5 : SYNAPSES

5.1 : MORPHOLOGICAL ASPECTS

Synapses are of two types; chemical or electrical. Electrical synapses are difficult to identify on electron microscopic micrographs, since they may consist of no more than two closely opposed membranes (approximately 2 nm), one from the pre-synaptic cell, and the other from the post-synaptic cell. There need be no visible membrane specialization. This type of synapse is uncommon in vertebrates, and hence will not be considered further.

A chemical synapse is classically described as the junction between the terminal region of an axon (pre-synaptic), and a neuronal dendrite (post-synaptic). In both the pre- and post-synaptic sides of the junction, there are membrane specializations. The nature of these specializations is described below. Synapses are not exclusive to axons and dendrites. Other types also exist; such as those between two dendrites (dendro-dendritic), between axon and soma (axo-somatic) or between two axons (axo-axonic). The synaptic specialization consists of a thickening of the membrane at the region of the junction.

Two populations of synapses were identified on the basis of differences in these thickenings (Gray 1959). Gray type I synapses were described as having a comparatively thick post-synaptic density relative to the pre-synaptic density, and were of a greater length than the Gray type II synapses. Gray type I were also shown to have a wider separation of the paramembranous densities, with a greater clarity of material lying in the gap between them. On this basis, Gray type I synapses are known as 'asymmetric', and Gray type II as 'symmetric'. Gray (1959) states that type I synapses do not make contact with neuronal cell bodies,

and that type II are restricted to dendritic trunks and neuronal soma. These are generalizations which do not hold true for all brain regions. Other authors have used a much expanded classification of synaptic types, often based upon vesicle shape (Hassler *et al* 1978).

The pre-synaptic terminal is the synaptic 'bouton'. It contains varying numbers of synaptic vesicles, some of which are closely associated with the pre-synaptic membrane thickening. The thickening can be seen to be composed of several triangular-shaped dense projections, spaced regularly at intervals of 40-45 nm. It is thought that these dense projections serve as sites of attachment for the synaptic vesicles (Jones 1981). The vesicles themselves contain a neurotransmitter, which is released into the synaptic cleft via exocytosis of the contents of the vesicles following their attachment between the pre-synaptic dense projections.

Uchizono (1965) observed that in the cat cerebellum, the majority of synapses targeted onto the soma of Purkinje cells, contained flattened vesicles in the pre-synaptic bouton, whilst those on dendrites in the molecular layer of the cerebellum contained round vesicles. Since it was known from physiological studies that the Purkinje cells were influenced by the presence of inhibitory synapses, and the dendrites of the molecular layer by excitatory synapses, it was concluded that excitatory synapses displayed round vesicles and inhibitory synapses flattened vesicles (Uchizono 1965). However, in the young avian brain no such distinction is possible, since synapses with flattened vesicles are either rarely seen (Nixdorf 1989) or not at all (Curtis *et al* 1989). The shape of synaptic vesicles has been shown to be correlated with age (Nixdorf 1989). In the *ectostriatum*, 5-day old Zebra-finches exhibit round vesicles in symmetric

synapses, whilst in adult Zebra-finches symmetric synapses are commonly associated with flattened vesicles (Nixdorf 1989). Occasionally, synapses are seen to contain vesicles of pleomorphic shapes (Curtis *et al* 1989). The pre-synaptic terminal also contains dense-cored vesicles and coated-vesicles. Dense-cored vesicles are thought to contain secretory peptides in granular form, which may act as 'local hormones', being released under conditions of intense axonal stimulation (Golding 1988). Coated vesicles on the other hand are formed by endocytosis of the pre-synaptic bouton membrane. By shedding its coat, the coated-vesicle may form a synaptic vesicle or become incorporated into the smooth endoplasmic reticulum. Synaptic vesicles are closely associated with microtubules, although microtubules cannot usually be seen in the synaptic bouton. Microtubules are characterized by the presence of tubulin, which has been shown to account for up to 25% of the soluble protein fraction of synaptosomes (Jones 1981). The bouton is also densely packed with a microfilamentous network, which has been shown to have actin, myosin and acto-myosin like properties (Jones 1981).

The neuron and its axon transmit action potentials tonically. It is therefore highly active, since the action potential requires the active transport of ions. In addition, the turnover of synaptic structural elements, neurotransmitters and neurochemicals, takes place at a remarkably high rate (Jones 1975). This is reflected by the presence within the synaptic bouton, of large numbers of mitochondria.

Many of the previous methods for quantifying the number of synapses in a given brain region, relied upon assumptions that the synapse was disc-shaped. A serial section study of synapses in the rat hippocampus, showed that this

assumption was invalid, since a large proportion of synapses were of complex shape (DeGroot and Bierman 1986). Previous studies of serially sectioned synapses, had revealed that a substantial number had deficiencies in the synaptic density, i.e had 'perforations' or holes (Cohen and Siekevitz 1978). These perforations tend to occur in the centre of large post-synaptic densities (Cohen and Siekevitz 1978). These perforations can occur in a wide range of synaptic types, and hence can be seen in synapses which are targeted onto both dendritic shafts and dendritic spines (Geinisman *et al* 1987a, 1987b).

The present study was primarily concerned with changes in number and size of synapses in the chick LPO, determined using the 'disector' technique (see Chapter 7, Section 2e). The disector technique relies upon detecting the edge (top or bottom) of the synapse. Since perforations tend to be present in the centre of synapses, it was not possible to quantify 'perforated synapses' without redefining the counting unit. Therefore, although perforated synapses may well be involved in the mechanisms of synaptic plasticity (Calverley and Jones 1990a), and hence of relevance to the process of memory formation, their role and function will not be discussed here. An example of the methodological difficulties encountered in quantifying perforated synapses is given by Calverley and Jones (1990b).

Synapses differ in their morphology with age. I have already reviewed the features common to synaptic plasticity, that is, the effect of environmental factors on the synaptic structure. Many of the developmental changes that accompany synaptic maturation are similar to those described for experimentally induced synaptic plasticity, since during maturation, the individual is subject to much environmental stimulation. One of the commonest findings in the developing brain, is the increase in synaptic number during early

life (Aghajanian and Bloom 1967, Nixdorf 1989). The exact timing and size of this increase varies considerably, depending upon the region of brain involved, and the type and quality of environmental stimulation (Jones 1975). There is also some synaptic elimination evident in much of the developing nervous system, both centrally and peripherally (Purves and Lichtman 1980). This process may provide a mechanism for the refinement of appropriate neural connections, and a loss of inappropriate ones.

In addition to changes in number, there are alterations in the morphology of each synapse. It has been proposed that there are three principal stages in the development of synapses (Jones 1975). Firstly, synaptic contact area is small and is associated with very few vesicles. The pre- and post-synaptic thickenings are of moderate but equal size, and in the absence of vesicles in the pre-synaptic site, are indistinguishable from desmosomes or *puncta-adherens*. The presence of vesicles is thus crucial in order to identify a given membrane specialization as a synapse. The most immature synapse has at least 3 synaptic vesicles associated with it, and hence this is a common number to be quoted as the minimum that must be present, in order to classify a particular density as a synapse (Stewart *et al* 1987, Curtis *et al* 1989). Vesicle number increases rapidly during the second stage of synaptic development, such that by the third stage, the pre-synaptic bouton may become completely packed with vesicles. The second stage is also characterized by the appearance of dendritic spines, and the development of asymmetry between many of the pre- and post synaptic densities. The size of the apposition grows throughout the second and third stages. The third stage, at least for mammalian synapses, is the formation of a spine apparatus (see Chapter 6). It is also thought that axodendritic synapses appear earlier than axo-somatic synapses (Jones 1975).

5.2 : FUNCTIONAL ASPECTS

It is now generally accepted that the action potential of a nerve axon, causes the release of neurotransmitter from the vesicles contained within the synaptic bouton (Jones 1981). The present thesis is concerned with the dynamic and modifiable aspects of synaptic functioning. There are two possibilities for such synaptic adaptation, or 'plasticity' as it is known. Firstly, the number of functioning synapses can increase or decrease. This can be achieved by either altering the absolute numbers of synapses present, or by changing the number of synapses which are active. It has been shown that in some brain regions there are ineffective synapses, which can be recruited into action following the loss of other active afferents (Wall 1977). The second possibility for synaptic change is via a modification of the effectiveness of communication between pre- and post-synaptic cells. This change in 'synaptic efficacy' may be brought about by either a change in the size of the synapse (the mean diameter of the post-synaptic thickening), the size of the synaptic cleft, the number of dense projections, or the number of synaptic vesicles.

In addition, the position of the synapse (i.e. whether it is targeted onto a neuronal cell body, dendritic shaft or dendritic spine) may influence the effectiveness of the synapse. A change in this position may be another mechanism whereby synaptic plasticity can take effect. There are likely to be a host of micro-molecular changes in the synapse during plastic change, such as the balance of chemical constituents, and membrane conductance levels, which will regulate synaptic function. These changes are however, outside the scope of the present investigation.

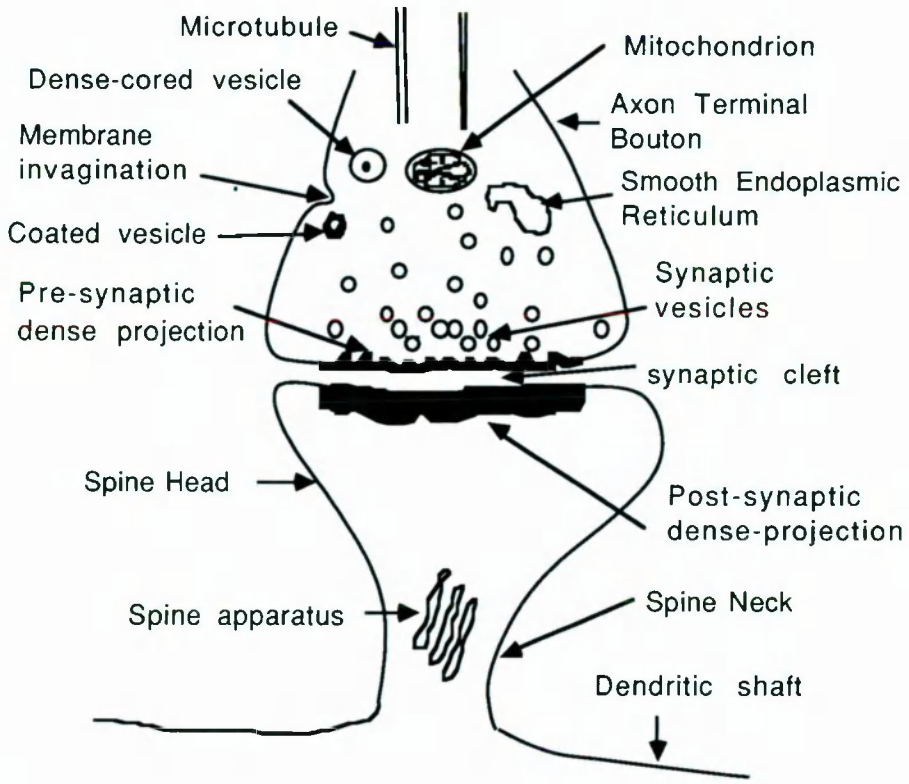


Fig.5.1a : Diagram of a typical axo-spinous synapse

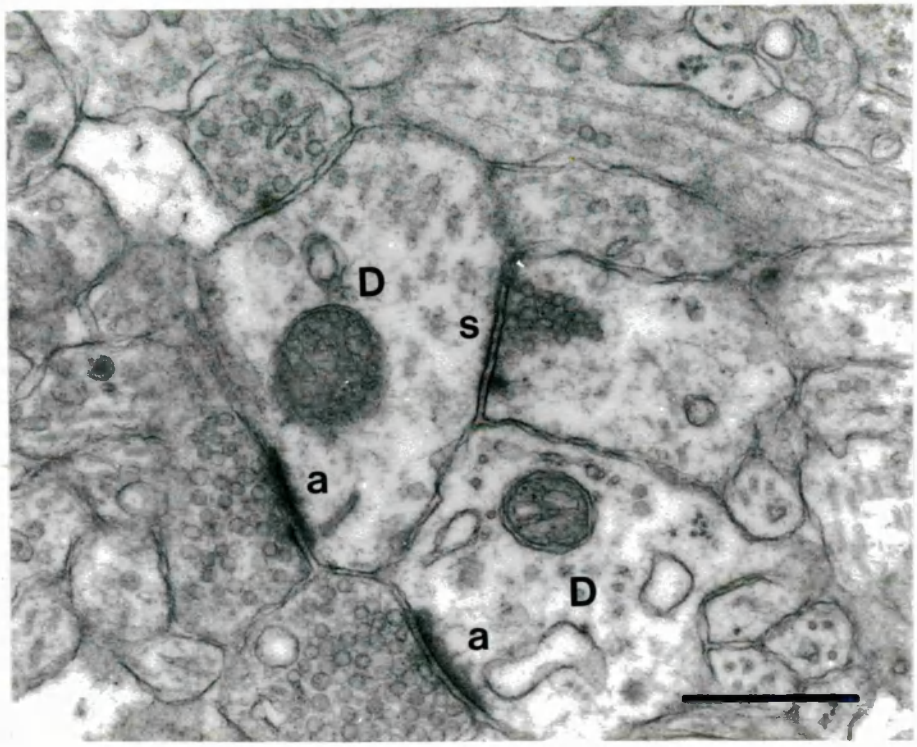


Fig.5.1b : Electron micrograph of chick LPO showing asymmetric (a) and symmetric (s) synapses targetted onto dendritic shafts (D). Scale bar represents 1μm.

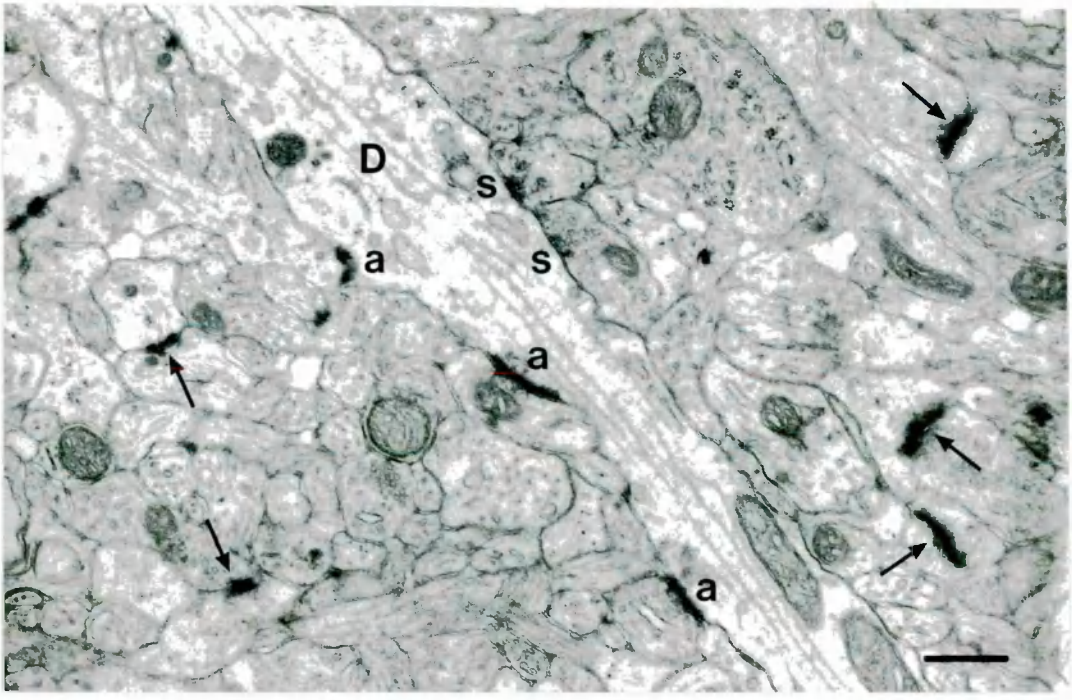


Fig.5.1c : Electron micrograph of chick LPO showing a dendritic shaft (D) which is the target for 3 asymmetric (a), and 2 symmetric synapses (s). Asymmetric spine synapses are also shown (arrows). Scale bar represents 1μm.

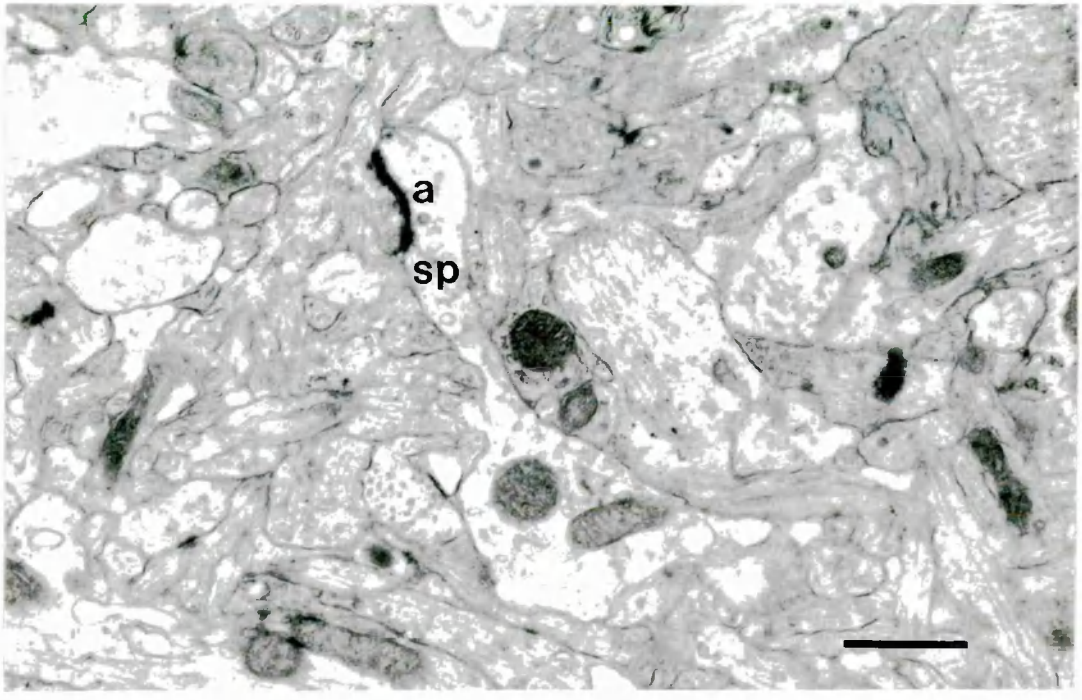


Fig.5.1d : Electron micrograph of chick LPO showing an axo-spinous synapse (a) targetted onto the head of a dendritic spine (sp). Mitochondria and smooth ER are visible within the dendrite. Scale bar represents 1μm.

CHAPTER 6 : DENDRITES

6.1 : MORPHOLOGICAL ASPECTS

The post-synaptic target is invariably the neuronal dendrite. A dendrite is a cytoplasmic extension of a neuronal cell body, of which a neuron has many. The dendrites are classified as being of two kinds; spiny or non-spiny, depending upon the presence of numerous outgrowths (spines) from the shaft of the dendrites. Aspinous dendrites lack this structure, and appear relatively smooth in Golgi-stained sections. The spines consist of a neck and a head (Fig. 5.1) of varying proportions (Landis 1985), and hence have a wide variety of shapes. The total length of the spines is usually no more than 2 μm. Occasionally, some spines lack a neck, and these are known a 'sessile' spines. A small proportion of spines, at least in the rat dentate gyrus, display a double head (Geinisman *et al* 1989). This double-head is usually in contact with two perforated asymmetric synapses. The cytoskeleton of the dendritic spine, which is responsible for the maintenance of spine shape, is composed of filaments that display actin-like qualities (Landis 1985).

It has been suggested that spines develop in response to the contact of a type I synapse on a dendritic shaft (Mates and Lund 1983a). Qualitative studies of the developing stellate neurons in the monkey visual cortex, showed that the presence of a type I synapse, appeared to initiate a swelling of the dendritic shaft at the point of contact. However, it has since been shown that in the developing mouse cerebellum there are many spines which lack an associated synapse (Landis 1985). The spines of dendrites in neonatal mouse cerebellum were similar in size, shape and cytoplasmic content, to those of mature mice, despite the fact that many of the developing spines lacked a synaptic contact (Landis

1985).

In mammalian dendritic spines, there is characteristically a cisternal membranous structure known as the spine apparatus, which is located at the base of the spine neck. The spine apparatus is not present in immature mammalian brain, nor in 'stubby' spines of mature brains (Calverley and Jones 1990a). It has not, as yet, been detected in the dendritic spines of any avian species (Nixdorf 1989), although most studies of dendritic ultrastructure have been performed on young birds (Curtis *et al* 1989, Patel 1988). This feature may therefore reflect the immature state of the tissue.

Another feature of mammalian spines is the presence of polyribosomes at the base of the spine (Steward and Falk 1985). These are involved in the synthesis of proteins associated with the synaptic density, and hence may be involved in the process of modulation of synaptic and spine ultrastructure (Calverley and Jones 1990a). Again however, there is no evidence from the literature that polyribosomes exist at the base of avian spines (Nixdorf 1989).

In addition to the dendritic spine, which forms the target for the majority of synapses, some synapses contact the dendritic shaft directly. It has been suggested from observations of synapses in the monkey visual cortex, that the spines receive type I (asymmetric) synapses , whilst the shafts and soma receive type II (symmetric) synapses (Anderson 1986, Mates and Lund 1983a). It is clear that while this is true as a generalization, it does not hold as a rule for all synapses, since there are examples of symmetric spine synapses, and asymmetric shaft synapses (Curtis *et al* 1989).

6.2 : FUNCTIONAL ASPECTS

The dendritic tree is the receptive field of the many incoming axons from other neurons, and provides the main structural feature for the formation of synapses. The dendritic surface area has been estimated to be as much as a 100 times the surface area of the soma (Rall and Segev 1987). The dendritic spine outgrowths contribute greatly to this areal expanse. It is thought however, that the dendritic spines have a more important role in the propagation of electrical impulses, than simply a structural one (Wickens 1988). The dendritic shafts are larger in diameter nearer the soma (Patel and Stewart 1988). The position of a synapse within the dendritic tree will determine its effectiveness, since there are resistances to the passage of electrical impulses along the dendritic shafts towards the soma (Holmes 1989). Clearly, the nearer the synapse is to the soma, the greater the probability that the impulse will be propagated to the axon hillock (Anderson 1986). The size (i.e. diameter) of the dendritic shaft will also have an effect on the resistances offered. For any given value of membrane conductance, there is an optimal diameter required to ensure propagation with least resistance (Holmes 1989). The spine may be a site where the action potential can be modulated before passing onto the dendritic shaft (Peters and Kaiserman-Abramof 1970). This is achieved by altering electrical resistances in the spine neck. This is in contrast to the dendritic shaft, which seems to serve merely to transmit a signal, rather than to modify it (Nixdorf 1989). It has been shown that the excitatory post-synaptic potential can be modified by changes in the structure of the spine (Pongracz 1985). This mechanism may be important in controlling the efficacy of the synaptic input, and hence may be involved in the regulation of the memory trace (Patel and Stewart 1988, Calverley and Jones 1990a, Coss and Perkel 1985).

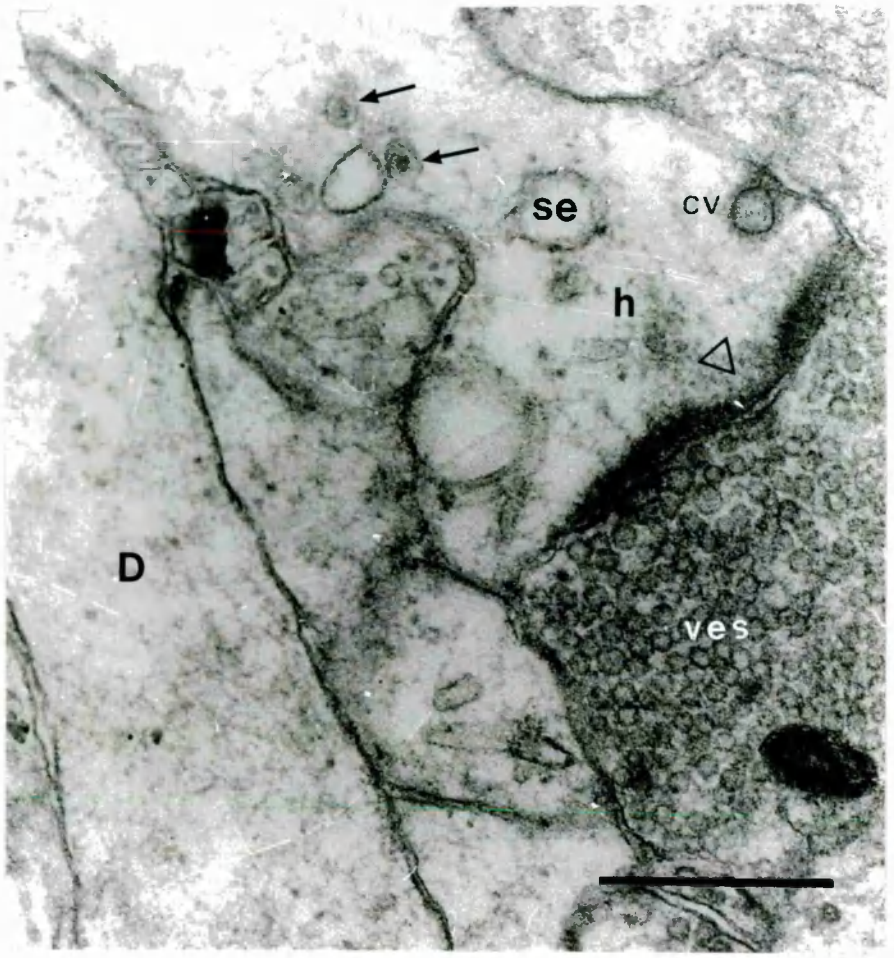


Fig.6.1 : Electron micrograph of chick LPO showing an asymmetric synapse with a perforation (open arrow). The synaptic bouton is packed with vesicles (ves). This synapse is in contact with the head of a dendritic spine (h), which contains smooth endoplasmic reticulum (se), a coated-vesicle (cv) and two dense-cored vesicles lying within the neck (arrow). The dendritic shaft (D) is seen on the left. Scale bar represents 1μm.

CHAPTER 7 : STEREOLOGY

7.1 : THE HISTORY OF NEUROMORPHOMETRY

In the early 1960's, a group of scientists were invited to establish a society with the aim of increasing the communication of ideas, regarding quantitation in the biological and material sciences. The first 'International Society of Stereology' meeting was held in Vienna in 1963. The term 'stereology' was coined to mean results correct in a true 3-dimensional sense. It is hence, the quantitative interpretation of 3-dimensional structures from 2-dimensional images. The first measurement regarding a stereological estimation of the volume density (V_V) however, is nearly 144 years old (Delesse 1847). This method involved weighing paper which had been cut to the size of the observed images. More efficient methods followed at the turn of the century, and were modified well into the 1930's. These methods were based in linear sampling and point counting methods, which still form the basis of many of the current particle size estimators (for review, see Weibel 1979). It is only recently however, that true unbiased methods of estimating number using planar sample probes, have been applied (Sterio 1984).

7.2 : ESTIMATION OF NUMBER

One of the most important parameters to measure in studies of synaptic plasticity, is number. The most obvious method by which neural circuitry could alter its activity and efficiency, is to alter their absolute number. However, it is also one of the most difficult parameters to measure, because in order to count something on a two-dimensional plane, such as that encountered on the electron micrograph, we need to know something of the object's three-dimensional shape. Although stereological techniques have been developed that allow quantitation

without assumptions regarding shape, these methods still rely on being able to unambiguously identify on two consecutive sections, a single profile. This often relies upon a prior knowledge of shape.

a) Unfolding techniques

Many previous quantitative studies of changes in synaptic number have been disadvantaged, due to the fact that they have used biased estimation procedures (Nixdorf 1989, Curtis *et al* 1989). Such studies have made unnecessary assumptions about synaptic shape. They have often assumed that synapses are characteristically disc-shaped. These assumptions cannot be relied upon, since synapses are often found to be non-uniform and of complex shape (DeGroot and Vrensen 1978). A single complex-shaped object may give rise to several profiles when cut transversely, and viewed in 2-dimensions. This problem is overcome if the shape is non-complex such as a sphere, or a cylinder, etc., since the section will only give rise to a single profile, although the size of the profile will be dependent upon the plane of sectioning (Fig.7.1a). A group of spheres transected in a random plane will yield a series of circular profiles of various sizes, depending upon whether or not a given sphere is cut near the equator . The mean profile diameter is therefore an under-estimation of the true mean profile diameter. A correction procedure known as the Schwartz-Saltykov procedure is used to provide an estimate of the true mean profile diameter. This states that;

$$Nv_j = 1/D \left[a_{j,j} (Na)_j - a_{j,j+1} (Na)_{j+1} - a_{j,j+2} (Na)_{j+2} \ldots - a_{j,k} (Na)_k \right]$$

where; $D = D_{max} / k$
N_a = Number per unit area
k = number of size classes
$a_{j,k}$ = coefficient in row j, column i of table (see Underwood 1970)

A discussion of this procedure is given by Williams (1977), and its theoretical basis by Underwood (1970).

It was reported recently that synapses, which were thought to be flat, disc-shaped objects (West and Greenough 1972), can have a complex shape with perforations in the membrane specialisation (DeGroot and Bierman 1983). The synapse is reported to adopt a more complex shape in response to training (Vrensen and Nunes-Cardoso 1981), or environmental stimulation (Greenough 1984). Verwer and DeGroot (1982) showed that conventional calculations of the numerical density of synapses (Nv_{syn}) using an 'unfolding' technique (see Underwood 1970), are inadequate when complex-shaped, perforated synapses are present.

In addition, counts of large objects, such as neuronal nuclei, will be over-estimated, and conversely small objects (e.g. synapses) will be under-estimated, since the probability of being transected by a 2-dimensional plane (a microscopic section), is proportional to the size of the object. Under-estimation can also occur as a result of the inability to view 'lost caps', which are sections through the edges of profiles too small to be seen. Another problem in quantitative microscopy is the over-projection of profiles below the section surface, onto the measuring plane. This is known as the 'Holmes effect' (Holmes 1927), and occurs due to the section having a positive thickness. All of these factors lead to bias of estimation of numerical density or number from 2-dimensional sections.

There are several formulæ used to estimate numerical density, which are all basically variations on a theme. Some of these are given below;

$$N_V = N_a / \bar{D} + t \qquad \text{(Abercrombie 1946)}$$

$$N_V = N_a.t / (4d/\pi) + t\text{-}2h \qquad \text{(DeHoff \& Rhines 1961)}$$

$$N_V = N_a / d$$ (Colonnier & Beaulieu 1985)

where; N_a = number per unit area, t = section thickness, 2h = twice the minimum height of particles, d = mean profile diameter

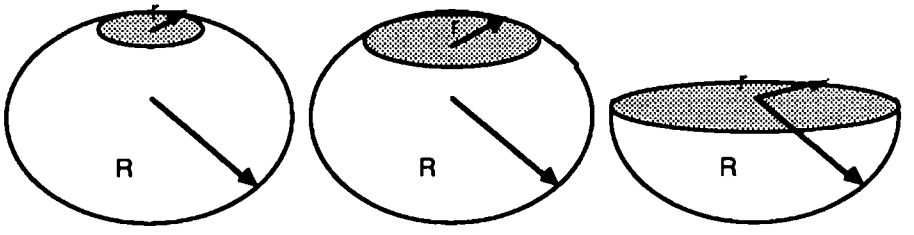


Fig.7.1a Spheres of equal size, with a radius of R, intersected by planar transects at differing levels. The profile radius (r) depends on the level at which the sphere is sectioned. Adapted by the author from Weibel (1979)

Recently, unbiased techniques have been described in the literature (for review, see Gündersen *et al* 1988a). These are the 'fractionator' (Gündersen 1986), the 'selector' (Cruz-Orive 1987), the 'serial section technique' (Cruz-Orive 1980), and the 'disector' (Sterio 1984).

b) The 'serial section' method

The serial section method, devised by Cruz-Orive (1980) is essentially a development from the formula of DeHoff and Rhines (1961), which stated that;

$$N_V = N_a / \bar{D}$$

where; N_a is the number of particles per unit area, $\bar{D}$ is the mean particle diameter. N_a is relatively easy to estimate from sections, but in order to estimate particle size from a random section, one needs to know something of the particle shape. Cruz-Orive devised a method whereby one need not know anything about shape, size or orientation of the particles under investigation (Cruz-Orive 1980). The method involves the determination of mean projected particle height, which is estimated via the use of serial sections. The method, as described in the original paper, is only valid for infinitely thin sections. The formula is as

follows;

$$N_V = N_a \cdot (1/\bar{H})$$

where; $\bar{H}$ is the estimated mean projected height of the particles. By taking account of section thickness (t), the method can be applied, without bias, to estimations of numerical density. The formula is then;

$$N_V = N_a \cdot (1/\bar{H} + t)$$

That is; the probability of finding a synapse profile in a given section, is estimated from the harmonic mean of the sum of synapse heights and section thickness. Estimation of mean synaptic height from serial sections is time-consuming and laborious, although short-cuts have been suggested (DeGroot 1988), It is hence not the method of choice.

c) The 'fractionator'

The fractionator allows absolute number to be estimated directly. Particles existing within a reference space (e.g. brain nuclei) are sampled with equal and known probability. The reference space is divided into pieces of arbitrary size and shape. A number of these pieces are then sampled. Sampling may be performed at several levels, such that the first sampling fraction may itself be sampled by cutting a smaller fraction. This process may be repeated many times depending upon the manageable size of the sampled pieces. An estimate of the number of particles (Q^-) within the final sample is then made, by use of serial sections. The total number of particles within the reference space can then be estimated as;

$$N = Q^- \cdot \text{fraction}_1 \cdot \text{fraction}_2 \cdot \text{fraction}_n \ldots\ldots$$

The efficiency of the technique is improved if each sample 'piece' contains roughly equal numbers of particles being measured.

It is a condition of the fractionator that all of the reference space is available for sampling, and particle estimation (Gündersen 1986). This is problematic at the periphery of the reference space, or the sample reference space, since there will be artificial boundaries created. Some particles may be sectioned such that they are contained partially within two separate reference volumes. They are therefore impossible to count unambiguously. Correction must be made for the corresponding un-sampled areas, within which these sectioned particles are contained. The LPO was the reference space of this particular exercise. The boundary of the LPO is indistinct, and contains within its upper portion, an area of separate function (homologous to area X of songbirds). This makes the use of the fractionator impossible in the present study, since the boundary cannot be ascertained with any degree of certainty. In addition, in order to quantify small particles such as synapses, one must make extensive fractionations, so that the final fractions can be sectioned exhaustively. This is considered impractical for studies where synaptic quantitation is involved (Calverley *et al* 1988).

d) The 'selector'

Both the 'serial section' method, and the 'disector' (described in next section), require a precise estimation of section thickness, which is notoriously difficult. Cruz-Orive devised a method for the determination of number which does not require the estimation of volume of reference space, within which the number estimation is made (Cruz-Orive 1987). Particles are sampled unbiasedly, using the 'disector' principle (see below), which selects particles irrespective of their size. This is important, since a random sample, say by a planar section, will sample particles proportional to their size. A large particle has a greater chance of being hit by the probe. The volume of each of the sampled particles is then estimated using point-sampled intercepts (Gündersen and

Jensen 1985). From the average of these values, an estimate of the population mean particle volume ($V_{N)}$ is obtained. If one then estimates the particle volume fraction (V_V), one can determine numerical density (N_V) using the following formula;

$$N_V = V_V / V_N$$

This method is problematic for use in the estimation of synaptic density. It is difficult to measure the volume fraction of synapses, since the synapse has a very small dimension in one direction; i.e across the synaptic cleft. In order to measure this dimension, and hence volume, one would need to magnify the synapse greatly. This is impractical, since the numbers of synapses, within a given brain region are many, and one would need to take numerous micrographs to obtain a reasonable sample. One other solution, would be to alter the counting unit, to include say, the pre-synaptic bouton. However, it has been shown that the PSD is the most reliable counting unit for such quantitative estimations (Mayhew 1979).

e) The 'disector'

The 'disector' was devised to counteract the drawbacks of the 'unfolding' method (Sterio 1984). It draws on no assumptions about the shape, size or orientation of particles, and hence is unbiased. It is defined as two parallel sections of known thickness (t) and separation (s), such that the distance between the upper plane of the two sections (h) is equal to t+s. This first section is the 'selected' section, and the other 'reference' section. Identified particles which lie within a sampling frame and which do not cross 'forbidden lines' (Gündersen 1977), are noted on the 'selected' section (see below for details). If that particle is absent on the subsequent 'reference' section, it is counted. The number of particles per unit volume (N_V) is then given by the following

formula;

$$N_V = \Sigma Q^- / h.A$$

where; ΣQ^- = total number of counted profiles, h = separation height between the two planes, A = area of reference space. The only assumptions of the disector are that a given profile must be unambiguously identifiable in the 'selected' section, and that h is small enough, so that no particle goes undetected between the disected planes. The latter assumption means that for synapses, h must be extremely small. In practical terms, this means taking planes which are separated by the width of a single section, ie. adjacent sections. It is therefore important that section thickness is accurately determined. It has been shown that the efficiency of estimation of numerical density of synapses using the 'disector' technique, is greater than the 'unfolding' techniques (Calverley *et al* 1988).

7.3 : EDGE EFFECTS: UNBIASED COUNTING RULE FOR 2D-SECTIONS.

Many of the counting methods rely on measurements taken from a restricted series of images obtained in two dimensions. These are usually in the form of micrographs, which present artificial boundaries within the tissue. The particles which one wishes to count, may or may not be contained entirely within a given micrograph. Some particles may only appear partially within the micrograph frame. The number of particles being counted is dependent upon the area (and subsequently volume) of tissue sampled. If one were to count all the profiles which lay wholly or partially within the frame, this would clearly lead to an overestimation of particles, since some profiles lying on the edge of the frame, are also in an area outwith the boundary used for the count. One therefore needs to devise a 'counting rule', such that say particles which lie partially within the micrograph on one side (e.g. top and right) are counted, and those on the other side (bottom and left) are not. The latter edges can be deemed 'forbidden

edges', since particles crossing these edges are not counted. However, some particles at the corner of the counting frame may appear as part of a profile, say at the right hand side of the micrograph, where it is counted, and reappear at the bottom of the frame, where it is excluded. There also exists the possibility of counting particles more than once, where they appear at both top and right hand edges, leading to an overestimation. The frame then, needs to be smaller than the visible part of the field, in order that one can ascertain the associations of the particle profiles. The distance between the edge and the frame must be at least the size of the largest particle encountered (Gündersen 1977). In addition, the 'forbidden lines' (since they are no longer edges) must be parallel, and extended to infinity (Gündersen 1977). Such a frame is shown in Fig.7.1b. This rule for counting irregular profiles using a two dimensional sample probe, is the only one known to be unbiased.

7.4 : THE ESTIMATION OF SIZE

A common requirement for quantitative biological studies, is to estimate changes in the size of cellular elements. An increase in size results in a corresponding increase in volume, therefore there is a requirement for an unbiased estimator of volume. Such an estimator has recently been described by Gündersen and Jensen (1985). The emphasis here is on *unbiased*, since almost all estimators prior to 1985 rely upon assumptions regarding particle shape (see Section 7.2a above). To estimate the volumes of particles from profiles which appear on random sections, is biased, since larger profiles have a greater probability of being sectioned. Hence one needs to weight the estimations according to particle size. Gündersen and Jensen (1985) describe a method whereby one measures the volume-weighted mean volume ($\bar{V}_V$) of particles, and hence fits the requirement outlined above, by being unbiased. This is obtained by direct moment estimation from point-sampled intercepts, such that;

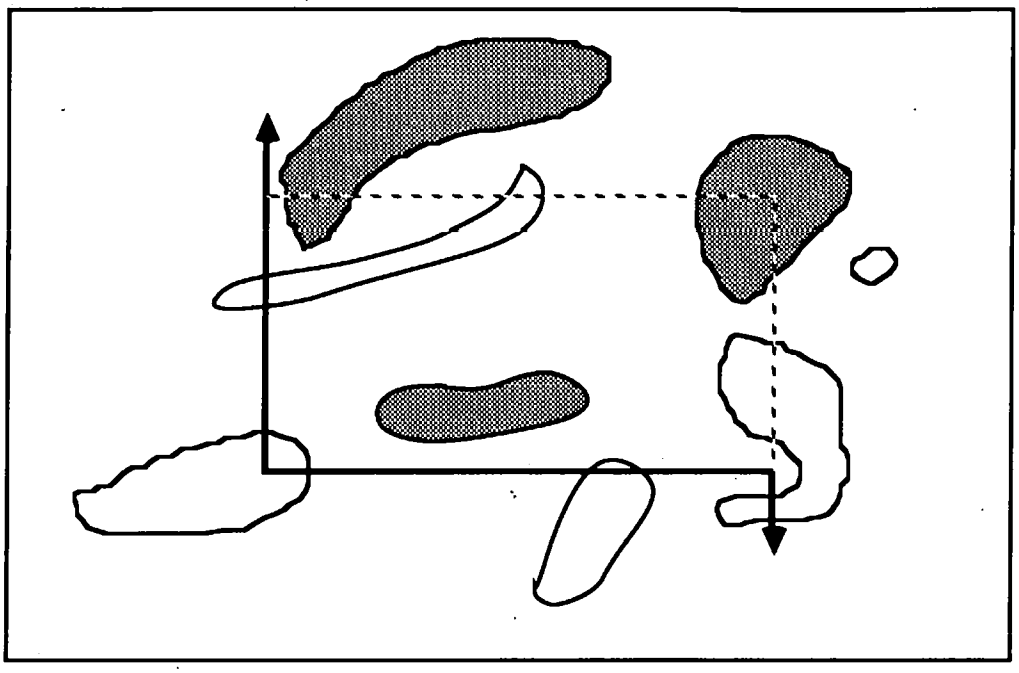


Fig.7.1b : The forbidden line. A diagram illustrating the application of the 'forbidden line' rule on a micrograph field containing a number of particles. Only particles contained entirely within the frame, and those intersected by the stippled line, but not by the bold line ('forbidden line'), which extends to infinity, are counted (i.e. hatched profiles).

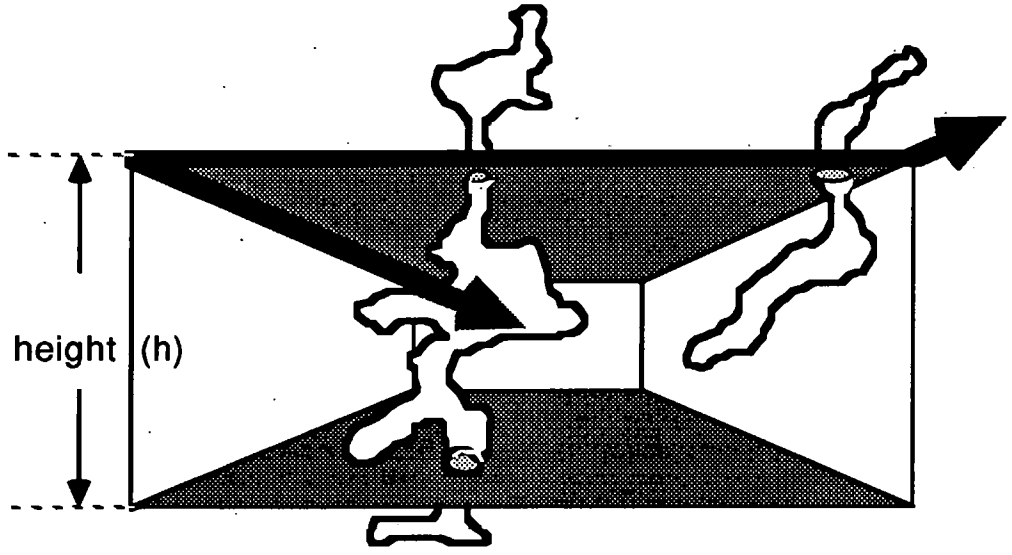


Fig.7.1c : The Disector. A disector of distance h, generating planar transects by cutting through particles in a containing reference space. The upper plane represents the 'selected' section, the lower plane the 'reference' section. If particles transect both planes, or cross the frame's forbidden line (dense line), they are not counted. Particles are only counted when they intersect the selected plane alone, and do not intersect the forbidden line. Adapted by the author from Gündersen (1986).

$$V_V = \pi/3 . l_o^3$$

where; l_o is the length of the linear intercept across the profile and through a random point which intercepts that profile. The volume-weighted mean volume is only unbiased if the direction of the line through the point-sampled intercept is isotropic with respect to the particles. This can be achieved if either the particles, or the sections, are isotropic. This is unusual however, and it is more likely than not, that they will be anisotropic. A solution to this problem was described by Baddeley *et al* (1986). They used 'vertical sections', all parallel to one arbitrary axis (the vertical). This is the case in the present studies using electron microscopic fields, since they are all parallel to one another. On such sections, one must use anisotropic directions. Practical details of this method are given in Chapter 10.

If a randomly orientated particle increases in volume, then the mean linear dimension of those particles will also increase. The most commonly used estimator of this kind, is that of mean projected particle height ($\bar{H}$) (Calverley *et al* 1988, Sterio 1984, Cruz-Orive 1980). That is, the average linear size of particles in a direction perpendicular to the plane of sections. This can be estimated from the ratio of profiles that appear in one section but not in a subsequent section (Q^-), to the total number of profiles present in the section plane (Q). Then it follows that;

$$\bar{H} = \Sigma Q.h / \Sigma Q^-$$

where; h is the distance between section planes. Changes in size may be restricted to two dimensions, such as increases in surface area (Sa). This can be estimated using line sampled intercepts (Weibel 1979), such that;

$$Sa = 2 . I_L$$

where; I = intersections between test lines and particles, L = total test line

length within the counting frame.

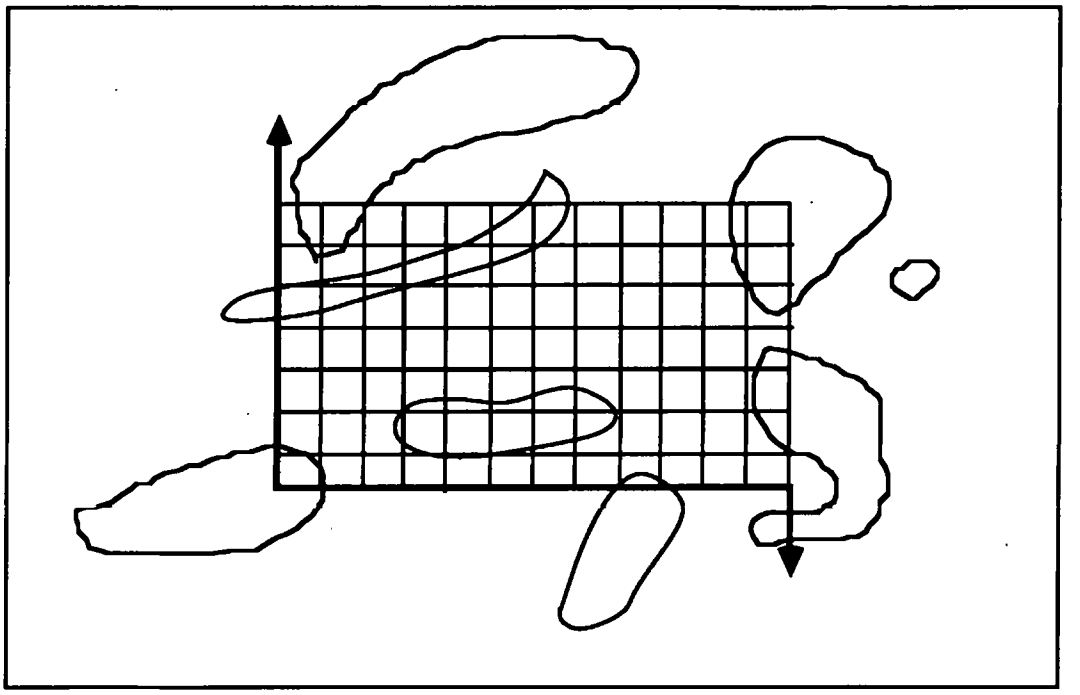


Fig.7.1d : Line sampled intercepts. An overlay test-grid is used to determine surface area (Sa). This is achieved by noting the number of intersections (I) which the particles make with test lines (single black lines) of length, L (corrected for magnification of micrograph). The forbidden lines (heavy arrowed lines) are not part of this test system, but may be used in the estimation of particle number (see Fig.7.1b). Formula for the calculation of Sa is given in the text.

CHAPTER 8 : PILOT STUDY - SAMPLING EFFICIENCY

8.1 : INTRODUCTION

In order to obtain an estimate of the number of synapses in the chick LPO, it is necessary to make measurements on tissue samples. Indeed, there is a hierarchy of sampling needed: i.e a sample of chicks, from which a sample of tissue blocks from the LPOs of those chicks, from which a sample of sections is taken, etc. This sampling scheme is carried down to the lowest level, which is the sample of micrograph fields from a given section. At each level, an assumption is made that the sample is representative of the stage above. There is however, a degree of error introduced with each assumption. A summation over all levels gives the error associated with the experimental design (systematic error). In addition, there is also an error involved when one assumes that the results truly reflect the population, since there is an inherent biological variation between individuals. All of these errors contribute to the overall observed variation in a given group. At all levels of the experimental design, some error is introduced due to the imprecise nature of measurements. The size of the error will reflect the precision of the measuring device or method employed. Thus, the sampling technique employed is of utmost importance with respect to reducing the magnitude of systematic error. In this thesis, systematic random sampling has been employed throughout. The benefits of systematic random sampling are considered fully elsewhere (Gündersen and Jensen 1987).

By using analysis of variance principles, it is possible to isolate the variations due to each of the sampling levels, and to differentiate this from the biological variation (Shay 1975). A knowledge of differences in the magnitude of error attributable to the sampling levels, will allow the experimenter to devise

the optimum sampling strategy. By applying the appropriate experimental design, one may therefore gain the maximal amount of biological information from the study. In all of the above types of error, the overall variance can be reduced by increasing the sample size, or by increasing the precision with which measurements at a particular level are made. However, these improvements are time consuming and costly. A compromise between an acceptable degree of error in estimation, and the cost of obtaining that accuracy must be balanced.

The purpose of this pilot study was to analyse the efficiency of the counting technique, and to ensure that there was an adequate representation of the tissue in the main study. One of the aims of the main study, was to describe the synaptic development of the LPO. Hence for the pilot study, an analysis of the efficiency of the counting method had to be performed over a wide range of developmental ages. To do this, both pre- and post-hatch material was needed.

8.2 : MATERIALS AND METHODS

Animals : Six embryos aged 16 days *in ovo* (E16) were excised from their shells, and given an intra-peritoneal injection of 0.05 ml sodium pentobarbitone (60 mg/ml). They were then perfused through the left ventricle of the heart with approximately 100 ml of a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (final osmolarity was approximately 1130 milliosmoles), using a peristaltic pump. Groups of six chicks, each of 1 day (day after hatching) (P1), and 9 days (P9), were used for the post-hatch period of the experiment. These chicks were given an intra-peritoneal injection of 0.1 ml of sodium pentobarbitone. They were then perfused in a similar manner to that described above for the pre-hatch chick embryos, however 20 ml of 0.9% NaCl (saline) preceded the fixative fluid. This served to

flush the vascular system of cells, and so prevent blood from clotting, which may have hindered the perfusion.

Tissue preparation : The brains were carefully dissected from the cranium, and were then immersed in fresh primary fixative overnight at 4°C. A coronal slab of fixed brain tissue containing the LPO, was obtained using a resin mould which had been constructed using stereotaxic co-ordinates, designed specifically for the purpose (Rose and Csillag 1985). Two pieces of LPO tissue were obtained (one rostral, one caudal), which were approximately 1 mm^3 in size. These tissue pieces were then processed for electron microscopy, using Spurr's resin as the embedding medium.

Microtomy : Once polymerized, the resin moulds yielded two 'blocks' of tissue for each brain hemisphere. These blocks were trimmed non-symmetrically, in order for unambiguous orientation in the microscope. This was achieved by cutting a square-shaped block face, and cutting the top left hand corner at 45°. Trimmed blocks were sectioned using a Reichert-Jung Ultramicrotome and diamond knife. Sections of a silver interference colour were thereby obtained (approximately 50-60 nm thickness). They were stretched with chloroform, and then picked up on single-hole copper grids coated with either formvar or pioloform. Sections were stained with uranyl acetate and lead citrate, and viewed in a Jeol 100S electron microscope at an accelerating voltage of 80 kV.

Microscopy : Ribbons of parallel serial sections were used in order that proper alignment of the fields of view could be obtained. If any section or its adjacent neighbour contained a large number of folds, it was eliminated from further investigation. Suitable candidates were initially screened using a stereo-

microscope, and then subsequently using the electron microscope at low power (x2000). Two suitable adjacent sections were thus selected for further study at this magnification. Once selected, analysis was performed to completion, in order that selection of sections could not be influenced by experimenter bias.

Because the sections had been cut in such a way that their orientation could be established, the first section of the two was found. This section was nominated the 'reference' section, and its neighbour was nominated the 'selected' section. However, by applying the 'disector' in reverse, these labels could be reversed. The top left hand corner of the 'selected' section was located at the nominal magnification of X2000. At this magnification, synapses are invisible on the microscope screen, and therefore no experimenter bias with regard to area selection could be introduced. The magnification was then increased to X6000. On the fluorescent screen of the microscope, there is a field delineator, which marks the boundary of the micrograph area. The top left hand corner of this frame was placed on the nearest small object (e.g. a mitochondrion), and a micrograph taken. This procedure enabled alignment of the micrographs taken on adjacent sections. The same field of view was found in the next adjacent (serial) section, and a micrograph of this area was also taken at X6000. These two micrographs represented a single disector, though by using one micrograph firstly as the 'selected' section, and then subsequently as the 'reference' section, sampling efficiency was doubled. Each micrograph was individually labelled with a sequential number, in order that unambiguous identification of the first section of a pair could be made. The magnification was again reduced to X2000, and the initial area on the first section located. From this point, the section was moved exactly 3 field widths to the right. Another disector was obtained as described above. If a field of view contained a section edge, the frame was moved down 3

frames, and the disector procedure repeated moving from left to right. This zig-zag pattern across the section was repeated until a minimum of 20 micrographs were obtained. No micrographs were taken of any area obscured by section folds, or staining precipitates. In this way, an unbiased systematic sample of almost the whole block face was obtained. 20 micrograph pairs were taken of each tissue block. Prints were made at final magnifications of approximately X20,000.

Section thickness : An estimation of section thickness was determined by the 'small fold method' (Small 1968). Folds on the sections are unavoidable when using a supporting film, such as formvar, or pioloform. In this instance however, they turn out to be of great benefit. The smallest folds possible on a section are known as 'minimal folds'. They are characteristic since they have parallel edges running for some distance, and at high power display a thin cleft in the centre of the fold. These sections are exactly twice the section thickness, since they are made by two opposing vertical 'pleats' viewed edge-on. One of the benefits of the 'small fold' method, is that it is a measurement made from electron microscopic images, and is therefore made under the influence of the electron beam. The measurement of synaptic density, and estimations of size are also made on sections placed under the electron beam. It is therefore desirable to measure section thickness under these same conditions.

An alternative method for calculating section thickness involves re-embedding and re-cutting the section (Yang and Shea 1975, Bedi 1987). This method was rejected because the section is liable to be subject to compression distortion due to the microtomy. The small fold method does not suffer from this drawback. Above all, the chosen method is simple, and gives a permanent record of thickness for each section.

Measurements of section thickness of six separate sections, were made firstly by interferometric methods (kindly performed by Dr.A.Warren, Sheffield University), and secondly by the 'small fold' technique. The microinterferometer used was the Vickers M86 (Goldstein & Hartman-Goldstein 1974). This instrument passes a laser beam of wavelength 632.8 nm through the section, and through a reference of empty background. The optical path difference (OPD) between these two can be related to the thickness of the section;

$$OPD = t\,(Ns-Nb)$$

where 't' is the section thickness, 'Ns' is the refractive index of the section, and 'Nb' is the refractive index of the background. The estimates produced by the two methods were within 5 nm of one another for any given section, showing the reproducibility and accuracy of the 'small fold' method.

Synaptic identification : Synapses were recognised by the presence of both pre- and post-synaptic dense projections, with at least 3 associated vesicles. An exception to this rule was made for synapses sectioned '*en face*', where either post-synaptic dense projections or pre-synaptic dense projections were seen as a lattice-like array. These profiles were also included in the analysis. The post-synaptic thickening has been shown to be the most reliable counting unit for synapses (Mayhew 1979).

Calculation of synaptic numerical density : The numerical density of synapses in the LPO (Nv_{syn}) was calculated using the formula;

$$Nv_{syn} = \Sigma Q^{-}_{syn} / h.A$$

where 'ΣQ^{-}' represents the sum of the synapses present in the counting frame of the 'selected' section but absent in the 'reference' section. 'h' is the distance between disector planes, and 'A' is the sample area. The sample area was defined

by the boundaries of the sampling frame, appropriately corrected for magnification. The bottom and left-hand boundaries of the frame were deemed 'forbidden lines' (Gündersen 1977). Any synapse crossing this line was automatically excluded from the count. These forbidden lines were placed at least 3 cm inside the boundaries of the micrograph.

Calculation of nested sample variance : The nested sampling regime employed in this study was;

ANIMALS ⟶ BLOCKS ⟶ DISECTORS ⟶ FIELDS

Each age group contained six chicks. Only the left hemisphere LPO from these chicks was analysed, since it was assumed that the efficiency of the sampling scheme would not differ between the hemispheres. From each hemisphere there were two blocks; one from the rostral LPO, and one from the caudal LPO. Each block gave rise to 2 disectors, which were effectively two adjacent (serial) sections. The first disector was obtained in one direction (i.e. from the first section to the second), and the second disector taken in the reverse direction (through the same spatial reference volume) (i.e. from the second section to the first). From each disector (section), there were 10 micrograph pairs taken, therefore each disector was represented by 10 fields of view.

The individual results of a given sampling level (e.g. number of synapses per field) provide a mean of the sampling level above (e.g. disectors). By comparing each estimate with the mean for all estimates obtained in this way, one can analyse the variance at that sampling level, through a calculation of the sums of squares (SS), and mean sums of squares (MS) (see Shay 1975, for details). The sample variances can be calculated as;

Sample variance of fields (Sf^2) = MS(f)

Sample variance of disectors (Sd^2) = $\frac{MS(d) - MS(f)}{nf}$

Sample variance of blocks: (Sb^2) = $\frac{MS(b) - MS(d)}{nf \times nd}$

Sample variance of animals (Sa^2) = $\frac{MS(a) - MS(b)}{nf \times nd \times nb}$

where; MS(a) is the mean sums of squares between animals, MS(b) is the mean sums of squares between blocks, MS(d) is the mean sums of squares between disectors, MS(f) is the mean sums of squares between fields, na is the number of animals, nb is the number of blocks, nd is the number of disectors, nf is the number of fields. All calculations were made on the basis of number of synaptic profiles fulfilling the disector principle (Q^-) per unit volume. Each field estimate was therefore adjusted to take account of the different magnifications, and different sample volumes. The variances are then expressed as a percentage of the total, using the following formulae;

Variance ratio of block estimates (R_b) = $\frac{(Sb^2/Nb)}{Os^2} \times 100$

Variance ratio of disector estimates (R_d) = $\frac{(Sf^2/Nb.Nd)}{Os^2} \times 100$

Variance ratio of field estimates (R_f) = $\frac{(Sf^2/Nb.Nd.Nf)}{Os^2} \times 100$

8.3 : RESULTS

16 days *in ovo* (E16) : The total observed variance (Os^2) between chicks was 2.67 X 10^{-3}. The block variance was 3.54 X 10^{-4}. The variance between disectors (Sd^2) was 8.45 X 10^{-5}, and the variance between fields (Sf^2) was 7.05 X 10^{-2}. (Table 8.3a). These variances between blocks and disectors contributed little to the overall variance (7% and 8% respectively). The field variance accounted for 14% of the total. However, the vast majority of the observed variance (71%) was due to the biological variation between chicks (Sa^2 = 1.85 X 10-^{3}). The results for this group can be seen in Table 8.3a.

1 day post-hatch (P1) : The observed variance (Os^2) = 2.16 X 10^{-3}, of which 1.35 X 10^{-3} (62%) was attributable to the variance between animals. The variation between blocks was 5.21 X 10^{-4}, accounting for 12% of the total. The variance between disectors (Sd^2) was 5.76 X 10^{-4}, and the variance between fields (Sf^2) was 3.30 X 10^{-2}. These latter two factors could explain only a quarter of the total observed variance (7% and 19% respectively). The results for the 1-day old group are shown in full in Table 8.3b.

9 days post-hatch (P1) : The observed variance was 5.64 X 10^{-2}. The variance between blocks was 2.24 X 10^{-2}. The variance between disectors was 9.24 X 10^{-3}, and the variance between fields was 5.01 X 10^{-1}. It could therefore be deduced that the variance due to chicks was 3.65 X 10^{-2}. Again, the inherent biological variation far outweighed the variance of the other factors. It accounted for 65% of the total observed. The variance between blocks was approximately 20% of the total, whilst that due to disectors was only 4%, and that due to fields, 11%. The results for the 9-day post-hatch group are displayed in Table 8.3c.

Table 8.3a : E16 Sampling variances

Animals (Sa^2)	Blocks (Sb^2)	Disectors (Sd^2)	Fields (Sf^2)	Total (Os^2)
1.85×10^{-3}	3.54×10^{-4}	8.45×10^{-4}	3.05×10^{-2}	2.67×10^{-3}
	1.77×10^{-4} (Sb^2/Nb)	2.11×10^{-4} ($Sd^2/Nb.Nd$)	3.81×10^{-4} ($Sf^2/Nb.Nd.Nf$)	
71	7	8	14	100 **Percent**

Table 8.3b : P1 Sampling variances

Animals (Sa^2)	Blocks (Sb^2)	Disectors (Sd^2)	Fields (Sf^2)	Total (Os^2)
1.35×10^{-3}	5.21×10^{-4}	5.76×10^{-4}	3.30×10^{-2}	2.16×10^{-3}
	2.60×10^{-4} (Sb^2/Nb)	1.44×10^{-4} ($Sd^2/Nb.Nd$)	4.12×10^{-4} ($Sf^2/Nb.Nd.Nf$)	
62	12	7	19	100 **Percent**

Table 8.3c : P9 Sampling variances

Animals (Sa^2)	Blocks (Sb^2)	Disectors (Sd^2)	Fields (Sf^2)	Total (Os^2)
3.65×10^{-2}	2.24×10^{-2}	9.24×10^{-3}	5.01×10^{-1}	5.64×10^{-2}
	1.12×10^{-2} (Sb^2/Nb)	2.31×10^{-3} ($Sd^2/Nb.Nd$)	6.25×10^{-3} ($Sf^2/Nb.Nd.Nf$)	
65	20	4	11	100 **Percent**

Optimal sampling design : In order to determine the optimal sampling design, it was necessary to estimate the cost (in time) of each item in the study. The values are given below, and should only be considered a rough approximate.

	Animals	Blocks	Disectors	Fields
Cost (mins)	385	160	100	10

The cost is the time taken in total, to include an extra animal, block, disector, or field in the study. It is an entirely subjective amount, and includes penalties for procedures which are difficult, costly (in financial terms), or for which there are restrictions on equipment use (e.g. microtomes, microscope, etc.). Time penalties were also added where occasional preparatory work was needed (e.g. coating of pioloform grids). These penalties seem reasonable, since the desire is to find the most efficient experimental design, and efficiency must be equated with ease of use. With this information, the following formulae were applied (after Gündersen and Østerby 1981);

$$Onf = \left[\frac{Sf^2}{Sd^2} \times \frac{Cd}{Cf} \right]^{0.5}$$

$$Ond = \left[\frac{Sd^2}{Sb^2} \times \frac{Cb}{Cd} \right]^{0.5}$$

$$Onb = \left[\frac{Sb^2}{Sa^2} \times \frac{Ca}{Cb} \right]^{0.5}$$

where; Onf is the optimal number of fields per disector, Ond is the optimal

number of disectors per block, Onb is the optimal number of blocks, Sa^2 is the variance due to animals, Sb^2 is the variance due to blocks, Sd^2 is the variance due to disectors, Sf^2 is the variance due to fields, Ca is the cost per animal (in minutes), Cb is the cost per block (in minutes), Cd is the cost per disector (in minutes), Cf is the cost per field (in minutes).

The corresponding values for chicks of varying ages are given below;

16-days *in ovo*

$$Onf = \left[\frac{3.05 \times 10^{-2}}{8.45 \times 10^{-4}} \times \frac{100}{10} \right]^{0.5} = 19$$

$$Ond = \left[\frac{8.45 \times 10^{-4}}{3.54 \times 10^{-4}} \times \frac{160}{100} \right]^{0.5} = 2$$

$$Onb = \left[\frac{3.54 \times 10^{-4}}{1.85 \times 10^{-3}} \times \frac{385}{160} \right]^{0.5} = 1$$

1-day post hatch

$$Onf = \left[\frac{3.30 \times 10^{-2}}{5.76 \times 10^{-4}} \times \frac{100}{10} \right]^{0.5} = 24$$

$$Ond = \left[\frac{5.76 \times 10^{-4}}{5.21 \times 10^{-4}} \times \frac{160}{100} \right]^{0.5} = 1$$

$$Onb = \left[\frac{5.21 \times 10^{-4}}{1.35 \times 10^{-3}} \times \frac{385}{160} \right]^{0.5} = 1$$

9-days post hatch

$$Onf = \left[\frac{5.01 \times 10^{-1}}{9.24 \times 10^{-3}} \times \frac{100}{10} \right]^{0.5} = 23$$

$$Ond = \left[\frac{9.24 \times 10^{-3}}{2.24 \times 10^{-2}} \times \frac{160}{100} \right]^{0.5} = 1$$

$$Onb = \left[\frac{2.24 \times 10^{-2}}{3.65 \times 10^{-2}} \times \frac{385}{160} \right]^{0.5} = 1$$

Progressive Means Test : In order to check whether the low sample sizes indicated were realistic, an analysis of the progressive means estimation was undertaken (Williams 1977). This consisted of making an estimate of Nv_{syn} from a single field ('selected' field), using the 'disector' method. A second field is further analysed from the same disector plane, and the mean estimate of Nv_{syn} between the two fields is calculated. This 'progressive mean' calculation is continued for approximately twenty fields. Each additional field gives an increased sample volume, and therefore a more reliable estimate of the 'true' value. There is a limiting factor, namely the inherent variation in the experimental design (which includes the variation between individuals). The 'true' population mean value can only be estimated within the bounds of biological variation. Increasing the sample size does not help to reduce this error of estimation. We must deduce the size of sample, which is a minimum to estimate the 'true' value within these confines, since this is the sample size which will give the most efficient estimate in terms of cost (time).

Results for each field were plotted as a percentage of the final estimate of

Nv_{syn}, after 20 fields were analysed. Mean estimates for each field were obtained from analysis of all blocks within a given age group of chicks. Confidence limits of ± 10% were set, within which, all progressive mean estimates and their standard errors had to fall. The minimum number of micrographs needed was taken as the lowest number of fields after which all subsequent estimates and their errors, fell within the set confidence limits. The results are shown in Figs. 8.1a,b,c.

Fig.8.1a

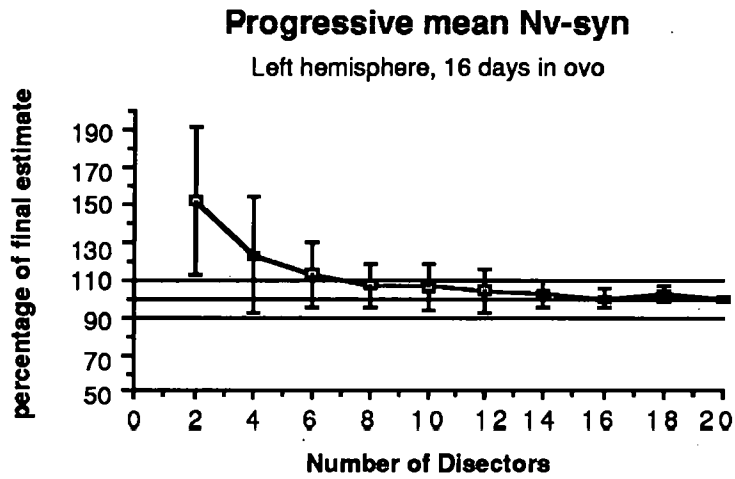


Fig.8.1b

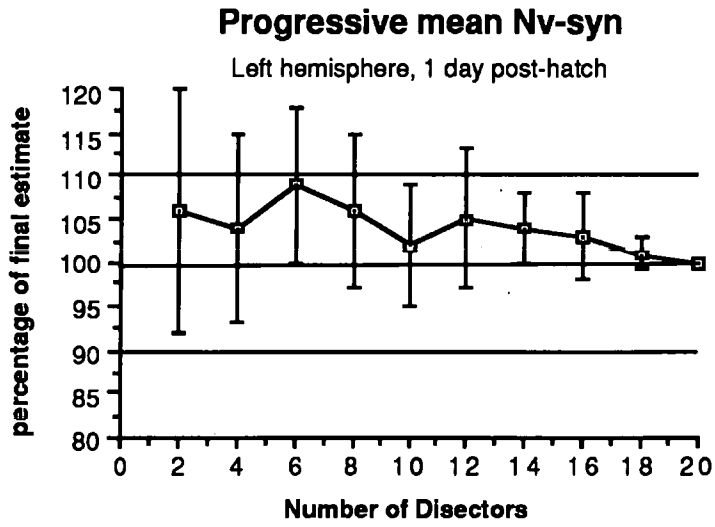
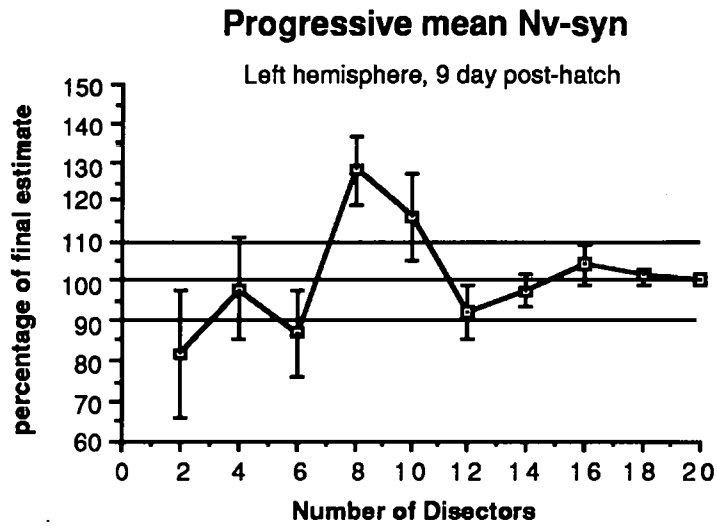


Fig.8.1c



Figs.8.1a,b,c : These graphs show the progressive estimates of Nv_{syn} ± SEM expressed as a percentage of the final estimate after 20 pairs of micrographs, which represent two disectors. The estimates are based upon counts of synapses in the left hemisphere LPO of chicks aged 16-days *in ovo* (a), 1 day post-hatch (b), and 9 days post-hatch (c). The minimum number of disectors required to bring the estimate and its SEM permanently within 10% of the final value is 14 in all 3 cases.

8.4 : DISCUSSION

It is clear that the majority of the observed variation, in all 3 age groups, is due to the inherent variation between animals. In this pilot study, the inter-animal variation accounted for between 62 and 71%. That is, the variation between individuals, is by far the greatest source of error in the experimental design. This is in accordance with previous findings for other experimental designs (Gupta *et al* 1983, Gündersen and Østerby 1981). Clearly, the magnitude of inter-animal variation will depend upon the measurements made, since variation is not uniform. In the present case, the variance is based upon synaptic density in the left hemisphere LPO, which varies substantially between individuals. If one had measured for example, body temperature, then the variance between individuals would be very low.

There is very little variation between blocks, which ranges from 7% of the total observed variance in the E16 group, to 20% in the P9 group. There may be a significant increase in the variance with developmental age. Embryonic chicks have few synapses, and as they develop new synapses are acquired. The rate at which they do this differs between individuals, since synaptic development is influenced by environmental conditions, which may not be experienced similarly by all chicks. It is possible that during this developmental phase, the LPO undergoes differentiation which may result in differences in synaptic density in different localities. Since the variance increase is small, this is not of major concern to the experimental design. Only 2 disectors were used in the present study, the variance between them was negligible (4-8% of total observed variance). This perhaps reflects the fact that the second disector is taken through the same volume as the first, only reverse in direction. Field variation contributed to the second largest source of experimental error, although in relative terms was still small (11-19% of the total observed variance). This suggests that increasing the number of fields, would be of relatively small benefit to the efficiency of the experimental design. This was confirmed in the progressive means test, which showed that by as few as 14 fields, the estimates of synaptic density were within ± 10% of the final estimate taken after analysis of 20 fieids. The synaptic counts per field increased with age, but were approximately 10 per field in the P9 group. Therefore, the sample size does not have be very large in order to make a reasonable estimate (i.e. with low error) of the population mean.

The optimum sampling design for the study was assessed for groups of chicks of varying ages. However, analysis has shown that the efficiency of the sampling

design does not alter significantly between the groups. The optimum number of blocks in all 3 cases was 1, although there was some rounding down performed in the calculation. It was decided therefore, that 2 blocks would be adequate for further studies. This result was welcome, since the analysis of blocks is labour-intensive. It implies that at least for the regions sampled (rostral and caudal), there is little variation in synaptic density, within a given LPO. That is, it is relatively homogeneous with respect to the density of synapses, which aids any quantitative study of this region. The optimal number of disectors per block was 1-2. Again, this is in line with the outlined sampling strategy undertaken in this pilot study, and will hence be continued in further work. The optimum number of fields ranged from 19 in the E16 group, to 24 in the P1 group, which is in close approximation with the numbers used in the present study (20). However, the progressive means test suggested that as few as 14 fields were sufficient to provide a reasonable estimate of the synaptic density in a given LPO. There is a constraint upon the number of micrographs that one can obtain for a given block, as the microscope camera system holds a maximum of 50 plates. Since disector pairs are required, this puts a limit of 25 fields as a maximum. With each batch of micrographs, a micrograph of a calibration standard, and a micrograph of a small fold, with accompanying calibration standard are also needed. This effectively limits the maximum field number to 23. Of course, it would be possible to insert a second plate cannister into the microscope, but this would not be cost effective, since the time required to perform such a procedure would be prohibitive.

CHAPTER 9 : DEVELOPMENTAL STUDY OF LPO SYNAPSES

9.1 : INTRODUCTION

It has been suggested previously in this thesis, and elsewhere (see Chapter 4 for review), that learning, and subsequent memory formation may result in an alteration of synaptic structure. It has been shown that the synapse is an extremely plastic structure, and is modifiable by a variety of experiential factors (for review, see Greenough 1985). Structural changes may occur through synaptic use or disuse (Rutledge 1976). One method of investigating these structural changes, is by examining the synapses during normal development, when they are used increasingly (cumulatively) with time. Early development is a period of intense information acquisition, and hence structural changes seen during this time, may in part, reflect plasticity due to memory.

There is a general agreement on the developmental course of certain synaptic characteristics of mammalian brain during early development. Synaptogenesis is one constant feature of early development, and is usually rapid and of large magnitude (Nixdorf 1989, Aghajanian and Bloom 1967, Mates and Lund 1983b, Jones and Cullen 1979). In addition to the increase in synapse number, there are changes in the size and shape of synapses. There appears to be a thickening of the post-synaptic density (DeGroot and Vrensen 1978, Markus *et al* 1987), and an increase in the number of vesicles per synapse (Devon and Jones 1981, Dyson and Jones 1980). However, there is widespread disagreement on other parameters, e.g. synaptic length (DeGroot and Vrensen 1978, Dyson and Jones 1980, Jones and Cullen 1979, Petit 1988, Markus *et al* 1987), cleft width (DeGroot and Vrensen 1978, Markus *et al* 1987), and changes in the number of pre-synaptic dense projections (DeGroot and Vrensen 1978, Nixdorf 1990b).

Comparatively little data has been obtained on the synaptic development of the avian brain, and in particular, that of the domestic chick. In common with mammalian synapses, the avian synapses also undergo a rapid increase in number during early development (Curtis *et al* 1989, Nixdorf 1990b, Bradley and Galal 1984, Bradley 1985). Synaptic size on the other hand, appears to be more stable, increasing little with age in the first few weeks post-hatch (Curtis *et al* 1989, Nixdorf 1990b, Rostas *et al* 1984). There is little data on synaptic size in pre-hatch chicks, but synaptic size has been shown to increase rapidly in the IMHV between 1 and 2-days post-hatch. Synaptosomal preparations from chick forebrain have also shown that PSD's increase in length between 16-days *in ovo*, and 2-days post-hatch.

Recent studies have suggested a role for the LPO in memory formation (for review, see Stewart 1990). It is therefore necessary to have some baseline data with which to compare the plastic changes that are brought about by a learning task, with those already established by normal development. The naive state of the nucleus may then be compared with post-hatch development, when experience and memory formation are prevalent.

9.2 : MATERIALS AND METHODS

Animals : Approximately 50 chick eggs (Ross I) were incubated in the department animal house. Hatched chicks were subsequently reared as a single group, and kept in communal brooders. Temperature was kept at a constant (38-40^{o}C) throughout the experiment. Eggs were rotated in the incubator. Hatched chicks were exposed to a constant 12 hour light/ 12 hour dark cycle. Chick embryos aged 16 days *in ovo* (E16) were excised from their shells. It has been

shown that by E16, neurons in the chick telencephalon have migrated to their final 'adult' positions (Tsai *et al* 1981a). This is also the minimum age at which the major post-synaptic density protein could be detected in sub-cellular fractions from chick brain (Rostas *et al* 1984). This protein is thought to be the main factor influencing synaptic thickness (Rostas *et al* 1984). It was therefore decided to select this age as a starting point for the developmental study. The E16 chicks were given an intra-peritoneal injection of 0.05 ml sodium pentobarbitone (60 mg/ml), and then perfused through the left ventricle of the heart with approximately 100 ml of a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (final osmolarity was approximately 1130 mOsm), using a peristaltic pump. Groups of chicks of 1 day (day after hatching) (P1), 9 days (P9), and 22 days (P22), were used for the post-hatch period of the experiment. These chicks were given an intra-peritoneal injection of 0.1 ml of sodium pentobarbitone (60 mg/ml). They were then perfused in a similar manner to that described above for the pre-hatch chick embryos, however 20 ml of 0.9% NaCl (saline) preceded the fixative fluid.

Tissue Analysis : The E16 and P9 groups both contained 5 chicks. One brain from each of the P1 and P22 groups was not adequately fixed, and hence this group contained 4 chicks. Since it was decided to restrict analysis to a single batch of chicks (from common parentage), it was not possible to increase these numbers. Each brain was dissected from it's cranium, and the hindbrain removed. The posterior surface of the forebrain was then attached to a metallic chuck with glue, which was then transferred to a Reichert-Jung vibratome. From the tip of the frontal lobe, a section of a random thickness (between 10 and 100 μm) was discarded. This section did not contain any part of the LPO, since

the LPO lies caudal to this plane. This procedure achieved a random starting position for subsequent sections. A section of approximately 100 µm thickness, was then taken and mounted onto a glass slide. A light micrograph of this 'thick' unstained section was made at a magnification of X35. This micrograph was used to estimate the volume of the LPO, and the procedure for this is described below. From this position, a section approximately 200 µm thick was cut, and was subsequently processed for electron microscopy. A further section of 100 µm thickness was cut and subsequently discarded. The next successive section was cut (100 µm thick), mounted on a slide and then photographed for volume analysis. Hence, a repetitive systematic cycle of sections were cut, some being used for volume estimation of the LPO, and others being processed for electron microscopy. This process was continued until the blade cut through through the optic lobes, or the section displayed lateral divergence of the ventricles, which occurs near the point of appearance of the optic lobes. This ensured that at least 12 micrographs were obtained from each brain for use in the volume analysis. From the samples of tissue processed for electron microscopy, two were selected at random for subsequent analysis. Quantitative analysis of synaptic numerical density within these blocks was made using the 'disector' principle, as described previously (Chapter 8, Section 2).

Volume estimation of chick LPO : The volume of the LPO of each hemisphere of individual brains, was estimated using Cavalieri's principle (Gündersen and Jensen 1987). The area of brain occupied by the LPO on each micrograph (obtained as described above) was estimated with the aid of a stereotaxic atlas (Salzen and Williamson, unpublished). The atlas was designed for use with chicks aged 0-2 weeks of age, with appropriate scales for each age. The approximate boundary of the LPO was drawn onto each of the micrographs. Since there is no

clear boundary, this was largely performed as a rough approximation, and an atlas was used as guidance (Saltzen and Williamson, unpublished). A test grid lattice, with intercept sample points separated by a distance of 1 cm, was overlayed in random orientation, onto the micrographs. The number of points which lay within the delineated LPO in each hemisphere was noted. The volume could be then calculated from the formula;

$$V = t.\ (A_1+A_2+....+A_m)$$

where; 'm' is the random number of systematic sections, of areas 'A_1,A_2,...,A_m'. 't' is the distance between section planes. Planar areas may be unbiasedly and efficiently estimated from point-sampled intercepts, such that;

$$A_{lpo} = (a/p).P_{lpo}$$

where; 'a/p' is the unit area between test points (corrected for magnification), and 'P' is the number of test points hitting the LPO. Combining the above two equations;

$$V_{lpo} = t.(a/p).P_{lpo}$$

Synaptic identification : Synapses were identified by the criterion described previously (Chapter 8, Section 2). They were further classified on the basis of the appearance of the post-synaptic thickening, and the nature of the post-synaptic target. Four groups of synapse were identified; asymmetric spine (ASp), asymmetric shaft (ASh), symmetric spine (SSp) and symmetric shaft (SSh). The 'spine' and 'shaft' relate to the appropriate region of the dendrite. The few axo-somatic synapses were classified along with the 'shaft' groups. Occasionally, it was impossible to assign a given synapse into one of the categories above, either because of the ambiguous nature of the target, or of the post-synaptic density. When this happened, they were simply allotted to a blanket group (ie. 'shaft', 'spine', 'symmetric' or 'asymmetric'), and were included in the analysis where possible. Very rarely, although identified as

'synaptic', no particular label could be applied. These 'unknowns' were counted for the purpose of measuring overall synaptic density, but were omitted from individual grouping analysis. The numbers of axo-axonic, dendro-dendritic, and axo-glial synapses were negligible. Where these occurred they were excluded from the analysis. The numbers of perforated synapses were also small, and impossible to count using the 'disector' method as used in this study. The disector can be a useful way of providing an unbiased sample, from which to estimate the proportion of perforated synapses. However, this would entail complete serial section of the chosen synapses, and would be extremely time-consuming and laborious. The disector principle as used in this study, relies upon sectioning through the periphery of the synapse, and therefore cannot be used to count perforations, which tend to be located in the centre of the synapse (Geinisman *et al* 1987a, 1987b, DeGroot and Vrensen 1978).

Calculation of synaptic numerical density : The numerical density of synapses in a given hemisphere of the LPO (Nv_{syn}) was calculated using the formula;

$$Nv_{syn} = \Sigma Q^{-}_{syn} / h.A$$

where; 'ΣQ^{-}' represents the sum of the synapses present in the counting frame of the 'selected' section but absent in the 'reference' section. 'h' is the distance between disector planes, and 'A' is the sample area. The sample area was defined by the boundaries of the sampling frame, appropriately corrected for magnification. The bottom and left-hand boundaries of the frame were deemed 'forbidden lines' (Gündersen 1977). Any synapse crossing this line was automatically excluded from the count. These forbidden lines were placed at least 3 cm inside the boundaries of the micrograph. In addition, the numerical densities of the synaptic groups were calculated using the same formula. Hence;

$$Nv_{Asymm} = \Sigma Q^-_{Asymm} / h.A \qquad Nv_{Symm} = \Sigma Q^-_{Symm} / h.A$$

$$Nv_{Spine} = \Sigma Q^-_{Spine} / h.A \qquad Nv_{Shaft} = \Sigma Q^-_{Asymm} / h.A$$

$$Nv_{ASp} = \Sigma Q^-_{ASp} / h.A \qquad Nv_{ASh} = \Sigma Q^-_{ASh} / h.A$$

$$Nv_{SSp} = \Sigma Q^-_{SSp} / h.A \qquad Nv_{SSh} = \Sigma Q^-_{SSh} / h.A$$

The values of numerical density for these eight groups were converted into percentage values of the total synaptic numerical density, for ease of comparison. By representing the data in this way, it was possible to readily identify whether any increase or decrease in synaptic density could be attributable to specific synaptic groups, and whether there was a re-distribution of synaptic types. This may occur in the absence of any overall change in synaptic number.

Calculation of synaptic number (N_{syn}) : Estimates of Nv_{syn} were converted into absolute number measurements (N_{syn}) using the formula;

$$N_{syn} = Nv_{syn} \cdot V_{LPO}$$

where; V_{LPO} is the estimate volume of LPO for a given hemisphere. Such estimations of number were made for all classes of synapse.

Estimation of synaptic height : An unbiased assessment of mean 'projected height' of the post-synaptic density (PSD) (an indicator of relative size), was made using a formula derived from the 'disector' principle (Sterio 1984);

$$\bar{H}_{syn} = \Sigma Q_{syn} . h / \Sigma Q^-_{syn}$$

where 'ΣQ_{syn}' is the number of synaptic profiles present in the counting frame of the 'selected' sections, 'ΣQ^-_{syn}' is the sum of the number of synaptic profiles present in the counting frame of the 'selected' sections but absent in the 'reference' sections, and 'h' is the distance between disector planes. The estimations of synaptic height were based entirely upon asymmetric synapse profiles, since symmetric synapse profiles were too few in number to make any

statistically valid measurements.

Statistical analysis : Students' T-tests were used to compare mean estimates of Nv_{syn}, Nv_{Asymm}, Nv_{Symm}, Nv_{Spine}, Nv_{Shaft}, Nv_{ASp}, Nv_{ASh}, Nv_{SSp} and Nv_{SSh}, based on results calculated from six animals per group. Paired Students' t-tests were used to compare mean Nv_{syn}, N_{syn}, and $\bar{H}_{syn}$ estimates of LPOs taken from the right and left hemispheres of the same individuals. A one-way analysis of variance (ANOVA) was used to test the effect of age on the estimated values of Nv_{syn}, N_{syn} and $\bar{H}_{syn}$. A two-way ANOVA was also used, to test the significance of the effects of age on the observed mean Nv_{syn}s, N_{syn}s and $\bar{H}_{syn}$s, and to test whether age influenced the hemispheres differently. Where appropriate, paired sample ANOVAs were made using a repeated measures multivariate analysis of variance (MANOVA) program of Statistical Package for Social Sciences (SPSS) on the Open University mainframe computer. Significance of the statistical tests was assumed when the probability of significance was greater than 0.95 (95% level).

9.3 : RESULTS

a) QUALITATIVE ANALYSIS

Pre-hatch (E16) : The tissue is characterised by large axonal and dendritic processes, separated by large interstitial spaces. Both asymmetric and symmetric synapses are present, though the proportion of the latter is less than 5% of the total. Astrocytes are abundant, and contain numerous polyribosomes. There were many post-synaptic thickenings devoid of any pre-synaptic component. These profiles have been likened to 'free-postsynaptic thickenings' described by Wolff (1978), and Spacek (1982). There were also profiles of pre- and post-synaptic thickenings in the absence of vesicles. 'Complete'

synapses appeared 'immature' in form, since they contained only a few synaptic vesicles. This made identification more difficult, though in the majority of cases classification was possible. No perforated synapses were seen in the tissue.

Early post-hatch (P1) : The interstitial space had become negligible by this age. The frequency of profiles resembling 'free post-synaptic thickenings' had reduced significantly, such that their presence in the post-hatch material was rare. Many more smaller axons were present. The dendrites had also reduced in size, and appeared more circular in profile. Synaptic densities appeared larger, and the synaptic boutons had more aggregates of synaptic vesicles. Occasionally, a perforation was seen, though these were rare.

Late post-hatch (P9, P22) : There was no qualitative difference between the P9 group, and the P22 group. The most obvious difference between this material and that from the early post-hatch, and pre-hatch groups, is the apparent increase in the number of synaptic profiles. There appeared to be an increase in the synaptic length, and an increase in its curvature.

b) QUANTITATIVE ANALYSIS : SYNAPTIC DENSITY

All synapses : The estimated mean values of Nv_{syn} for each hemisphere of chick LPO at various ages, are shown in Table 9:1. There is a 7-fold increase in the density of synapses in the left hemisphere LPO, between 16 days pre-hatch and 9 days post-hatch, and a corresponding 10-fold increase in the right hemisphere. There is a subsequent 36% reduction in synaptic density over the following 13 days in the left hemisphere, but no corresponding fall in density in the right hemisphere. The mean value of Nv_{syn} at 9-days post-hatch in the left hemisphere LPO, is significantly higher than that in the right hemisphere

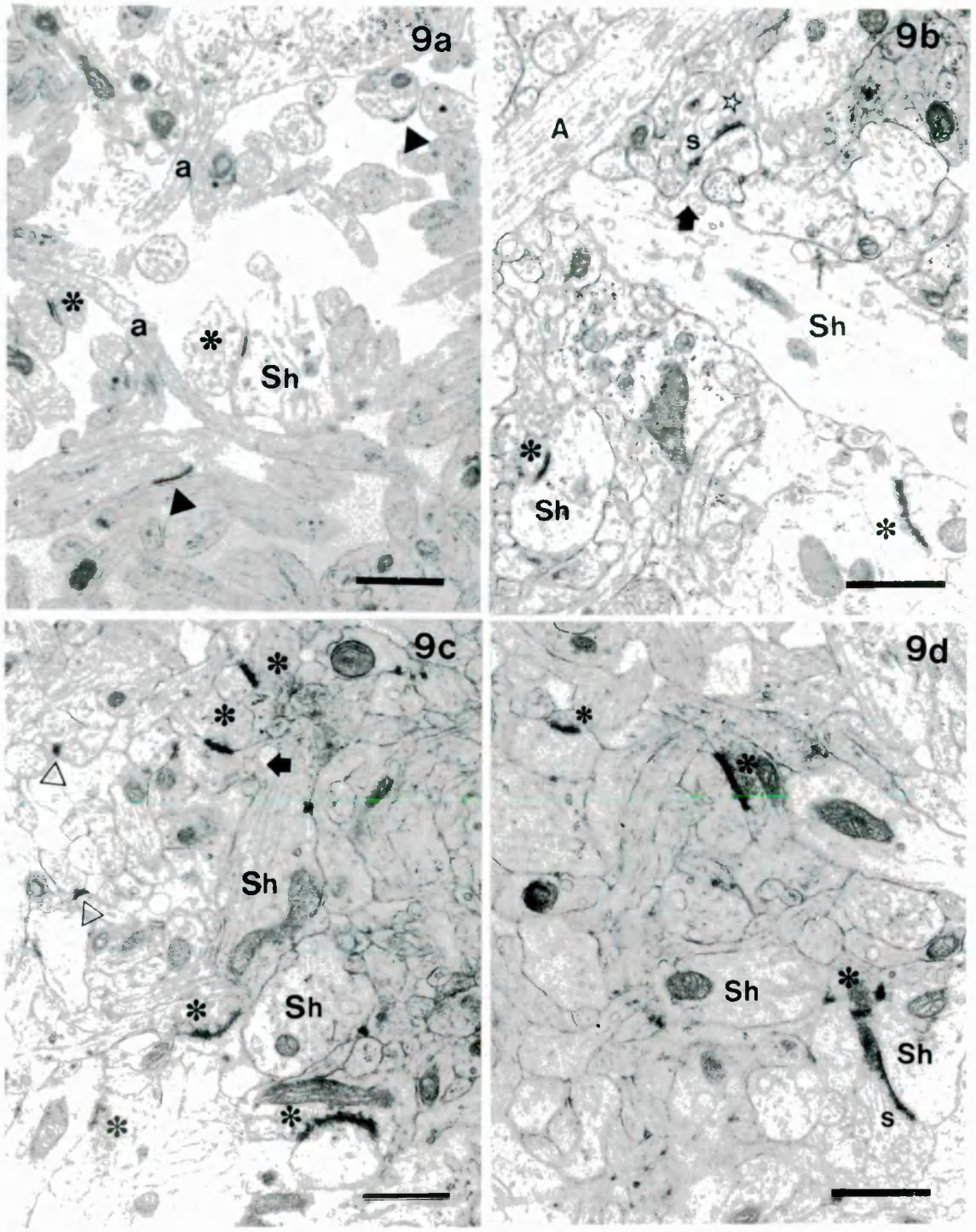


Fig.9a : 16 days in ovo: Synapses are associated with few synaptic vesicles. There are numerous membrane thickenings (arrowheads) devoid of any presynaptic component. **Fig.9b : 1 day post-hatch:** Spines (arrow) receive mainly asymmetric synapses. **Fig.9c : 9 days post-hatch:** A dendritic spine (arrow), and synaptic-like dense projections. (open arrowheads) are shown. **Fig.9d : 22 days post-hatch:** There is no qualitative difference between the tissue of this age group and that of 9-days post-hatch. **Key:** *-asymmetric synapse, s-symmetric synapse, a-axons, A-Astrocyte process, Sh-Dendritic shaft, Scale bar = 1μm.

(Paired t-test: t=5.67 (df=4) $p<0.05$). A one-way ANOVA (Table 9:3) also shows the significant effect that age has on the density of synapses in both left and right hemisphere LPO (Left hemisphere: $F_{3,14}=39.00$, $p<0.05$, Right hemisphere: $F_{3,14}=26.79$, $p<0.05$). A two-way ANOVA (Table 9:4) confirmed the significant increase in synaptic density with age ($F_{3,28}=62.46$, $p<0.05$), and that this increase is significantly different between hemispheres ($F_{1,28}=8.98$, $p<0.05$). An interaction effect of age and hemisphere was shown ($F_{3,28}=5.59$, $p<0.05$). This reflects the divergent rates of growth in synaptic density during the post-hatch period, between the two hemispheres (Fig.9:1).

Asymmetric synapses : The vast majority of synapses in the chick LPO are of the asymmetric type. It is not surprising therefore to see that the results of the statistical tests mimic those for 'All synapses' (above). The comparison of estimated mean values of Nv_{Asymm} (see Appendix 3) between hemispheres is significant for the 9-day old group only (Paired t-test: t=6.63 (df=4) $p<0.05$) (Table 9:2a). A one-way ANOVA (Table 9:3) demonstrates a significant increase in Nv_{Asymm} in both right ($F_{3,14}=8.71$, $p<0.05$) and left hemisphere LPO ($F_{3,14}=10.38$, $p<0.05$). A two-way ANOVA (Table 9:4) shows significant effects of age ($F_{3,28}=73.74$, $p<0.05$), and hemisphere ($F_{1,28}=9:54$, $p<0.05$). There is also a significant interaction of these two effects ($F_{3,28}=6.28$, $p<0.05$). The results are displayed graphically in Fig.9:2a.

Symmetric synapses : A small, but significant increase in Nv_{symm} was seen in both hemispheres with increasing age (One way ANOVA: Left hemisphere: $F_{3,14}=10.38$, $p<0.05$, Right hemisphere: $F_{3,14}=10.76$, $p<0.05$) (Table 9:3). The mean estimated Nv_{Symm} values however, were not significantly different between hemispheres at any given age (Paired t-test, Table 9:2a). A two-way

ANOVA showed significant effects of age ($F_{3,28}$=20.94, $p<0.05$), but no significant hemisphere or interaction effects (Table 9:4). The mean values are shown graphically in Fig.9:2b, and also given in Appendix 3.

Asymmetric and Symmetric synaptic ratios : When one views the results in terms of the proportion of asymmetric and symmetric synapses as a percentage of the total (Fig.9:2c,d), one can see that the proportion of asymmetric synapses increases steadily over the time period of study, whilst the proportion of symmetric synapses undergo a corresponding decrease. Although statistical analysis was not applied to these results[1], it can be seen that there is no apparent difference in this trend between the hemispheres.

Spine synapses : The large majority of synapses were targeted onto dendritic spines, as can be seen by comparing the estimated mean values of Nv_{Spine} (given in appendix) with the mean estimated values of Nv_{syn} (Table 9:1). As a consequence, the pattern of significance of the statistical tests, mimics that seen for Nv_{syn}. The 9-day old chicks show a hemispheric difference in estimated mean Nv_{Spine}, with the left hemisphere having a greater density of synapses than the right (Paired t-test: t=5.51 (df=4), $p<0.05$) (Table 9:2a). Both hemispheres have a significant increase in synaptic density over the time period of study (One-way ANOVA: Left hemisphere; $F_{3,14}$=49.65, $p<0.05$, Right hemisphere; $F_{3,14}$=22.17, $p<0.05$) (Table 9:3). A two-way ANOVA (Table 9:4) shows that as well as a significant increase in Nv_{Spine} with age ($F_{3,28}$=66.56, $p<0.05$), there is also a difference in this respect between the hemispheres ($F_{1,28}$=12.62, $p<0.05$) with a significant interaction effect of hemisphere with age ($F_{3,28}$=6.36, $p<0.05$). A graph of these results is shown in Fig.9:3a, and

[1]Analysis requires arcsine transformation of raw data (Bishop 1971).

the mean values ± SEM are given in Appendix 3.

Shaft synapses : There were no significant differences between mean estimated values of Nv_{Shaft} of right and left hemispheres at either of the ages studied (Table 9:2a). There was however, a significant increase in these means with age (see Appendix 3), up to 9-days post-hatch, in both left ($F_{3,14}=5.12$, $p<0.05$), and right hemisphere ($F_{3,14}=6.21$, $p<0.05$) using a one-way ANOVA as the statistical test (Table 9:3). A two-way ANOVA, comparing the effects of age with hemisphere also showed a significant contribution of the effects of age ($F_{3,28}=10.41$, $p<0.05$) (Table 9:4). As suggested by the paired t-tests (Table 9:2a), the two-way ANOVA showed no significant effect of hemisphere ($F_{1,28}=0.15$, $p\geq0.5$) or any interaction of hemisphere with age ($F_{3,28}=0.86$, $p\geq0.05$). The results are displayed in graphically in Fig.9:3b.

Spine and Shaft synaptic ratios : By plotting the results of Nv_{Spine} and Nv_{Shaft} as percentages of the total synaptic density (Fig.9:3c,d), one can see an apparent hemispheric asymmetry in the distribution of these ratios. The left hemisphere has a larger ratio of spine synapses than the right, and consequently, the right hemisphere has a larger ratio of shaft synapses than the left. These differences are only seen in the post-hatch period. There is no detectable asymmetry in the pre-hatch chicks. The actual differences in ratio in the post-hatch chicks are very small (approximately 5%), though of course these cannot be proven using the chosen statistical tests, since the values are not absolute. This can be done however, by using an arcsine transformation on the data (Bishop 1971), but this was considered unnecessary in the present case, since more extensive statistics has been performed on the absolute values. Another interesting point to note, is that the proportion of a given synaptic type (spine or

shaft) remains remarkably constant throughout the post-hatch period, but is subject to modification in the late pre-hatch period. This occurs in spite of the hemispheric asymmetry that exists in the ratio of spine and shaft synapses post-hatch.

Asymmetric Spine synapses : Approximately 75% of synapses in the right hemisphere LPO and approximately 85% in the left hemisphere LPO, are of the asymmetric spine type (see Appendix 3 for mean values). Since this is the vast majority of synapses, the pattern of significance of the statistical tests again mimics that of the results of Nv_{syn}. There is a hemispheric asymmetry of Nv_{ASp} at 9-days post-hatch (Paired t-test: $t=7.10$ ($df=4$) $p<0.05$) (Table 9:2b). This is the result of a greater increase in Nv_{ASp} in the left hemisphere compared to the right with age, between 1- and 9-days post-hatch (Fig.9:4a). This asymmetry disappears by 22-days post-hatch due entirely to a decrease in synaptic density, mainly from the asymmetric spine type. This divergence of densities between the two hemispheres is reflected in the statistical significance of the interaction effect of a two-way ANOVA, comparing hemisphere with age ($F_{3,28}=7.91$, $p<0.05$) (Table 9:4). The hemispheric asymmetry is present at 9 days post-hatch only. This however, is sufficiently large as to cause a significant hemisphere effect in the two-way ANOVA, despite the fact that the test reflects the variance of the sample from chicks of all ages ($F_{1,28}=15.64$, $p<0.05$) (Table 9:4). There is also a significant increase in Nv_{ASp} over the period of study in both left (One-way ANOVA: $F_{3,14}=54.00$, $p<0.05$) and right hemisphere (One-way ANOVA: $F_{3,14}=22.12$, $p<0.05$) LPO (Table 9:3).

Asymmetric Shaft synapses : The density of asymmetric shaft synapses increased from 16 days *in ovo* until 9 days post-hatch, in both hemispheres (Fig.9:4b) (see Appendix 3). Statistical analysis of the change in density of these

synapses over the period of study, revealed significant changes in both left (One-way ANOVA: $F_{3,14}$=4.68, $p<0.05$) and right hemispheres (One-way ANOVA: $F_{3,14}$=15.08, $p<0.05$) (Table 9:3). A comparison of means at the various ages of study (Paired t-test, Table 9:2b) showed no statistically significant differences. A two-way ANOVA (Table 9:4) was also unable to find any significant effect of hemisphere ($F_{1,28}$=0.50, $p\geq0.05$), nor a significant interaction of hemisphere with age ($F_{3,28}$=1.77, $p\geq0.05$).

Asymmetric Spine and Asymmetric Shaft synaptic ratios : There is a hemispheric asymmetry in the ratio of asymmetric spine, and asymmetric shaft synapses in the LPO (Fig.9:4c,d). There is a greater preponderance of asymmetric spine synapses in the left hemisphere, and a corresponding greater ratio of asymmetric shaft synapses in the right hemisphere. Again, the ratio of both of these synaptic types is remarkably constant during the post-hatch period, but differs somewhat from the ratio of synaptic types present in the late embryonic phase. The percentage of asymmetric spine synapses is lower in both hemispheres during the late embryonic phase. This is balanced by a correspondingly greater proportion of asymmetric shaft synapses during this phase. There is no hemispheric asymmetry in these ratios before hatching.

Symmetric Spine synapses : A one-way ANOVA (Table 9:3) shows a significant increase in the density of symmetric spine synapses from 16 days *in ovo* until 9 days post-hatch (see also Appendix 3 for mean values). The test is significant for both left hemisphere ($F_{3,14}$=7.26, $p<0.05$) and right hemisphere LPO ($F_{3,14}$=6.58, $p<0.05$). There were no significant differences between the hemispheres with regard to the mean estimated values (Paired t-tests, Table 9:2b). A two-way ANOVA also showed no significant effect of

hemisphere on the observed values of Nv_{SSp} ($F_{1,28}$=0.02, p≥0.05), nor a significant interaction of hemisphere with age ($F_{3,28}$=0.17, p≥0.05) (Table 9:4). The mean values and standard error of the means are given in Appendix 3, and shown graphically in Fig.9:5a.

Symmetric Shaft synapses : There were no significant differences between the hemispheres with respect to the estimations of mean Nv_{SSh}, using paired t-tests to compare means (Table 9:2b). The density of symmetric shaft synapses remained constantly low throughout the period of study (One-way ANOVA, Table 9:3). A two-way ANOVA revealed no significant effects of age, hemisphere or interaction of these effects (Table 9:4). The results are shown in Fig.9:5b, and the means ± SEM are given in Appendix 3.

Symmetric Spine and Symmetric Shaft synaptic ratios : The proportions of symmetric spine and symmetric shaft synapses, expressed as percentages of the total synaptic population, are both less than 10%. The ratio decreases steadily during the developmental period under study, such that by 22 days post-hatch, there is approximately a 5% reduction in the ratio (Figs.9:5c,d). This decrease occurs in both right and left hemisphere LPO.

Table 9:1 : Mean ± SEM Nv_{syn}

Age	Left Hemisphere	Right Hemisphere	Paired t-value
E16	0.23 ± 0.12	0.11 ± 0.07	0.83 (4) ns
P1	0.57 ± 0.09	0.64 ± 0.06	0.50 (3) ns
P9	1.72 ± 0.11	1.06 ± 0.11	5.67 (4) *
P22	1.10 ± 0.10	1.06 ± 0.10	0.20 (3) ns

ns - not significant, * - significant at p<0.05, df in parenthesis

Fig.9.1

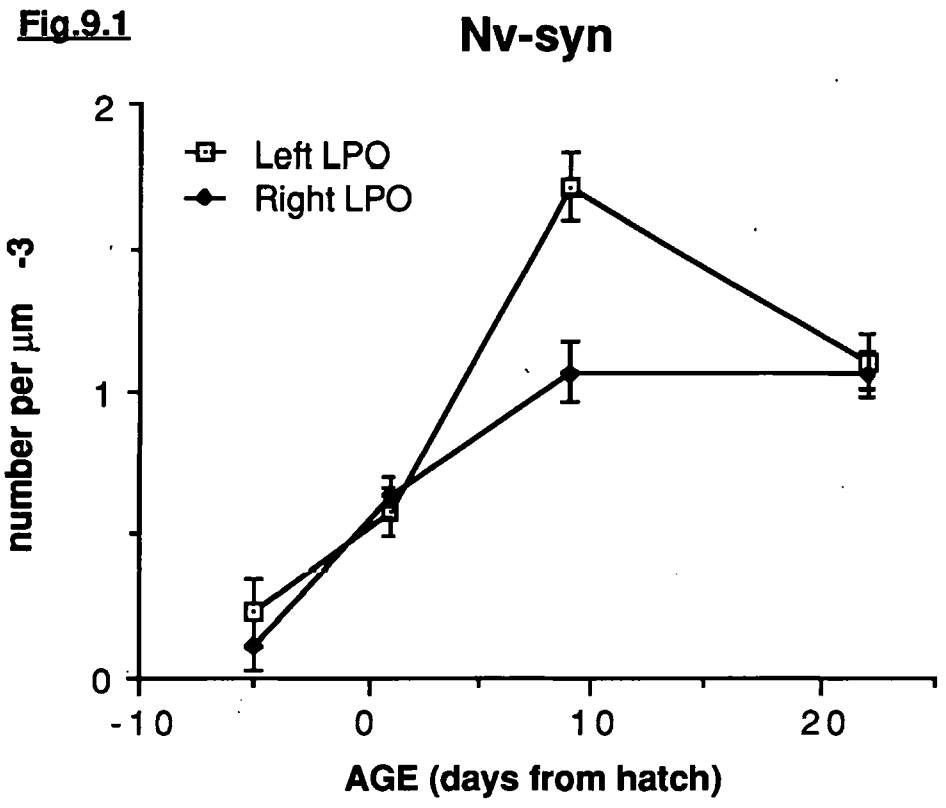


Fig.9:1 Mean numerical density of synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Table 9:2a : Paired t-test of left versus right hemisphere LPO synaptic density during development.

	Asymm	Symm	Spine	Shaft
E16 df=4	0.87 (ns)	0.92 (ns)	0.69 (ns)	0.47 (ns)
P1 df=3	0.58 (ns)	0.12 (ns)	0.21 (ns)	1.06 (ns)
P9 df=4	6.63 (*)	1.14 (ns)	5.51 (*)	0.34 (ns)
P22 df=3	0.17 (ns)	0.85 (ns)	0.79 (ns)	0.96 (ns)

Results are given as paired t-values,

* - significant at $p<0.05$, ns - not significant

see Appendix 3 for means ± SEM

Table 9:2b : Paired t-test of left versus right hemisphere LPO synaptic density during development.

	ASp	ASh	SSp	SSh
E16 df=4	0.78 (ns)	1.19 (ns)	0.59 (ns)	0.78 (ns)
P1 df=3	0.15 (ns)	0.79 (ns)	0.20 (ns)	0.32 (ns)
P9 df=4	7.10 (*)	0.84 (ns)	0.26 (ns)	0.65 (ns)
P22 df=3	0.76 (ns)	1.16 (ns)	1.00 (ns)	1.41 (ns)

Results are given as paired t-values,

* - significant at $p<0.05$, ns - not significant

see Appendix 3 for means ± SEM

Table 9:3 : One-way ANOVA of the Effects of Age on the Numerical Density of Various Synaptic Types

Type	Left Hemisphere	Right Hemisphere
All	39.00 (*)	26.79 (*)
Asymm	10.38 (*)	8.71 (*)
Symm	10.38 (*)	10.76 (*)
Spine	49.65 (*)	22.17 (*)
Shaft	5.12 (*)	6.21 (*)
ASp	54.00 (*)	22.12 (*)
ASh	4.68 (*)	15.08 (*)
SSp	7.26 (*)	6.58 (*)
SSh	0.83 (ns)	2.20 (ns)

Results are given as F-values, df=3,14

* - significant at $p<0.05$, ns - not significant

Table 9:4 : Two-way ANOVA of the Effects of Age and Hemisphere on the Numerical Density of Various Synaptic Types

Type	Age df=3,28	Hemisphere df=1,28	Interaction df=3,28
All	62.46 (*)	8.98 (*)	5.59 (*)
Asymm	73.74 (*)	9.54 (*)	6.28 (*)
Symm	20.94 (*)	2.29 (ns)	0.17 (ns)
Spine	66.56 (*)	12.62 (*)	6.36 (*)
Shaft	10.41 (*)	0.15 (ns)	0.86 (ns)
ASp	69.28 (*)	15.64 (*)	7.91 (*)
ASh	17.47 (*)	0.50 (ns)	1.77 (ns)
SSp	13.50 (*)	0.02 (ns)	0.17 (ns)
SSh	2.07 (ns)	0.37 (ns)	0.52 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

Fig.9.2a

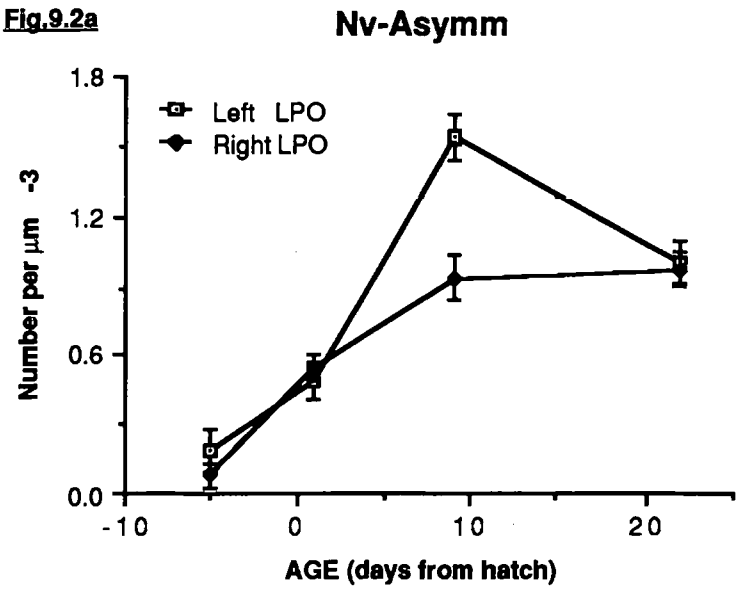


Fig.9.2b

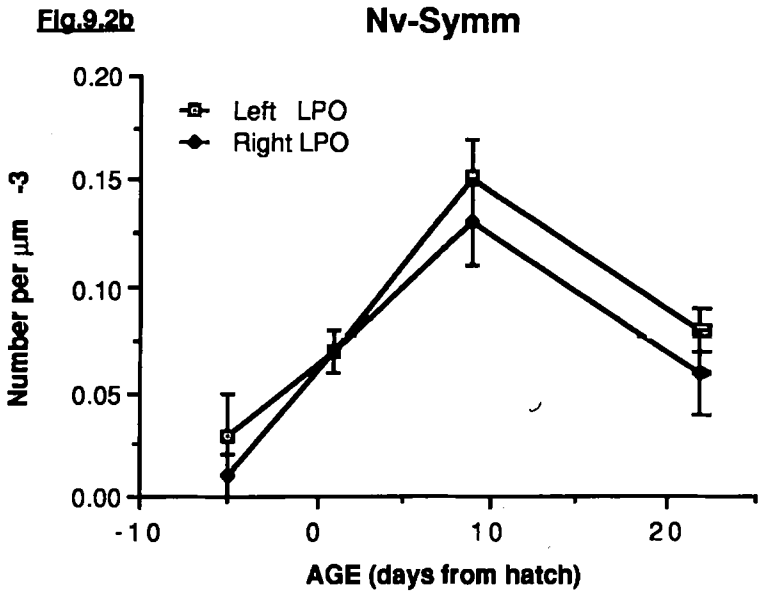


Fig.9:2a,b Mean numerical density of asymmetric and symmetric synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.2c

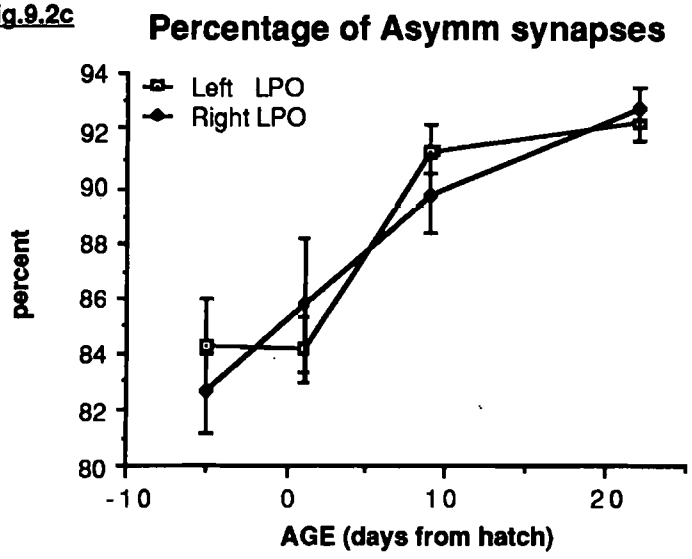


Fig.9.2d

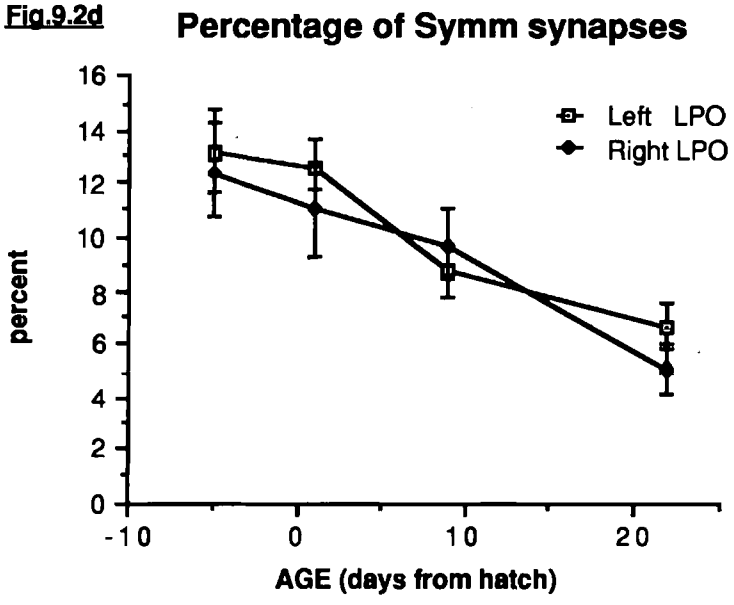


Fig.9:2c,d Mean number of asymmetric and symmetric synapses as a percentage of the total ± SEM, of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.3a

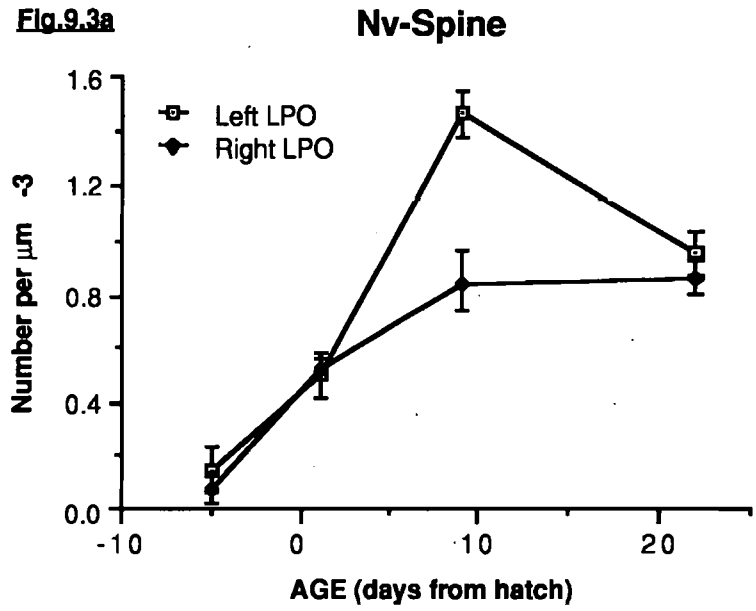


Fig.9.3b

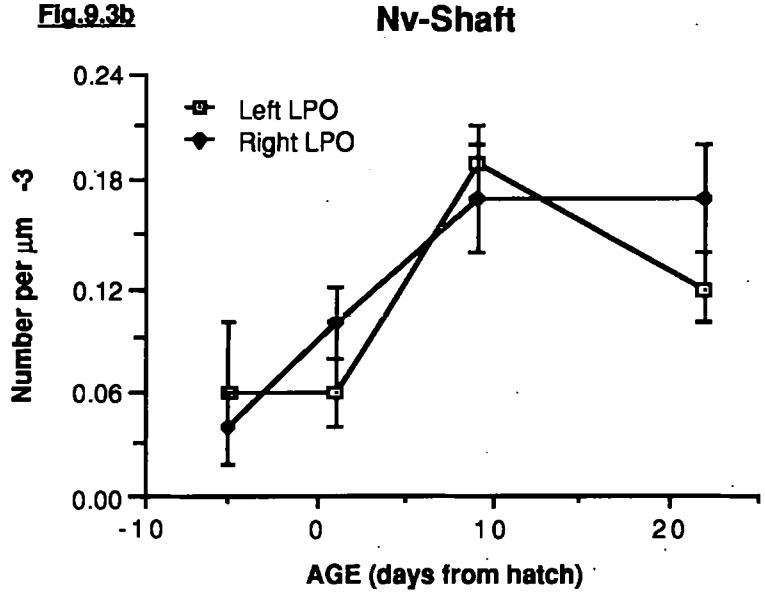


Fig.9:3a,b Mean numerical density of spine and shaft synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.3c **Percentage of Spine Synapses**

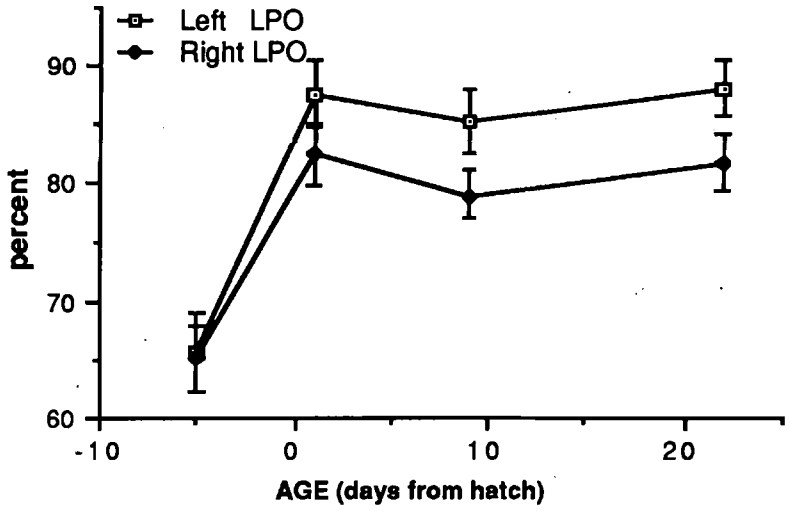


Fig.9.3d **Percentage of Shaft Synapses**

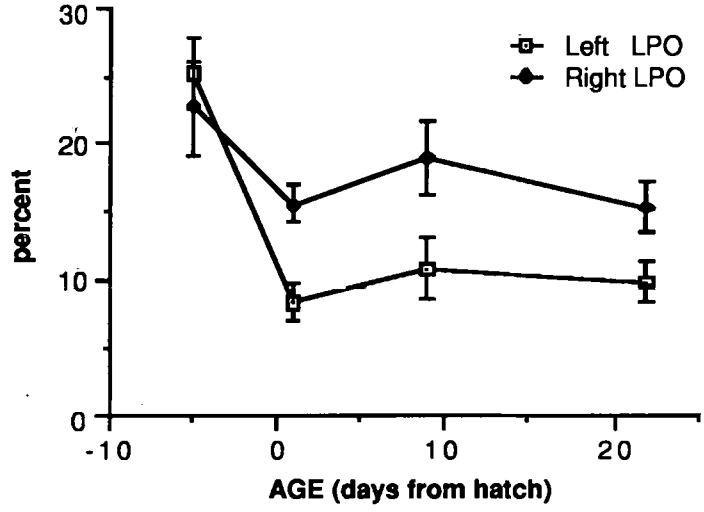


Fig.9:3c,d Mean number of spine and shaft synapses as a percentage of the total ± SEM, of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.4a

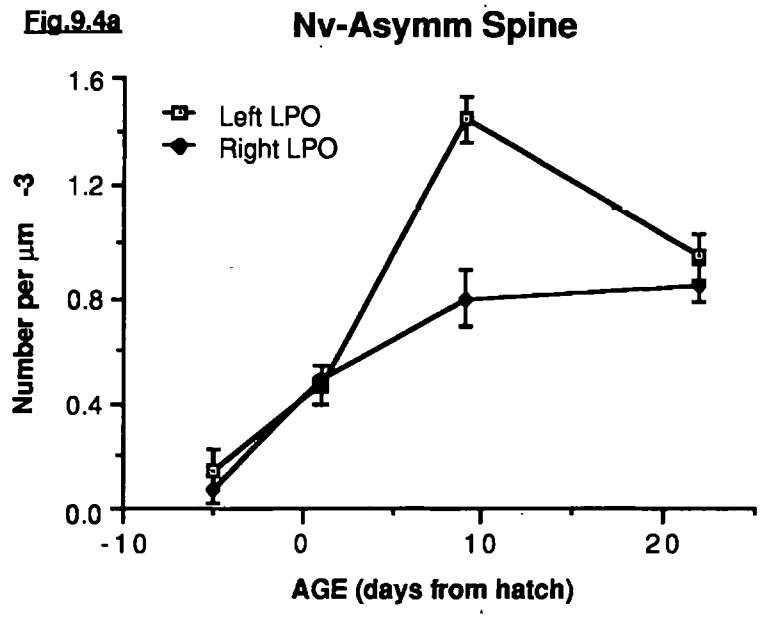


Fig.9.4b

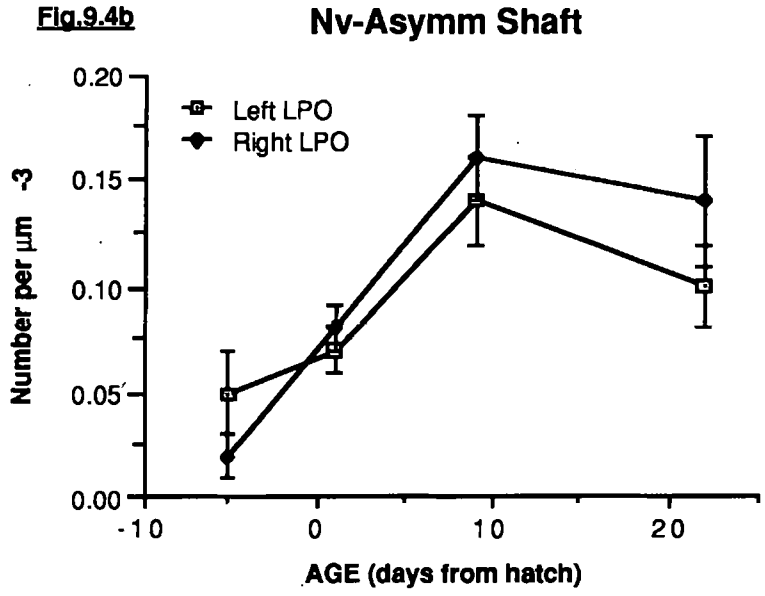


Fig.9:4a,b Mean numerical density of asymmetric spine and asymmetric shaft synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.4c

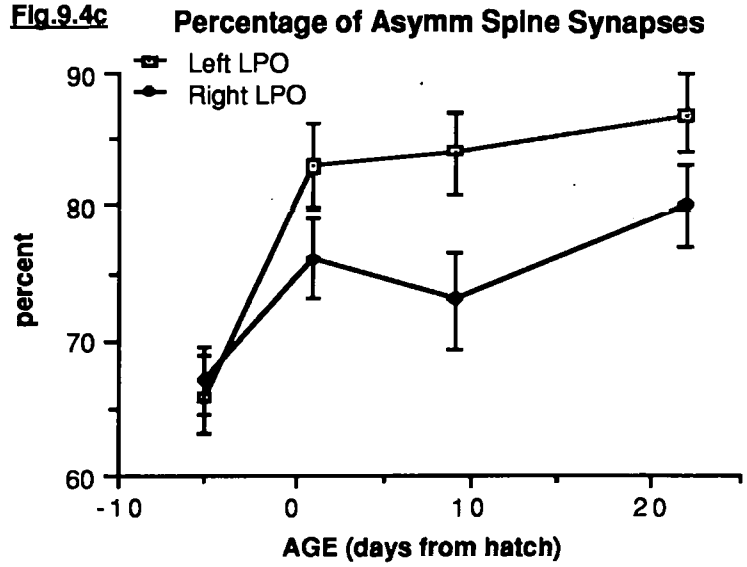


Fig.9.4d

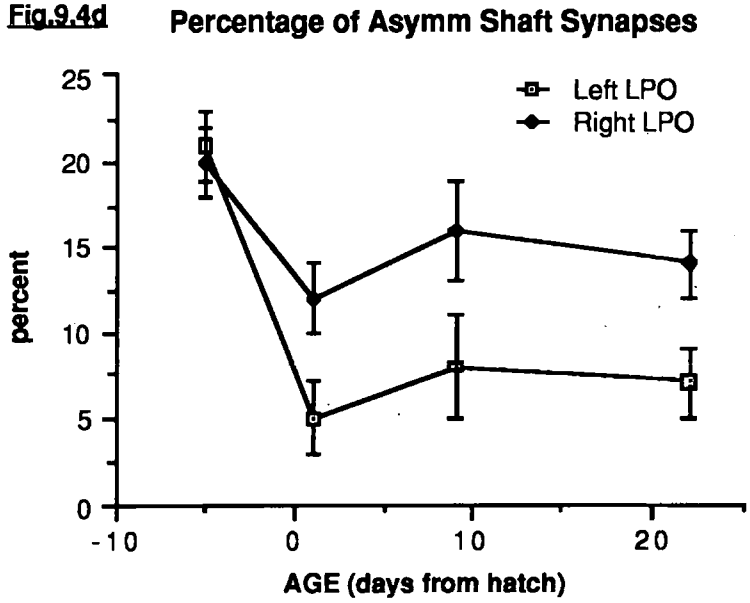


Fig.9:4c,d Mean number of asymmetric spine and asymmetric shaft synapses as a percentage of the total ± SEM, of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.5a

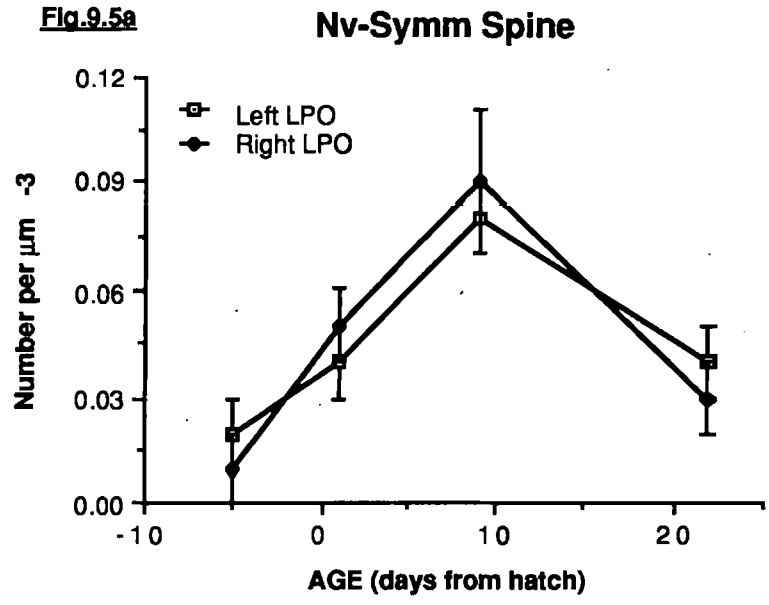


Fig.9.5b

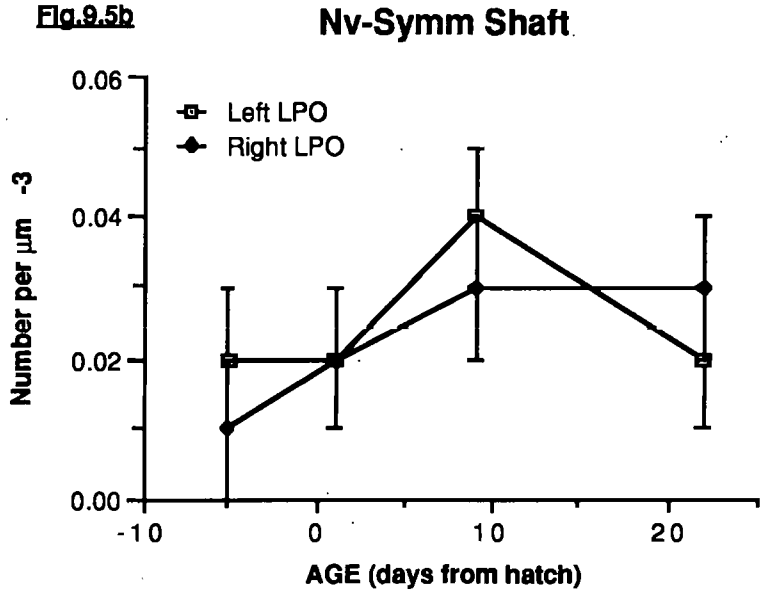


Fig.9:5a,b Mean numerical density of symmetric spine and symmetric shaft synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.5c

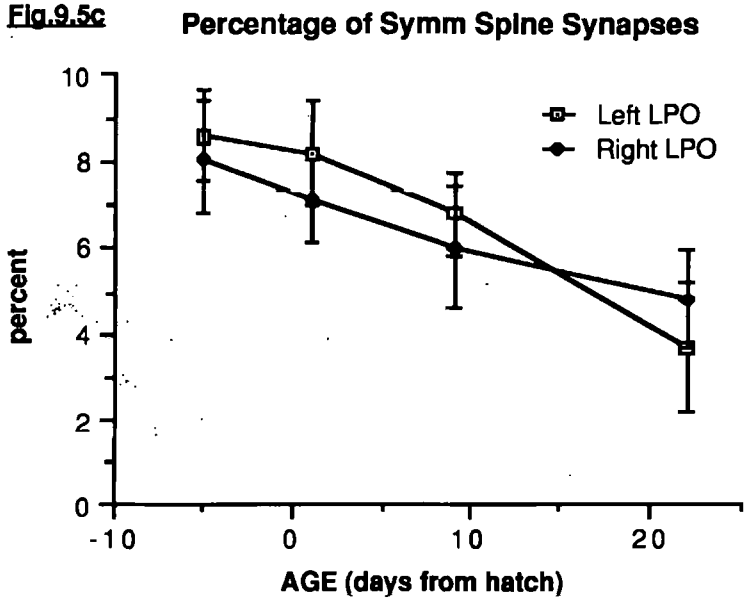


Fig.9.5d

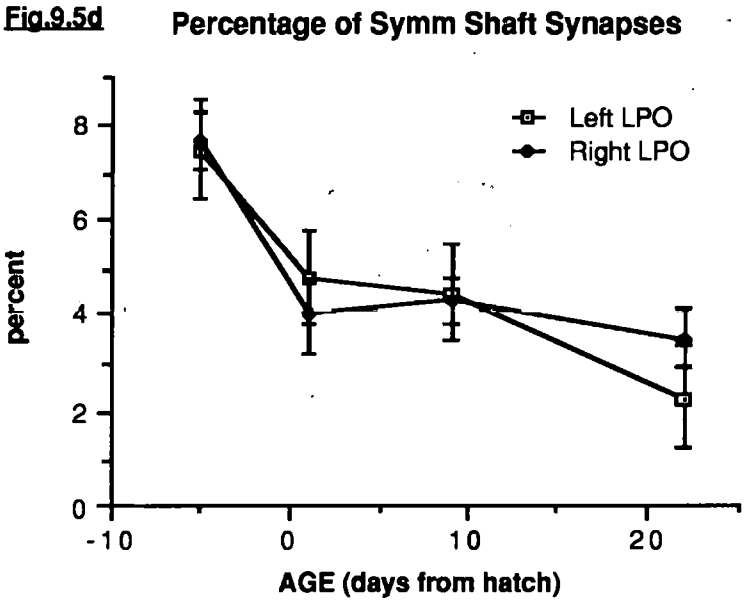


Fig.9.5c,d Mean number of symmetric spine and symmetric shaft synapses as a percentage of the total ± SEM, of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

c) QUANTITATIVE ANALYSIS : LPO VOLUME ESTIMATION

The volume of LPO tissue did not increase significantly over the period of development between 16-days *in ovo*, and 22 days post-hatch (Fig.9:6a). Mean values of V_{LPO} are shown in Table 9:5a. Using a paired t-test to compare mean volume of left and right hemisphere LPO of chicks of a given age, no statistically significant differences could be found (Table 9:5a). There was no significant increase in LPO volume of either right (One-way ANOVA: $F_{3,20}=0.72$, $p\geq0.05$) or left hemisphere (One-way ANOVA: $F_{3,20}=2.87$, $p\geq0.05$) (Table 9:5b). A two-way ANOVA (Table 9:5c) showed that LPO volume remained constant during the developmental period under investigation ($F_{3,40}=2.78$, $p\geq0.05$). There was also no significant effects on the estimation of LPO volume, due to differences between the hemispheres ($F_{1,40}=0.16$, $p\geq0.05$). There was a good correlation between the volumes of right and left hemispheres of each brain ($R=0.86$, $p<0.05$), and that a straight line of least squares passes close to the origin (Fig.9:6b).

Table 9:5a : Mean ± SEM LPO Volume (mm^3)

Age	Left Hemisphere	Right Hemisphere	Paired t-value
E16	0.56 ± 0.11	0.44 ± 0.11	1.28 (5) ns
P1	0.77 ± 0.12	0.60 ± 0.10	1.10 (5) ns
P9	0.75 ± 0.11	0.95 ± 0.14	1.13 (5) ns
P22	0.76 ± 0.14	0.70 ± 0.14	0.29 (5) ns

ns - not significant, df in parenthesis

Table 9:5b : One-way ANOVA of the Effects of Age on LPO Volume

	Left Hemisphere	Right Hemisphere
F-value	0.72 (3,20)	2.87 (3,20)

df in parenthesis

Table 9:5c : Two-way ANOVA of the Effects of Age and Hemisphere on LPO Volume

	Age	Hemisphere	Interaction
F-value	2.78 (3,40)	0.16 (1,40)	0.91 (3,40)

df in parenthesis

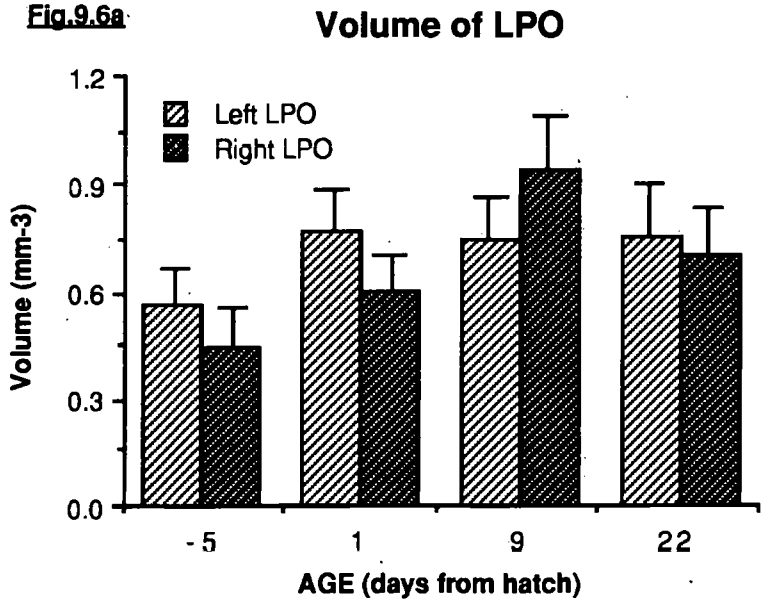


Fig.9:6a Mean volume ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on 6 chicks.

Fig.9.6b **Hemisphere Volume Correlation**

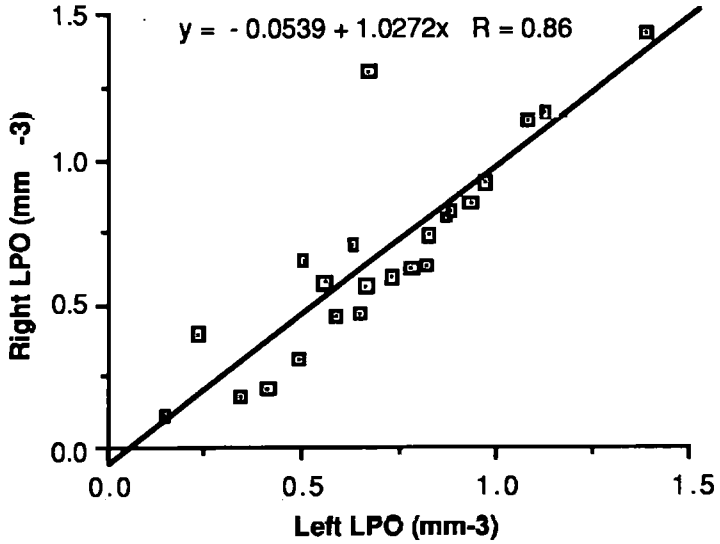


Fig.9:6b Volume correlation plot of volume from left (x-axis) and right hemisphere (y-axis) LPO of individual chicks. A line of least squares which represents the best straight line fit is drawn. As the gradient of this line is approximately 1, one can conclude that the hemispheres are approximately equal in size. A regression co-efficient (R) of 0.86 confirms that there is a good correlation of volume between hemispheres.

d) QUANTITATIVE ANALYSIS : SYNAPTIC NUMBER

All synapses : The mean values of N_{syn} are given in Appendix 4. Since the volume of LPO did not significantly alter during the early developmental period, the results for the total number of synapses, reflect the pattern seen for synaptic density. Hence a statistically significant difference in N_{syn} exists at 9-days post-hatch between left and right LPO (Paired t-test: $t=2.85$ ($df=4$) $p<0.05$) (Table 9:6a). There is no significant difference in this respect at any of the other ages studied. A one-way ANOVA (Table 9:8) revealed significant increases in N_{syn} in both left ($F_{3,14}=48.75$, $p<0.05$) and right hemispheres ($F_{3,14}=36.99$, $p<0.05$) with age. The difference between hemispheres at 9 days post-hatch (Table 9:6a, Fig.9:7), is sufficient to cause a statistically significant effect of hemisphere on the observed mean values of N_{syn}, using a two-way ANOVA ($F_{1,28}=6.82$, $p<0.05$) (Table 9:9), but not a significant interaction of

hemisphere with age ($F_{3,28}$=1.13, p≥0.05) as was seen in the results of Nv_{syn} (Table 9:4, Fig.9:1).

Asymmetric synapses : The mean values of N_{Asymm} are given in Appendix 4, and are displayed graphically in Fig.9:8a. There is an increase in this parameter with age (One-way ANOVA: Table 9:8) in both left ($F_{3,14}$=57.15, p<0.05) and right hemisphere ($F_{3,14}$=41.11, p<0.05). A hemispheric asymmetry exists at 9-days post-hatch, with the left containing 1.3 times as many asymmetric synapses as the right (Paired t-test: t=3.16 (df=4) p<0.05) (Table 9:7a). A two-way ANOVA revealed significant effects of both age ($F_{3,28}$=58.05, p<0.05) and hemisphere ($F_{1,28}$=7.00, p<0.05), but no interaction of these effects ($F_{3,28}$=1.24, p≥0.05) (Table 9:9).

Symmetric synapses : There were no statistically significant differences between the hemispheres with respect to the estimated mean N_{Symm} values (Table 9:7a). A two-way ANOVA also failed to reveal any hemispheric differences (Table 9:9). However, the number of symmetric synapses of both hemispheres increased with age over the period of study (One-way ANOVA: Left hemisphere, ($F_{3,14}$=13.67, p<0.05, Right hemisphere, ($F_{3,14}$=13.24, p<0.05)) (Table 9:8), at least until the age of 9-days post-hatch. As can be seen in Fig.9:8b, there was a substantial decrease in N_{symm} between 9- and 22-days post-hatch, in both hemispheres. Mean values of N_{Symm} ± SEM are displayed graphically in Fig.9:8b, and values are given in Appendix 4.

Spine synapses : The mean estimated values of N_{Spine} are shown in Fig.9:9a, and are also given in Appendix 4. The estimated means derived from left and right hemispheres of individual brains were compared using a Paired t-test (Table

9:7a). A statistically significant difference was seen at 9-days post-hatch (t=2.87 (df=4) $p<0.05$). There was an increase in N_{Spine} up to 9-days post-hatch, beyond which there was a subsequent decline (Fig.9:9a). The overall changes in N_{Spine} were analysed using a one-way ANOVA for both left ($F_{3,14}=58.76$, $p<0.05$) and right hemisphere ($F_{3,14}=26.21$, $p<0.05$), where a significant change was seen in both cases (Table 9:8). Although there was a significant alteration in N_{Spine} in both hemispheres (Two-way ANOVA: $F_{3,28}=78.74$, $p<0.05$), the pattern was different between the hemispheres (Two-way ANOVA: $F_{1,28}=9.38$, $p<0.05$) (Table 9:9). There was however no interaction of both age and hemisphere on the estimates of N_{Spine} (Two-way ANOVA: $F_{3,28}=1.51$, $p\geq0.05$) (Table 9:9).

Shaft synapses : Although the number of Shaft synapses increased with age (taking into account an overall reduction between 9- and 22-days post-hatch) (One-way ANOVA: Left hemisphere, $F_{3,14}=11.21$, $p<0.05$, Right hemisphere, $F_{3,14}=26.21$, $p<0.05$) (Table 9:8), there was no significant difference in the estimated values of N_{Shaft} between the hemispheres (Paired t-test, Table 9:7a, Two-way ANOVA: $F_{3,28}=36.27$, $p<0.05$, Table 9:9). The developmental profile of this particular synaptic group can be seen in Fig.9:9b, and mean values ± SEM are given in Appendix 4.

Asymmetric Spine synapses : The majority of synapses are of the asymmetric spine type. The mean values of N_{ASp} are given in Appendix 4. A comparison of left and right hemisphere N_{ASp} mean values using a Paired t-test (Table 9:7b), revealed that the left hemisphere has a significantly greater number of asymmetric spine synapses than the right hemisphere, at 9-days post-hatch (t=3.97 (df=4) $p<0.05$). This hemispheric difference is also shown

in a two-way ANOVA (Table 9:9) comparing the effects of hemisphere and age on the observed values of N_{ASp} ($F_{1,28}$=12.08, $p<0.05$). The number of asymmetric spine synapses increases steadily until 9-days post-hatch, when there is a subsequent decrease. This change in the estimated values of N_{ASp} with age is detected with both a two-way ANOVA ($F_{3,28}$=80.87, $p<0.05$) (Table 9:9), and a one-way ANOVA of the effects of age on both left ($F_{3,14}$=62.34, $p<0.05$) and right hemisphere ($F_{3,14}$=25.67, $p<0.05$) (Table 9:8). There was no interaction of hemisphere with age (Table 9:9). The results are shown graphically in Fig.9:10a.

Asymmetric Shaft synapses : Although the estimated mean number of asymmetric shaft synapses of right and left LPO did not differ significantly at either of the ages studied (Table 9:7b), there was an overall hemispheric difference detected using a two-way ANOVA, comparing the effects of age and hemisphere on the estimated values of N_{ASh} ($F_{1,28}$=5.28, $p<0.05$) (Table 9:9). There was no interaction of hemisphere with age ($F_{3,28}$=2.90, $p\geq0.05$). An overall increase in N_{ASh} over the period of study, was seen in both left hemisphere (One-way ANOVA: $F_{3,14}$=11.31, $p<0.05$) and right hemisphere (One-way ANOVA: $F_{3,14}$=26.53, $p<0.05$) (Table 9:8). The results are displayed graphically in Fig.9:10b, and are also given in Appendix 4.

Symmetric Spine synapses : The mean estimated number of symmetric spine synapses are similar in both left and right hemisphere LPO (Paired t-test: Table 9:7b). The estimates are however influenced by the age group involved (Two-way ANOVA: $F_{3,28}$=21.51, $p<0.05$) (Table 9:9). There is an increase in N_{SSp} up to 9 days post-hatch, and a subsequent fall in N_{SSp} after 9-days (Fig.9:11a). Table 9:8 shows the effect of age on N_{SSp} using a one-way ANOVA for left ($F_{3,14}$=8.86, $p<0.05$) and right hemisphere ($F_{3,14}$=12.78, $p<0.05$)

separately. The mean values of N_{SSp} ± SEM are given in Appendix 4.

Symmetric Shaft synapses : The number of symmetric shaft synapses remains fairly stable throughout the period of study (Table 9:7b, Table 9:8). There is however a small increase between 1- and 9-days post-hatch in both hemispheres (Fig.9:11b). This increase is large enough to cause a significant effect of age ($F_{3,28}$=6.94, $p<0.05$) when analysis is made using a two-way ANOVA (Table 9:9). There is no effect due to hemisphere ($F_{1,28}$=1.08, $p\geq0.05$) or interaction of hemisphere with age ($F_{3,28}$=0.40, $p\geq0.05$). Paired t-tests of the estimated values of N_{SSh} in right and left hemispheres of chicks of the same age, reveal no significant differences (Table 9:7b). The results are shown graphically in Fig.9:11b, and mean values ± SEM are given in Appendix 4.

Table 9:6 : Mean ± SEM N_{Syn}

Age	Left Hemisphere	Right Hemisphere	Paired t-value
E16	1.31E8 ± 6.50E7	4.92E7 ± 3.04E7	1.06 (4) ns
P1	4.41E8 ± 7.12E7	3.82E8 ± 3.65E7	0.63 (3) ns
P9	1.29E9 ± 8.49E7	1.00E9 ± 1.08E8	2.85 (4) *
P22	8.34E8 ± 7.42E7	7.46E8 ± 6.67E7	0.74 (3) ns

ns - not significant, * - significant at $p<0.05$, df in parenthesis

Table 9:8 : One-way ANOVA of the Effects of Age on the Number of Various Synaptic Types

Type	Left Hemisphere	Right Hemisphere
All	48.75 (*)	36.99 (*)
Asymm	57.15 (*)	41.11 (*)
Symm	13.67 (*)	13.24 (*)
Spine	58.76 (*)	26.21 (*)
Shaft	11.21 (*)	26.21 (*)
ASp	62.34 (*)	25.67 (*)
ASh	11.31 (*)	26.53 (*)
SSp	8.86 (*)	12.78 (*)
SSh	3.85 (ns)	3.47 (ns)

Results are given as F-values, df=3,14

* - significant at $p<0.05$, ns - not significant

Fig.9.7

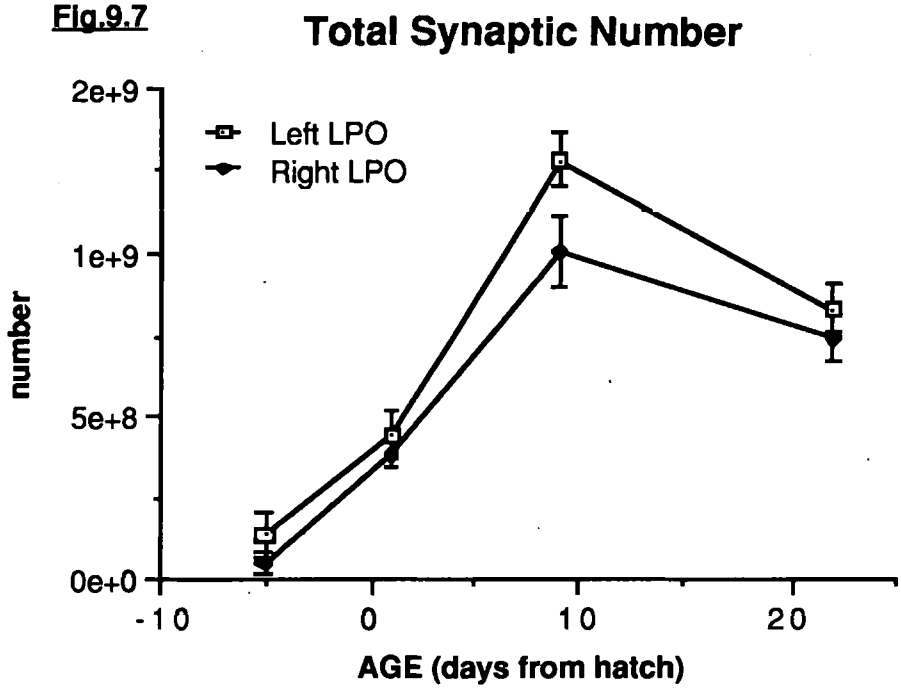


Fig.9:7 Mean number of synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Table 9:7a : Paired t-test of left versus right hemisphere LPO synaptic number during development.

	Asymm	Symm	Spine	Shaft
E16 df=4	1.11 (ns)	1.09 (ns)	0.94 (ns)	1.27 (ns)
P1 df=3	0.58 (ns)	1.09 (ns)	0.94 (ns)	1.64 (ns)
P9 df=4	3.16 (*)	0.71 (ns)	2.87 (*)	1.63 (ns)
P22 df=3	0.71 (ns)	1.54 (ns)	1.40 (ns)	1.22 (ns)

Results are given as paired t-values,

* - significant at $p<0.05$, ns - not significant

see Appendix 4 for means ± SEM

Table 9:7b : Paired t-test of left versus right hemisphere LPO synaptic number during development.

	ASp	ASh	SSp	SSh
E16 df=4	1.01 (ns)	1.31 (ns)	0.83 (ns)	1.02 (ns)
P1 df=3	1.01 (ns)	2.53 (ns)	0.58 (ns)	0.86 (ns)
P9 df=4	3.97 (*)	2.17 (ns)	0.69 (ns)	1.29 (ns)
P22 df=3	1.34 (ns)	1.09 (ns)	0.58 (ns)	0.73 (ns)

Results are given as paired t-values,

* - significant at $p<0.05$, ns - not significant

see Appendix 4 for means ± SEM

Table 9:9 : Two-way ANOVA of the Effects of Age and Hemisphere on the Number of Various Synaptic Types

Type	Age df=3,28	Hemisphere df=1,28	Interaction df=3,28
All	85.19 (*)	6.82 (*)	1.13 (ns)
Asymm	58.05 (*)	7.00 (*)	1.24 (ns)
Symm	26.88 (*)	3.13 (ns)	0.03 (ns)
Spine	78.74 (*)	9.38 (*)	1.51 (ns)
Shaft	36.27 (*)	2.07 (ns)	2.13 (ns)
ASp	80.87 (*)	12.08 (*)	2.43 (ns)
ASh	35.85 (*)	5.28 (*)	2.90 (ns)
SSp	21.51 (*)	0.00 (ns)	0.59 (ns)
SSh	6.94 (*)	1.08 (ns)	0.40 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

Fig.9.8a

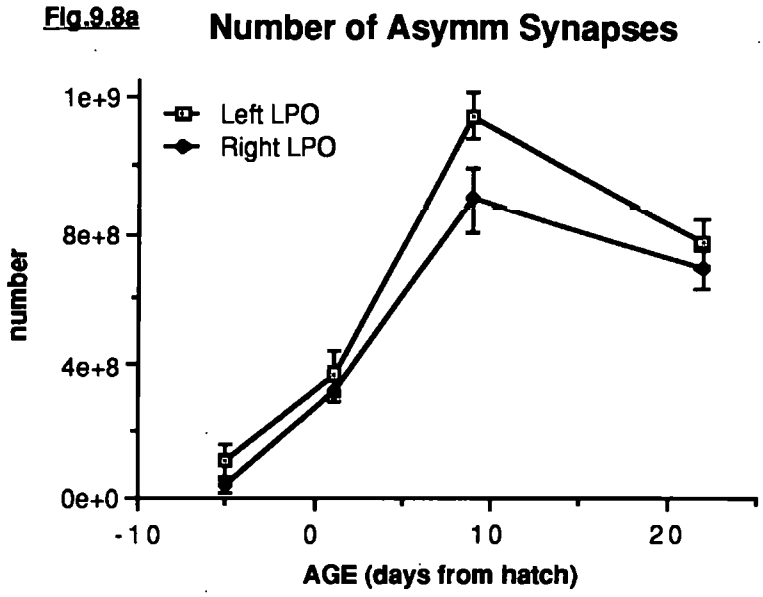


Fig.9.8b

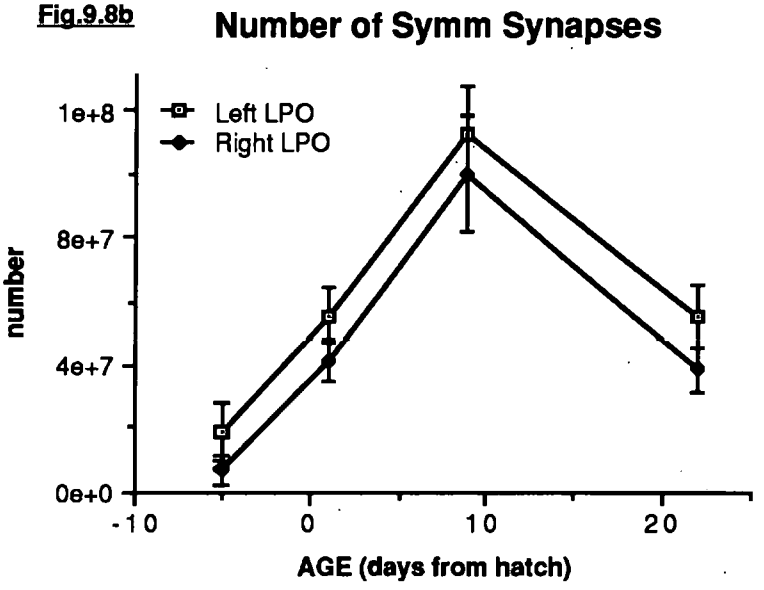


Fig.9:8a,b Mean number of asymmetric and symmetric synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.9a

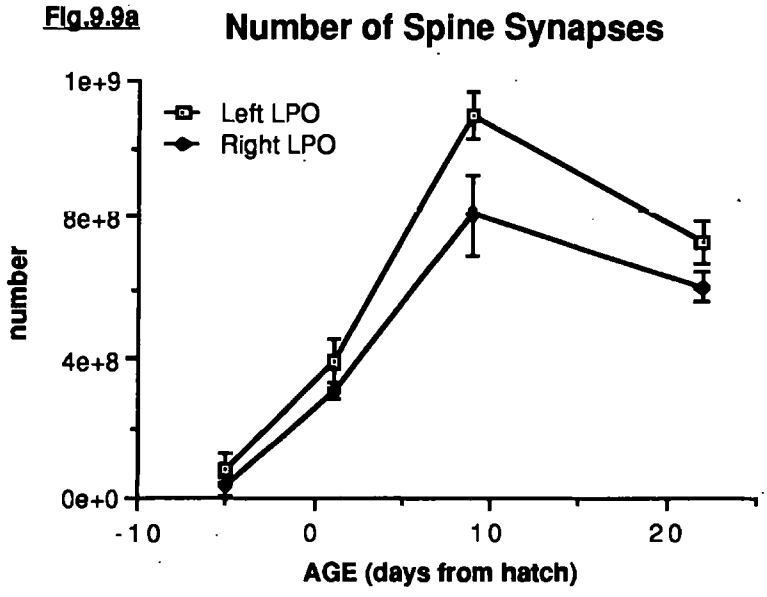


Fig.9.9b

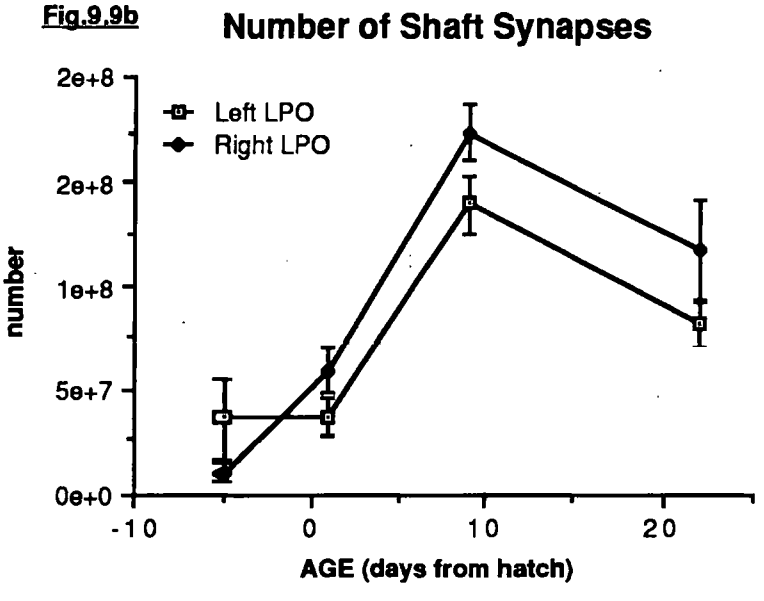


Fig.9:9a,b Mean number of spine and shaft synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.10a

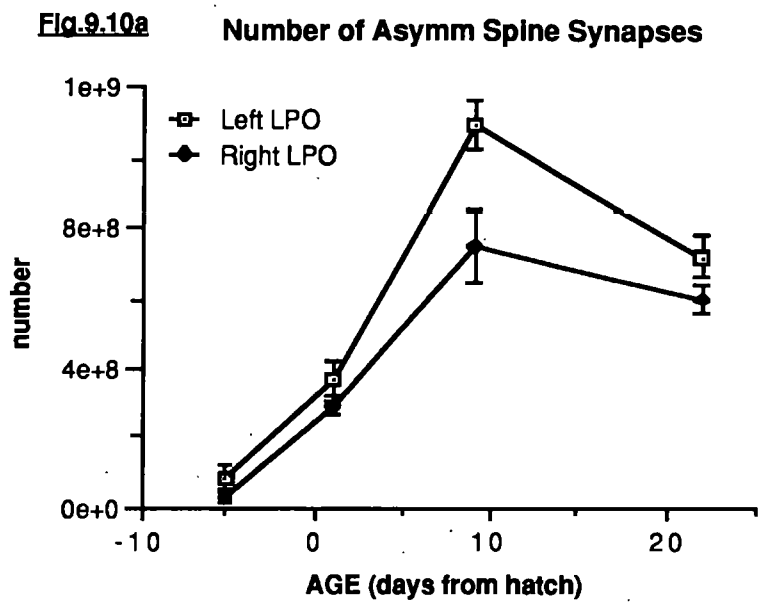


Fig.9.10b

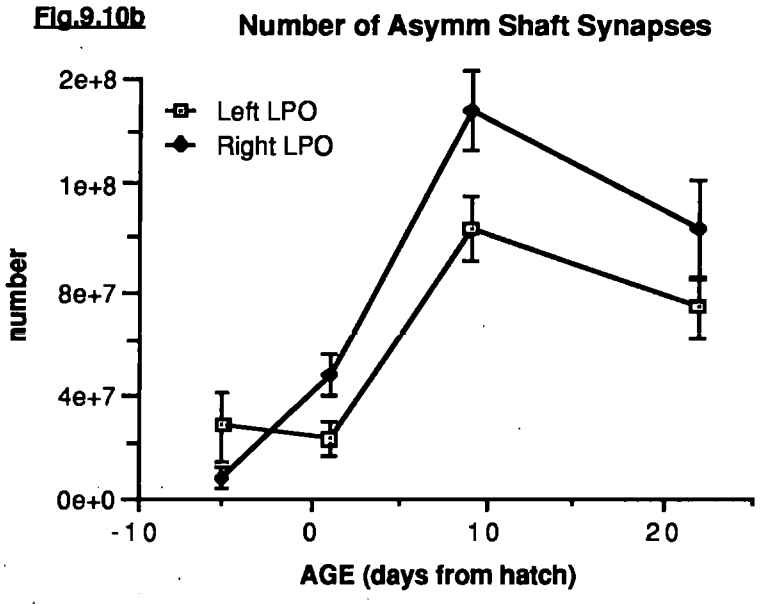


Fig.9:10a,b Mean number of asymmetric spine and asymmetric shaft synapses ± SEM of right and ieft hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

Fig.9.11a

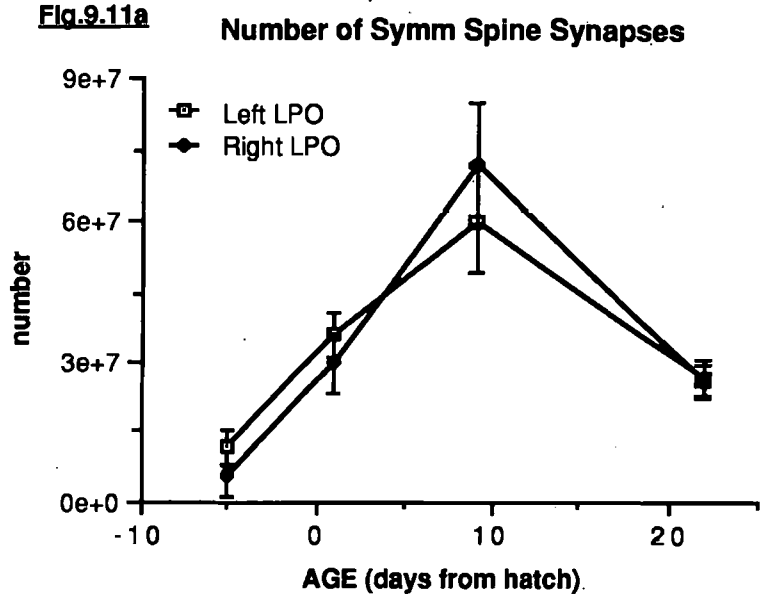


Fig.9.11b

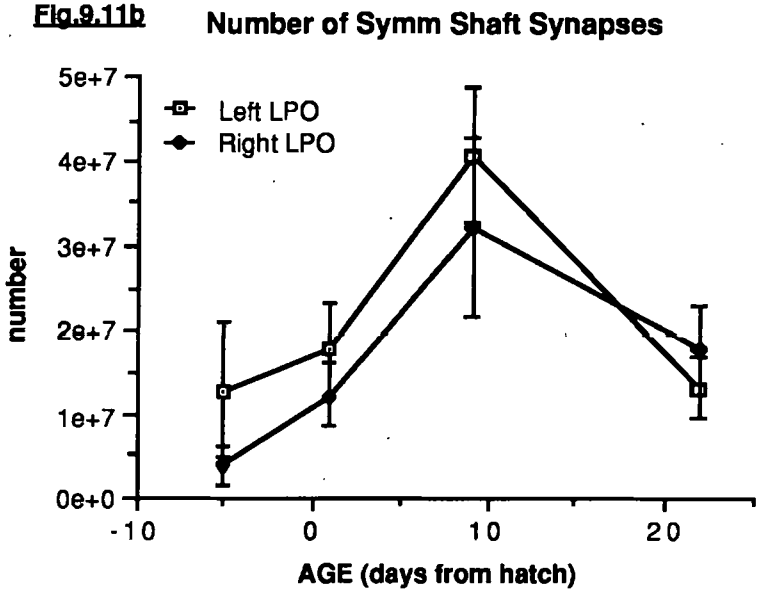


Fig.9:11a,b Mean number of symmetric spine and symmetric shaft synapses ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

e) QUANTITATIVE ANALYSIS : SIZE ESTIMATIONS

The mean projected synaptic height ($\bar{H}_{syn}$) was used as an estimator of synaptic size. The mean values of synaptic height ± SEM, for both right and left hemisphere of chicks of varying ages, are given in Table 9:10a, and in Appendix 5. A t-test for paired replicates was used to determine the significance of the differences obtained between the hemispheres (Table 9:10a). No statistically significant differences were found. However, a one-way ANOVA showed a significant increase in $\bar{H}_{syn}$ with increasing age, in both hemispheres (Left hemisphere: $F_{3,14}=9.58$, $p<0.05$, Right hemisphere: $F_{3,14}=18.20$, $p<0.05$) (Table 9:10b). A two-way ANOVA showed that the increase in size with age was similar in both hemispheres, since there was no significant effect of hemisphere on the observed values of $\bar{H}_{syn}$ ($F_{1,28}=0.29$, $p\geq0.05$) (Table 9:10c). There was also no interaction of hemisphere with age ($F_{3,28}=0.44$, $p\geq0.05$). The significant values obtained in these tests are largely due to a large increase in size between 1- and 9-days post-hatch (Fig.9:12). $\bar{H}_{syn}$ does not change significantly either between 16-days *in ovo* and 1-day post-hatch, or between 9- and 22-days post-hatch, in either hemisphere (Fig.9:12).

Table 9:10a : Mean ± SEM $\bar{H}_{Syn}$ (nm)

Age	Left Hemisphere	Right Hemisphere	Paired t-value
E16	198 ± 17	212 ± 12	0.83 (4) ns
P1	217 ± 30	203 ± 20	0.45 (3) ns
P9	317 ± 30	349 ± 22	0.72 (4) ns
P22	359 ± 17	353 ± 23	0.28 (3) ns

ns - not significant, df in parenthesis

Table 9:10b : One-way ANOVA of the Effects of Age on $\overline{H}_{Syn}$

	Left Hemisphere	Right Hemisphere
F-value	$9.58^{*}(3,14)$	$18.20^{*}(3,14)$

* - significant at $p<0.05$, df in parenthesis

Table 9:10c : Two-way ANOVA of the Effects of Age and Hemisphere on $\overline{H}_{Syn}$

	Age	Hemisphere	Interaction
F-value	$25.66^{*}(3,28)$	$0.29\ (1,28)$	$0.44\ (3,28)$

* - significant at $p<0.05$
df in parenthesis

Fig.9.12

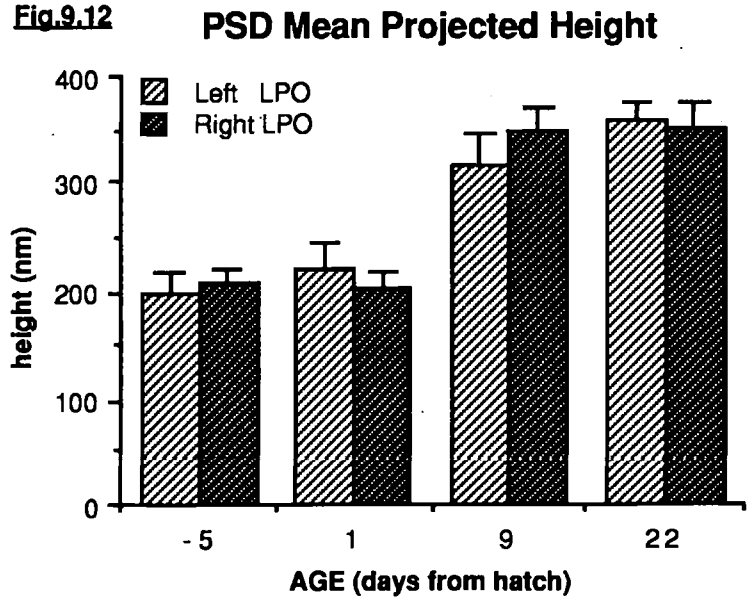


Fig.9:12 Mean projected synaptic height ± SEM of right and left hemisphere LPO during late pre-hatch and early post-hatch development. Each estimate is based on at least 4 chicks (see text for details).

9.4 : DISCUSSION

Qualitative Results

The pre-hatch material was characterized by the presence of large amounts of interstitial space. One possibility is that this space may be an artefact caused by tissue processing, which causes disruption of the delicate embryonic tissue. However, since it is a consistent finding in many studies of early brain development (Curtis *et al* 1989, Parnavelas *et al* 1978), despite varying fixation protocols, this is unlikely. It is more likely that it is indicative of the mobility of the cellular components during early synaptogenesis. Rostas *et al* (1984) have shown that 16 days of age, *in ovo*, is the earliest that synaptic proteins can be isolated from gel preparations. It is hence likely to be a time of intense activation, and to coincide with the period of formation of new synapses.

There is some controversy over the timing of synaptic events during synaptogenesis (for review, see Jacobson 1978). In the present study, at E16 there were many 'free' post-synaptic profiles. There were fewer pre- and post-synaptic thickenings without attached synaptic vesicles, as well as a few 'immature' synapses (pre- and post-synaptic membrane specialisations, with only a few vesicles in the pre-synaptic bouton). There were no synapses that resembled the 'mature' form (containing many synaptic vesicles), such as those seen in the post-hatch chicks, despite the fact that the majority of synapses in the E16 tissue had a prominent post-synaptic thickening. These results are consistent with the suggestion that it is the post-synaptic density that is formed first, followed by the pre-synaptic specialisation, although one cannot make any firm conclusions from such observations. The local conditions at the axon terminal, and in particular the nature of the post-synaptic neuron, must play an important part in dictating the formation and differentiation of the synapse. It

may be hypothesized, that only when a 'correct' pre-synaptic and post-synaptic specialization have established contact, does vesicle formation occur, and development of synaptic function progress.

The synapses of the LPO in the pre-hatch period appear to be very 'immature'. It was suggested previously, that the LPO may co-ordinate learned motor responses (see Chapter 3, section 2c). Since the *in-ovo* chick is not able to explore its environment from within the egg, it may not require a well developed LPO. It has been suggested that before proper maturation of the 'immature' synapse, the synapse has to be active (Burry *et al* 1984, Rutledge 1976), and hence the synapses of the pre-hatch LPO may not yet be 'activated'. Tissue from the P1 group showed a number of free-postsynaptic densities, and some 'immature' synapses. These had completely disappeared by P9, adding weight to the hypothesis described above.

Synaptic Density And Number

The results have been given for both synaptic density and synaptic number, since some of the results from the former have been published previously (Hunter and Stewart 1989). The data given here is much more extensive, and provides a useful comparison of techniques. Many papers have been published purely on the basis of density measurements (Nixdorf 1989, Curtis *et al* 1989, Dyson and Jones 1980, Geinisman *et al* 1986, Jones and Cullen 1979, Blue and Parnavelas 1983b, Bradley 1985) and it is worthwhile considering the validity of some of the results so produced. The present data show that Nv values are valid for the analysis of chick LPO, mainly because the LPO does not appear to grow in size during the period of development studied. This may not be the case in other brain regions, or in regions of brains of other species. One should always aspire

to provide data on total number rather than density. I will proceed therefore, to discuss the data obtained from number estimations rather than density estimations, unless there are differences in the statistical analysis or factors worthy of consideration.

The large increase in synaptic number during early development has been reported for a number of brain regions, in a number of different species (Nixdorf 1989, Aghajanian and Bloom 1967, Mates and Lund 1983b, Jones and Cullen 1979). It has also been reported for developing synapses in culture (Burry and Lasher 1978). It is perhaps remarkable that so few synapses are formed at E16, since the chick is precocious at birth (hatch), only 5 days away.

The number of synapses increased steadily in both hemispheres, until the estimated means reached a peak at P9. There may or may not have been a subsequent increase between P9 and P22, since no data were obtained between these two ages. However, at P22, there was a net reduction of synapses compared with P9. It is interesting to note that a quantitative study of the chick IMHV, also showed a steady increase in synaptic density between E16 and P9 (Curtis *et al* 1989). In a second study, Bradley (1985) reports an increase in synaptic density in the chick IMHV between 0 (hatch), and 7 days. In the chick *ectostriatum* also, there appears to be a period of synaptogenesis which lasts up to 20-days post-hatch (Nixdorf 1989), but not into adulthood (100 days). The density of synapses has been shown to decrease between 7- and 30-days post-hatch in the chick *hyperstriatum accessorium*, and IMHV (Bradley 1985). Curtis *et al* (1989) did not investigate the development of the IMHV beyond 9-days post-hatch, so their data are entirely consistent with that of Bradley (1985). Nixdorf (1989) however, failed to find any evidence of synaptic

elimination in the chick *ectostriatum*. Synaptic elimination has been reported previously for other developing systems (Purves and Lichtman 1980). There may be a correlation between the degree of synaptic loss, and the extent of neuronal death which has been characterised for developing neural systems (Curtis *et al* 1989). These features appear to be related to an ability of the developing nervous system to modify and refine its connections (Burry *et al* 1984). Such features are commonly connected with responses to environmental events, leading to adaptive plasticity within a given fibre network for a learned behaviour (Petit 1988).

Studies of the pigeon visual system (retina and optic tectum) have shown that the adult pattern of GABAergic neurons (i.e. adult pattern of GABA immunoreactivity) is reached around day 9 (Bagnoli *et al* 1991). Hence, the time course for neuronal and synaptic maturation may be similar in a number of brain regions in avian species.

There was an asymmetry of synaptic number at 9-days post-hatch, but not at the other ages studied (Fig.9:7). It is uncertain what this asymmetry represents, although it has been shown that the chick brain displays many functional and morphological asymmetries (Fersen and Günturkün 1990, Gaston 1980, Howard *et al* 1980, Horn *et al* 1983, Mench and Andrew 1986, Rogers 1986, 1989, Stewart *et al* 1984). It is worth noting that the size of the difference is much less in the N_{syn} estimate, in comparison to the Nv_{syn} estimate. This illustrates the importance of volume measurement in such quantitative studies. It is interesting to note that the density of dendritic spines in the 1-day old chick LPO is approximately 0.8 - 1.0 per μm in both left and right hemispheres (Lowndes *et al* unpublished data), i.e. there is no hemispheric asymmetry of dendritic

spine density at 1-day post-hatch, a finding which is consistent with the present results.

The numbers of synapses in each class increased significantly with age, with the exception of symmetric shaft synapses (Table 9:8). Between 75% and 85% of synapses in the chick LPO are of the asymmetric spine type. Therefore it is clear that the results of estimated mean values of the number of asymmetric, spine, and asymmetric spine synapses, will closely resemble those observed for the total synaptic population. The difference in synaptic number between the hemispheres, is mainly due to differences in the estimated numbers of asymmetric synapses. There are no significant effects of hemisphere on the observed means due to symmetric synapses (Table 9:9). If one were to rely entirely on the estimates obtained using density measurements, then one may have concluded that the asymmetry was restricted to asymmetric spine synapses, since a two-way ANOVA showed no significant effects of hemisphere on the estimated values, for either 'shaft', or 'asymmetric shaft' synapses (Table 9:4). However, the results obtained using absolute numbers, show a significant hemisphere effect for asymmetric shaft synapses (Table 9:9).

The present data are consistent with studies of synaptic quantitation in the zebra-finch *ectostriatum*, where 90% of synapses were of the asymmetric type (Nixdorf 1989). However, there appears to be a radical difference in this respect when one examines the proportion of those synapses targeted onto dendritic spines. The young zebra-finch (20-days old) has 40% of axo-spinous synapses and 60% of axo-dendritic synapses. The proportion of axo-spinous synapses is further reduced by 50%, between 20- and 100-days post-hatch. Since the synaptic density remains constant throughout this developmental period, the author concludes that axo-spinous synapses are converted into axo-

dendritic synapses (Nixdorf 1989). This would not appear to be the case for the chick LPO, where the present study shows that the large majority (between 80- and 90%) are axo-spinous, and the proportion of these is maintained at a fairly constant level, throughout post-hatch development. A study of synapses in the IMHV also shows that the majority of synapses were of the axo-spinous type (Bradley 1985). The present data has indicated that there may be some modification of the ratio of axo-spinous and axo-dendritic synapses, between pre- and post-hatch.

Examination of the data for each class of synapse as a percentage of the total shows that between the late pre-hatch and early post-hatch period, there is a shift in the proportion of asymmetric spine and asymmetric shaft synapses. The direction of this shift is such that the proportion of asymmetric spine synapses increases, whilst the proportion of asymmetric shaft synapses decreases. This pattern is not seen amongst the symmetric synapses. These observations are consistent with the hypothesis that asymmetric shaft synapses are capable of transformation into asymmetric spine synapses. The data can in no way however, prove this hypothesis. This method of synaptic development, where the presence of a synapse on a dendritic shaft causes the development of a dendritic spine at that same location, has been suggested as the pattern of synaptic development for the visual cortex of both rat (Miller and Peters 1981) and primate (Mates and Lund 1983a).

The numbers of symmetric synapses were consistently low at all ages studied. This is consistent with a number of studies on synaptic structure in the avian brain (Curtis *et al* 1989, Nixdorf 1989). It is possible that there was an under-estimation of number in the present study, since identification was not

easy. In mammals, synaptic vesicle shape is often used to help in the identification of synaptic type. Asymmetric synapses have characteristic round vesicles, whilst symmetric synapses have flattened or ellipsoidal vesicles (Gray 1959). Nixdorf (1989) has shown that the classification of synapses on the basis of vesicle morphology is not possible for avian species, since vesicle shape for a given class of synapse changes with age. In juvenile zebra-finches (5-10 days of age), vesicles of symmetric synapses were round. In 5-day old zebra-finches only 0.02% of symmetric synapses were associated with flattened vesicles. However, in the adult (100-days of age) this proportion rose to 80%. At no time, was vesicle shape an absolute indicator of synaptic type. In the domestic chick IMHV no flattened vesicles were found in tissue sampled from birds aged between 16-days *in ovo* to 9-days post-hatch (Curtis *et al* 1989). This was also the case for the present study, where all synaptic vesicles were either round or pleomorphic. The identification of symmetric synapses was therefore based entirely on the nature of membrane specialization. Since this type of synapse is characterized by a modest thickening of the post-synaptic density, identification can be difficult.

The percentage of synapses that were symmetric was approximately 10-15% of the total synapse number in the pre-hatch material. This value dropped to approximately 4-8% in the P22 group, and there was a steady decline in both hemispheres between these two ages. This is in close agreement with a study of synaptic parameters in the developing IMHV (Curtis 1989). Curtis *et al* found that the percentage of symmetric synapses fell from 15-20% to 7-10%, over the same period as the present study. It is important to stress that this is a relative measure, since the actual numbers of symmetric synapses continue to increase up to 9-days post-hatch. As a consequence of a decreasing ratio of

symmetric synapses, the proportion of asymmetric synapses increases over the same period. The possibility arises that some symmetric synapses may have the capacity to become asymmetric, perhaps as more proteins are laid down at the post-synaptic site as development progresses. This is quite feasible, since it has been shown that the concentration of a major post-synaptic density protein increases 3-5 fold during early development of the chick forebrain (Rostas *et al* 1984).

It is possible that some symmetric synapses were mis-categorized, and should have been included in the asymmetric group. This could occur in a small minority of cases where the section plane traverses the edge of an asymmetric synapse. It can be seen from the profile of most asymmetric synapses, that the post-synaptic thickening tapers at the periphery. If a section should cut a synapse at this point, its profile would resemble that of a symmetric synapse. Due to the randomness of sectioning, the probability of sectioning through a synaptic edge is proportional to the size of the synapse. However, its probability of being counted using the 'disector' is independent of size, but is never-the-less 50-50, since the subsequent section will either be away from the synapse (where the synapse will be counted), or towards the synapse (where it is likely to be sectioned again (since section thickness is less than the size of the smallest synapse)). In either event, the probability of such encounters are small, and are unlikely to distort the results.

Synaptic Size

Previous estimates of synaptic size have been made using biased techniques that rely on 'unfolding methods', and assume that synapses are disc-shaped (Nixdorf 1989, Stewart *et al* 1984, Curtis *et al* 1989). It has been shown that

synapses have varying shapes, and such assumptions are invalid (DeGroot and Bierman 1986). Indeed, it has been shown that only 25-30% of synapses in the LPO are indeed 'flat' (Stewart *et al* 1987). No previous study of synaptic size in the chick brain has been made using unbiased techniques such as the 'disector'. The data obtained in the present study, has therefore no direct comparison. Values of mean synaptic size obtained for the LPO are consistently lower than those obtained using 'unfolding' methods for the 1-day old chick IMHV (Stewart *et al* 1984, Curtis *et al* 1989), and for the 1-day old chick LPO (Stewart *et al* 1987). No data exists, at present, for comparison of other ages. The 'disector' method of synaptic size determination, makes only an estimate of mean projected synaptic profile height, and does not correct for orientation of the synapse within the disector planes. If a synapse lies obliquely within the section, or at a plane perpendicular to the section planes (i.e. horizontally), then its estimated size will decrease in proportion to its angle of orientation. Hence estimations of synaptic height are not estimators of synaptic length, and direct comparison of techniques is not possible. Synaptic height estimations will always be less than estimations of synaptic length, assuming that they are drawn from the same population of synapses.

There was a significant increase in the mean estimated height of the synapses between 1- and 9-days post-hatch. A similar finding for synaptic length has been reported for synapses in the IMHV (Bradley 1985), where an increase in synaptic length was seen between 1- and 2-days post-hatch. These mean lengths subsequently decreased gradually to 30-days post-hatch. Bradley (1985) separated synapses into spine and shaft synapses, without taking into account their PSD symmetry or hemisphere of origin. He reported that shaft synapses were consistently longer than spine synapses by 50-80 nm. The present study

did not take into account possible differences between synaptic types. The number of symmetric synapses was extremely low, hence estimation of mean symmetric synapse size would not have been statistically viable. The analysis was therefore only carried out for the asymmetric type. There remains the possibility that observed synaptic size changes during early development, may be restricted to one or more class of synapse. Curtis *et al* (1989) have suggested that at least for the chick IMHV, it is the symmetric synapse which increases in size during the the early post-hatch period. Other workers have shown that symmetric synapses are subject to substantial size fluctuations during development, and that no such fluctuations are seen for asymmetric synapses (Blue and Parnavelas 1983b).

Chick forebrain synaptosomal preparations of chicks aged 16-days *in ovo*, were shown to have a PSD junctional length of 170 ± 110 nm (Rostas *et al* 1984). This value increased to 298 ± 86 nm in synaptosomal fraction preparations from the forebrains of 2-day old chicks. This value was not significantly different to the 'adult' value of 304 ± 39 nm (Rostas *et al* 1984).

One theory of synaptic development is that synapses undergo a process of 'splitting', whereby a large synapse divides, hence generating two small synapses (Dyson and Jones 1984). This may account for the presence of 'perforations' in synapses in developing and adult brain tissue (Calverley and Jones 1990b). Where such a mechanism of synaptic plasticity exists, estimates of mean synaptic size may not not reveal such modifications. Hence mean size estimates are of limited value in assessing the nature of synaptic change during development.

CHAPTER 10 : TIME-COURSE OF SYNAPTIC PLASTICITY FOLLOWING MEMORY FORMATION

10.1 : INTRODUCTION

It has been suggested that the formation of memory in the 2-day old chick involves three stages; short-term (STM), intermediate-term (ITM), and long-term (LTM) (Gibbs and Ng 1977). These phases are sequentially linked and depend upon particular neurochemical events. STM lasts between 5 and 10 minutes following learning. ITM is fully developed by 15 minutes, and decays after 30 minutes. LTM is present after 30 minutes, and is persistent at least 24 hours after learning (Gibbs and Ng 1977). In reality, these phases show considerable overlap, and it may be that memory formation is a continuum in which the 'stages' are simply identifiable parts of a sequence of linked events.

The formation of each of these three memory stages can be inhibited by pharmacological intervention, using different drugs. STM can be disrupted by injections of LiCl, KCl or glutamate, which cause depolarization of neuronal membranes (Gibbs and Ng 1979). It may be that STM stage is characterized by hyperpolarization due to increased membrane conductance of K^+. ITM is disrupted by the administration of Na^+/K^+ ATPase inhibitors, such as ouabain and ethacrynic acid (Gibbs and Ng 1977). LTM is characterized by protein synthesis, since inhibitors of protein synthesis, such as anisomysin or cycloheximide, inhibit the formation of LTM. The injection of a protein synthesis inhibitor, emetine, into the hyperstriatum, has been shown to inhibit memory formation in the 2-day old chick, 90 minutes following training (Patterson *et al* 1986). Gibbs and Ng (1977) have also suggested that the intermediate phases between memory stages, may be seen as 'dips' (lower percentage) in avoidance

task responses at 12 and 55 minutes post-training. These 'dips' were also reported following studies of avoidance behaviour to strong and weak aversive stimuli (Rosenzweig *et al* 1989). These occurred at 1, 15 and 60 minutes following training. If there are indeed separate phases, it may be possible to correlate morphological changes in the brain within the proposed time-scale.

Patterson *et al* (1988) induced amnesia in chicks by injecting pharmacological agents (10 µl), 5 minutes pre-training, into either left IMHV or right lateral *neostriatum*. They found that the time-course of amnesia was different between the two regions. Hence, there may not be a single time-course of memory formation, rather it may depend upon the area of brain processing or storing the information. In the IMHV, injections of glutamate (50 mM) caused amnesia 5 minutes post-training. Ouabain (0.027 mM) produced amnesia between 15 and 30 minutes post-training, and emetine hydrochloride (2.25 mM) resulted in amnesia between 75 and 90 minutes post-training. Corresponding times for these injections into the right lateral *neostriatum* were; 5 minutes (glutamate), 30-45 minutes (ouabain), and 60-90 minutes (emetine). Hence the differences between the two brain regions were only seen in the ITM stage, which was 15 minutes later in the right lateral *neostriatum*.

Patterson *et al* (1988) showed that chicks were able to show memory for the avoidance task one minute post-training, despite having been injected with an inhibitor of STM (glutamate) 5 minutes pre-training. This would suggest that there is a stage of memory which precedes STM. Patterson *et al* termed this phase a 'sensory buffer'. This however, is an unnecessary phrase, since a 'sensory buffer' means a storage of information acquired by experience; in other words a 'memory'.

The present thesis is concerned with the role of the LPO in memory formation. The LPO however, is not thought to play a part in all three stages of memory (Serrano *et al* 1988), but may rather only be involved in the intermediate stage (Serrano *et al* 1988) or long-term stage of memory formation (Stewart *et al* 1990). Given the proposed hypothesis regarding the time-course of memory formation in the young chick, it was decided to investigate the nature of morphological changes in the lobus parolfactorius, using the passive avoidance task used in the experiments described above. Morphological changes are thought to be the basis for the formation of long-term memory (Rosenzweig and Bennett 1984a, 1984b). Indeed, a review article on the subject recently stated; "... The timekeeping steps for a long-term memory trace may be specified by morphological changes at the level of the synapse." (Bailey and Chen 1990).

Passive avoidance learning in the young chick has previously been shown to cause morphological, biochemical and physiological alterations in several forebrain nuclei (for review, see Rose 1985b, 1986), including the LPO (Stewart *et al* 1987). The most marked plastic change of synapses in the LPO following passive avoidance learning, is that of an increase in synaptic density, 24 hours after training. It has been postulated that the increase in the density of synapses is one of the factors contributing to the establishment of long-term memory in the chick (Stewart 1990). Studies of lesions of the LPO suggest that this nucleus is indeed involved in the long term storage of memory (Gilbert *et al* in press), since lesions of the LPO cause amnesia for the avoidance task, only if given post-training and not pre-training. It is presumed that by 24 hours, the memory trace has entered the 'long-term' phase (Gibbs and Ng 1977). It has

further been shown that the IMHV is involved in the acquisition of memory (Patterson *et al* 1989). Recall of the passive avoidance task is not affected by post-training lesions, but is affected by pre-training lesions. It is of note that the density of synapses in the IMHV, either 12 hours (Bradley and Galal 1987) or 24 hours following passive avoidance learning, is not significantly different to that of controls (Stewart *et al* 1984). Together with the data from the LPO studies, this suggests that an increase in synaptic density may represent a morphological change necessary for the formation of the long-term store of the memory trace (Stewart 1990). It has also been shown that synaptic length in the right hemisphere of the LPO, is greater than that in the left (by a factor of approximately 10%) (Stewart *et al* 1987). This difference in size is reversed after training, such that the left hemisphere is 10% greater than the right.

An analysis of the time-course of structural changes of neurons in *Aplysia californica*, following long-term sensitization has been reported (Bailey and Chen 1989b). It was shown that certain morphological changes were transient (1-2 days) (increase in PSD length, increase in number of synaptic vesicles), and others were longer-lasting (3 weeks) (number of neuronal varicosities, and an alteration in synaptic number).

The available data in the literature do not present information on synaptic events in chick brain after, or before, the 24 hour period following passive avoidance learning, with the exception of a study by Bradley and Galal (1987) who described changes in synaptic density in the IMHV, 12 hours following training. The present study was therefore designed to map the time-course of changes in synaptic density, and size, in the chick LPO following this training procedure. In addition, as a secondary aim to the synaptic investigations, it was

thought appropriate to quantify changes in the mean volume of both pre-synaptic (bouton), and post-synaptic (dendritic shaft and dendritic spine) components.

10:2 : MATERIALS AND METHODS

Animals : Approximately 40 chicks (Ross I) of both sexes, were hatched in communal brooders kept at a constant temperature of 38-40°C. A 12 hour light/12 hour dark cycle was in operation. On the first day after hatching (P1), five pairs of chicks were placed in separate 20 X 25 X 20 cm aluminium pens, each illuminated with a 25W red light. One chick in each pen was marked with ink, and was designated to either Control group or experimental group at random. Chicks were given 30 minutes in order to adapt to their new surroundings.

Training protocol : All training procedures were performed at approximately 9:30 am-10:30 am on a given day. The experiment was conducted over several weeks. Codes were given for each chick, allowing the subsequent experimental procedures to be performed 'blind'. There were three successive pre-training trials, each separated by 10 minutes. This involved presenting a small white bead (3 mm dia.) about 2-3 cm from the chicks beak. Only chicks which pecked at this bead on all three occasions were included in the study. After a further 10 minutes, one chick from each pen was presented with a small chrome bead (4 mm dia.) coated with methyl anthranilate. After pecking this bead, the chicks showed a characteristic disgust response (head shaking, bill wiping, rapid swallowing and emitting distress calls). About 10% of the chicks failed to show this response, and were not included further in the study. The second chick from each pen was presented with an identical bead, except that it was coated with water. These chicks showed no observable behavioural response to the pecking experience. Chicks failing to peck the water-coated bead during the training procedure were omitted from further study. Food and water was then available

ad libitum. After 30 minutes, chicks were presented with a water coated bead for 10 seconds. It was observed whether or not, this bead was avoided. Approximately 80% of the chicks who had previously pecked at the methyl anthranilate-coated bead (M-trained), avoided the bead. All of the chicks who had previously pecked at a similar water-coated bead (Controls), re-pecked. M-trained chicks who re-pecked were excluded from further investigation. The pair of chicks from each pen were removed 0.5, 5.5, 11.5, 23.5, and 47.5 hours after training respectively, and transferred to a separate room, to which they were allowed to adapt for 30 minutes. Hence groups of chicks were obtained that had been trained either 1, 6, 12, 24, or 48 hours previously. Temperature was maintained at approximately 38-40^{o}C throughout. This procedure was undertaken in order to minimize the stress that the chicks might undergo, and so helped to reduce non-specific effects of the experimental design. Chicks were then anaesthetized with sodium pentobarbitone (0.1 ml, 60mg/ml), and perfused intra-cardially with a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer, using a dual peristaltic pump.

Tissue analysis : Tissue was obtained and processed for electron microscopy as detailed previously (Chapter 8, Section 2). Two differences in method are noteworthy. Firstly, sections were cut at approximately silver-gold interference colour (approximately 70 nm). This was done to increase the effective sample size of synapses, since more synapses would be absent in the reference section in comparison to the previous study, assuming synaptic size was comparable. In order to further enhance the sampling efficiency, micrograph prints were made at final magnifications of approximately X12,000, rather than X20,000 as were made previously (Chapter 8, Section 2). These modifications were made purely on the basis of practical experience gained on completion of the 'developmental

study' (Chapter 9).

Section thickness : An accurate estimation of section thickness was determined by the 'small fold method' (Small 1968). Mean section thickness was 68nm ± 3nm (SEM) (n=60).

Synaptic identification : Synapses were identified, and categorized as stated previously (Chapter 8, Section 2 and Chapter 9, Section 2).

Estimation of Nv_{syn} : Synaptic density was calculated using the formula;

$$Nv_{syn} = \Sigma Q^{-} / h.A$$

where; Q^{-} = synapses present in 'nominated' section, but absent in the 'reference' section,, h = distance between disector planes, A = sample area. Since adjacent sections were used for the disectors, h was equal to the section thickness. A counting frame was overlayed onto each micrograph in turn. This frame was approximately three-quarters of the size of the micrograph. The left-hand and bottom margins of the frame were deemed 'forbidden lines' (Gündersen 1977). Any particle touching or crossing this line was automatically excluded from the analysis. The forbidden lines extended to infinity, such that any particle being measured had to be unambiguously identified, and its course defined. This presented no significant problem, since most of the particles being counted or measured were small. The dendritic shafts were the exception to this, and this point is raised in the discussion. The use of forbidden lines ensured that there were no 'edge effects' (Gündersen 1977). The frame was used to set the sample area for each 'disector'. Magnification was obtained from a cross-grating replica with 2160 lines/mm, a micrograph of which was taken with every set of 'disectors'. Corresponding estimates of Nv_{ASp} (asymmetric spine synapses),

Nv_{ASh} (asymmetric shaft synapses), Nv_{SSp} (symmetric spine synapses) and Nv_{SSh} (symmetric shaft synapses) were calculated for each hemisphere.

Estimation of LPO volume (V_{LPO}) : The volume of both right and left hemisphere LPO of M-trained and Control chicks, was made using the Cavalieri principle, described in Chapter 9, Section 2. Since the brains of chicks used in the time-course study were also to be used by another investigator, who would subsequently analyse morphological changes in the IMHV, it was not possible to make volume estimations of the LPO from these brains. To do so, would involve sectioning of the IMHV, and would render subsequent IMHV analysis invalid. A pilot study was therefore performed on chicks not used in the final morphometric analysis. These were six in number (3 Control, 3 M-trained). They were treated in an identical manner to the chicks which were used for quantitative studies. The chicks were killed by cardiac perfusion fixation, 24 hours after training, using a solution containing 2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer (approximately 100 ml). The brains were then dissected from the cranium, prior to being immersed fixed with fresh fixative at 4°C, in which they were left overnight. Procedures for volume estimation were performed as described before (Chapter 9, Section 2).

Estimation of mean synaptic height ($\bar{H}_{syn}$) : In addition to counts of synapses present in the counting frame of the 'nominated' section but absent in the 'reference' section (Q^-), the total number of synaptic profiles (Q_{tot}) present within the counting frame of each of the micrographs was also noted. This information could be used to estimate the mean projected synaptic height ($\bar{H}_{syn}$), the linear size estimator of the average vertical length of the projected synapses, irrespective of their orientation. This is calculated as follows;

$$\bar{H}_{syn} = Q_{tot} \cdot h / Q^{-}$$

where; 'h' is the section thickness. The results of mean estimated synaptic height are recorded in nanometres (nm).

Estimation of synaptic contact surface area (Sa_{syn}) : The area of the PSD determines in part, the probability that a given action potential will be transferred between one neuron and another. Any change in this parameter therefore may be an indication of a change in efficacy of the synapse. The surface area of the PSD is a more accurate indicator of changes in size, compared to synaptic height, which is a linear measurement. The changes in size that are hypothesized to occur in synapses following learning (Bailey and Chen 1990, Stewart *et al* 1984, Bradley *et al* 1985b, Bradley and Galal 1987), may only be of small magnitude, and may be missed by either one measurement alone. By estimating both projected height and surface area, it was thought that a more accurate portrayal of any plastic size changes could be detected. To measure synaptic surface area, an overlay test grid was placed within the counting frame. This grid contained a series of parallel lines horizontally and vertically. The spacing between lines was 2 cm, and the counting frame was 80 X 140 cm. This gave 28 intersections between the test grid lines (excluding the 'forbidden lines' at left and bottom margins) (see Fig.7.1d). These intersections served as test points. The test line length (l) per test point was therefore 4 cm (horizontal + vertical). This was adjusted for magnification, accurately assessed for each set of micrographs. The synaptic contact surface area (Sa_{syn}) was calculated using the following formula;

$$Sa_{syn} = 2 \cdot I/L$$

where; I = intersections between test lines and synaptic contacts (PSDs), L = total test line length within the counting frame. All values given in the results

are a summation over the total number of micrographs for a given hemisphere of an individual brain.

Estimation of Volume Weighted Mean Volumes : If a random point on a random section hits a particle profile, one may measure the length (l) of the liner intercept across the profile, and through the point. If the direction of the intercept is isotropic in 3-dimensional space then;

$$V = (\pi/3).l^3$$

where; 'V' is the particle volume. Since the probability of hitting a particle with a random point is strictly proportional to the volume of the particle, one must make estimates using appropriately weighted values. The weighting is based on the particle volume itself, such that one measures the volume weighted mean volume ($\bar{V}v$);

$$\bar{V}v = \pi/3.\bar{l}^3$$

where the mean cubic length is used as the estimator. The volume weighted mean volumes of the synaptic bouton ($\bar{V}v_{bouton}$), dendritic spine ($\bar{V}v_{spine}$) and dendritic shaft ($\bar{V}v_{shaft}$) were estimated in the present study.

To measure the intercept lengths (l), a frame was overlayed onto each micrograph in turn. This overlay contained a series of angle indicators with reference to the origin at the bottom left of the frame. These angle indicators were numbered from 0 to 97 (97 being the largest 2-digit prime number). The spacing between the numbers is related to the sine of the angle. A random number between 0 and 97 defined the first direction. A second overlay with a series of parallel lines spaced 2 cm apart was aligned along the axis of the given direction, from the origin to the chosen number. A series of point intercepts marked on the parallel lines at a spacing of 4 cm served as sample intercepts from which the

lengths would be measured. The starting position of the points on the parallel lines was entirely random. The radius of particles hit by points within the reference frame, was measured along the axis of the direction indicator described above. Measurements were made in both directions from the sample point to the boundary of the particle. Both 'nominated and 'reference' sections were used for the measurements, although the direction axis was changed between the two. This was done by adding a constant (37) to the direction axis. If this value exceeded 97, 97 was subtracted from the number obtained so that the values were cycled between 1 and 97. This was repeated for each subsequent micrograph in the set, for each hemisphere of a given brain. This allowed complete randomness despite using samples from adjacent serial sections. The mean length of line raised to the third power was obtained using a digitizer linked to a microcomputer. Analysis was made from the LPOs of Control and M-trained chicks 1, 24 and 48 hours after training only.

Statistical analysis : Students' T-tests were used to compare mean estimates of Nv_{syn}, Nv_{ASp}, Nv_{ASh}, Nv_{SSp}, Nv_{SSh}, $\overline{H}_{syn}$, Sa_{syn}, $\overline{V}v_{bouton}$, $\overline{V}v_{spine}$, and $\overline{V}v_{shaft}$, based on results calculated from six animals per group. Paired Students' T-tests were used to compare mean Nv_{syn}, $\overline{V}v_{bouton}$, $\overline{V}v_{spine}$, $\overline{V}v_{shaft}$, $\overline{H}_{syn}$ and Sa_{syn} estimates of LPOs taken from the right and left hemispheres of the same individuals. A two-way analysis of variance (ANOVA) was used to test the significance of the effects of training on the observed means, and to test whether this training influenced the hemispheres differently. Two-way ANOVAs were also used on data at each of the times studied (i.e. 1, 6, 12, 24, and 48hrs), to assess whether there were any effects or interaction of effects at a given time after training. Where appropriate, paired sample ANOVAs were made using a repeated measures multivariate analysis of variance (MANOVA) program of Statistical Package for Social Sciences (SPSS) on the Open University mainframe computer.

A one-way ANOVA was used to compare the estimates obtained for a given hemisphere with increasing time. These tests were performed separately for both Control and M-trained chicks. Significance of the statistical tests was assumed when the probability of significance was greater than 0.95 (95% confidence limits).

10.3 : RESULTS

a) LPO VOLUME ESTIMATION (V_{LPO})

The volume of LPO from the brains of chicks used in the pilot study to this experiment (i.e. not from the same brains used to estimate morphological parameters), was estimated for each hemisphere of Control (n=3) and M-trained chicks (n=3). The mean values obtained for V_{LPO} ± SEM are given in Table 10:1a. The estimated means for left and right hemisphere of Control chicks, were not significantly different to the previously estimated means for the 1-day old group from the developmental study, using a Student's t-test (Table 9:1a) (left hemisphere: t=0.59 (df=7) p>0.05, right hemisphere: t=0.54 (df=7) p>0.05). There was no significant difference between the hemispheres of either Control (Paired t-test : t=1.22 (df=2) p>0.05) or M-trained chicks (Paired t-test : t=1.31 (df=2) p>0.05) (Table 10:1a). Training caused no significant alteration in the volume of the LPO in either left (T-test: t=0.36 (df=4) p>0.05) or right hemisphere (T-test: t=0.21 (df=4) p>0.05). A two-way ANOVA (Table 10:1b) provided no evidence of an effect of training on the estimated mean LPO volume ($F_{(1,8)}$=0.02, p>0.05). There was also no interaction of effects between hemisphere and training ($F_{(1,8)}$=0.17, p>0.05). On the basis of these results, it was considered that in the circumstances it was justifiable to restrict subsequent quantitative analysis to density measurements alone.

Table 10:1a : Mean $V_{LPO} \pm$ SEM (mm^3)

Hemisphere	Control	M-trained	t-value
Left	0.66 ± 0.14	0.72 ± 0.11	0.36 (4) ns
Right	0.69 ± 0.11	0.66 ± 0.11	0.21 (4) ns
Paired t-value	1.22 (2) ns	1.31 (2) ns	

ns - not significant, df in parenthesis

Table 10:1b : Two-way ANOVA of Effects of Hemisphere and Training on V_{LPO}

Hemisphere	Training	Interaction
$0.02_{(1,8)}$	$0.02_{(1,8)}$	$0.17_{(1,8)}$

Results displayed as F-values, df in parenthesis

b) SYNAPTIC DENSITIES

Total synaptic density (Nv_{syn}) : The estimated mean synaptic densities of both Control and M-trained chick LPO are shown in Fig.10:1, and also in Appendix 6. Analysis of the corresponding values for Nv_{syn} for right and left hemisphere LPO using a Paired t-test, showed that there was no detectable difference between the hemispheres with respect to synaptic density (Table 10:1a). A two-way ANOVA was used to compare the effects of training and hemisphere on the observed values of Nv_{syn} at each of the given times after training (Table 10:2b). Statistically significant effects of hemisphere were seen after 24 hours ($F_{1,20}=4.98$, $p<0.05$). Training had a significant effect on Nv_{syn}, 24 hours ($F_{1,20}=10.72$, $p<0.05$), and 48 hours after the avoidance task

($F_{1,20}$=13.69, p<0.05). There was no observable interaction of these effects. There was a steady increase in mean Nv_{syn} with time after training, in both Control and M-trained chicks, of approximately 60-70%. This observation was tested statistically using a one-way ANOVA (Table 10:7). It showed a significant effect of time on Nv_{syn} in the left hemisphere of Control chicks ($F_{4,25}$=8.14, p<0.05), right hemisphere of Control chicks ($F_{4,25}$=8.29, p<0.05), left hemisphere of M-trained chicks ($F_{4,25}$=17.91, p<0.05), and right hemisphere of M-trained chicks ($F_{4,25}$=24.21, p<0.05). A two-way ANOVA on the pooled data (Table 10:8a) also showed significant effects of time after training on the observed Nv_{syn} values (left hemisphere: $F_{4,50}$=23.26, p<0.05, right hemisphere $F_{4,50}$=28.34, p<0.05). There was a significant difference in the extent of this increase between M-trained and Control chicks, in both hemispheres (left hemisphere : $F_{1,50}$=6.78, p<0.05, right hemisphere : $F_{1,50}$=8.96, p<0.05 (Table 10:8a)). There was a significant interaction of the effects of time and training in the left ($F_{4,50}$=2.72, p<0.05), but not the right hemisphere. A two-way ANOVA of the effects of time after training and hemisphere (Table 10:8b) again showed significance of time in both Control ($F_{4,50}$=14.80, p<0.05) and M-trained chicks ($F_{4,50}$=41.05, p<0.05), showing that synaptic density increases as a consequence of normal development. In Control chicks however, the increase is similar in both hemispheres (Table 10:8b), whereas M-trained chicks show a greater increase in the left hemisphere, such that a significant hemisphere effect on Nv_{syn} is seen in the two-way ANOVA ($F_{1,50}$=4.50, p<0.05) (Table 10:8b).

Asymmetric spine synapses (Nv_{ASp}) : The mean densities ± SEM of asymmetric spine synapses at the various times after training, are shown in Fig.10:2, and also in Appendix 6. Paired t-tests did not reveal any differences in

Nv_{ASp} between the hemispheres of Control or M-trained chicks (Table 10:3b). An unpaired t-test was used to compare the mean Nv_{ASp} values of the left and right hemisphere of Control and M-trained chicks between 1 and 48 hours after training (Table 10:3a). No significant differences were found. A two-way ANOVA of these parameters at the different times following training (Table 10:3b), revealed a significant effect of training at 12 hours ($F_{1,20}=4.41$, $p<0.05$) and 24 hours ($F_{1,20}=5.46$, $p<0.05$). The effect of training at 48 hours fell just short of the significance level ($F_{1,20}=3.90$, $0.05>p<0.10$). There was no significant difference due to hemisphere, or interaction of hemisphere and training. Since the majority of synapses are of the asymmetric spine type, there is a close comparison of results of Nv_{ASp} with those of Nv_{syn}. Hence, a one-way ANOVA of the effects of time after training on the observed mean Nv_{ASp} values, shows significant effects in all four groups (left hemisphere Control: $F_{4,25}=3.65$, $p<0.05$, right hemisphere Control: $F_{4,25}=6.00$, $p<0.05$, left hemisphere M-trained: $F_{4,25}=9.02$, $p<0.05$, right hemisphere M-trained: $F_{4,25}=9.87$, $p<0.05$) (Table 10:7). These significances are due to an increase in Nv_{ASp} in these groups with time. This is also reflected in significant effects of time after training in a two-way ANOVA on the observed Nv_{ASp} values (Table 10:8a,b). In addition, these tests show significant effects of training on the estimates obtained for the right hemisphere LPO, but not the left. Hemisphere, *per se*, did not influence the observed values of Nv_{ASp} (Table 10:8b).

Asymmetric shaft synapses (Nv_{ASh}) : Estimates of mean Nv_{ASh} are shown in Fig.10:3. It can be seen that the mean estimates obtained from the right hemisphere were always greater than those from the left (Appendix 6, Fig.10:3). However, this difference was only statistically significant at 24 hours after training in the Control chicks (Paired t-test: $t=4.61$ ($df=5$) $p<0.05$), and

48 hours after training in the M-trained chicks (t=3.27 (df=5) $p<0.05$) (Table 10:4a). It was just outside the level of significance ($0.05>p<0.10$) at both 6 and 48 hours after training in the Control chicks. There was no significant difference between M-trained or Control chicks in either the left or right hemisphere, at any of the times examined after training (Table 10:4a). However, a two-way ANOVA (Table 10:4b) showed a significant effect of hemisphere on Nv_{ASh} at 48 hours after training ($F_{1,20}=11.55$, $p<0.05$). Both a one-way ANOVA (Table 10:7) and a two-way ANOVA (Table 8a,b) showed that the estimated mean values of Nv_{ASh} did not alter significantly with time after training. Hence training did not have a significant influence on Nv_{ASh} in either left (two-way ANOVA: $F_{1,50}=0.01$, $p>0.05$), or right hemisphere (two-way ANOVA: $F_{1,50}=0.00$, $p>0.05$). The observed hemispheric asymmetry, which is apparent in the graph of Nv_{ASh} with time (Fig.10:3), was confirmed statistically using a two-way ANOVA of the effects and time and hemisphere on the observed mean Nv_{ASh} (Table 10:8b). There was a significant hemisphere effect in both Control ($F_{1,50}=9.70$, $p<0.05$) and M-trained chicks ($F_{1,50}=7.33$, $p<0.05$). Mean values of Nv_{ASh} ± SEM are given in Appendix 6.

Symmetric spine synapses (Nv_{SSp}) : Fig.10:4 shows the mean values ± SEM of Nv_{SSp}, and these are also given in Appendix 6. It can be seen that the estimated means are relatively constant between both Control and M-trained, and between left and right hemisphere. This was confirmed using a one-way ANOVA on the observed values, which showed no statistical significance of time after training. Using a t-test to compare the mean Nv_{SSp}s , no significant differences were seen either between the hemispheres, or between M-trained and Control chicks (Tables 10:5a). A series of two-way ANOVAs showed no significant effects due to hemisphere or training and no significant interaction of these effects (Table 10:5b) at any given time after training. When the data from each time

period was pooled and analysed using a two-way ANOVA (Table 10:8a,b), no significant effects of either time and training (Table 10:8a) or time and hemisphere (Table 10:8b) were seen. There was also no significant interaction of these effects on the mean values of Nv_{SSp} observed.

Symmetric shaft synapses (Nv_{SSh}) : The results of mean ± SEM density of symmetric shaft synapses are shown in Fig.10:5, and are also given in Appendix 6. The graph shows no clear pattern of change in Nv_{SSh} following training. Indeed, a one-way ANOVA shows no significant overall change in Nv_{SSh} with time after training, in either left or right hemisphere of Control or M-trained chicks (Table 10:7). A t-test was used to compare the estimated means between both Control and M-trained, and between left and right hemisphere (Table 10:6a). No statistically significant differences were found. A series of two-way ANOVA was used to test the effects of both training and hemisphere on the observed mean Nv_{SSh} values at various times after training (Table 10:6b), but this did not reveal any significant effects. When this data was pooled, and an ANOVA performed on the resultant groups, there were still no significant effects of time and hemisphere on the observed values (Table 10:8b), but training did have an effect on Nv_{SSh} in both left ($F_{1,50}=5.08$, $p<0.05$) and right hemisphere ($F_{1,50}=4.25$, $p<0.05$) (Table 10:8a).

Table 10:2a : T-tests of Mean Synaptic Density of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10		Paired t-test : df=5	
	Control versus M-trained		Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.87 (ns)	0.42 (ns)	0.73 (ns)	0.57 (ns)
6	0.10 (ns)	0.42 (ns)	0.82 (ns)	1.10 (ns)
12	1.41 (ns)	1.28 (ns)	1.64 (ns)	0.96 (ns)
24	3.02 (*)	1.66 (ns)	1.84 (ns)	1.11 (ns)
48	2.33 (*)	2.88 (*)	0.43 (ns)	0.67 (ns)

Results are given as t-values, see Appendix 6 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:2b : Two-way ANOVA of the Effects of Training and Hemisphere on Nv_{syn}

Hours After Training	Hemisphere	Training	Interaction
1	0.03 (ns)	0.14 (ns)	0.87 (ns)
6	2.38 (ns)	0.03 (ns)	0.11 (ns)
12	2.26 (ns)	3.63 (ns)	0.02 (ns)
24	4.98 (*)	10.72 (*)	0.73 (ns)
48	0.01 (ns)	13.69 (*)	0.75 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

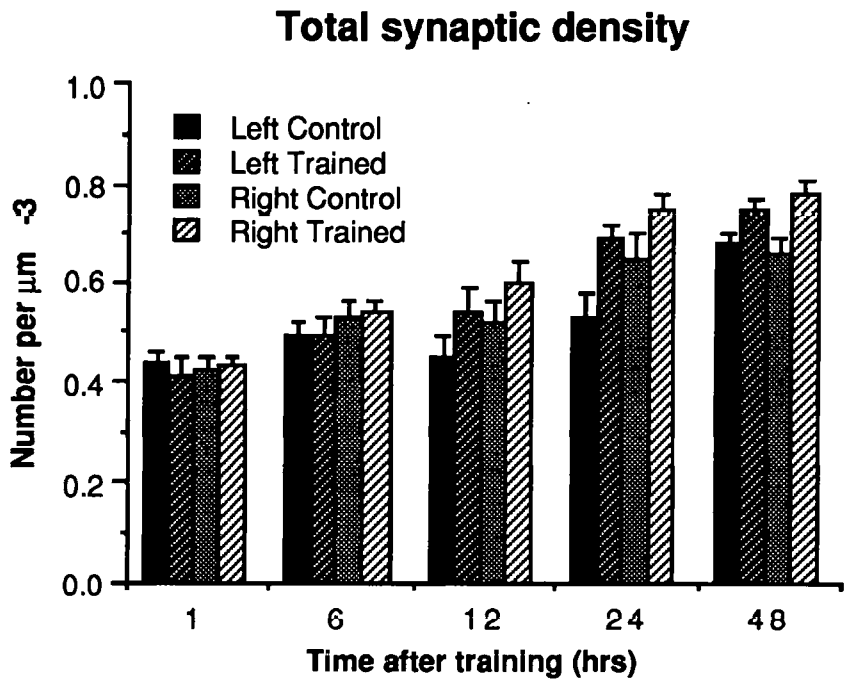


Fig.10:1 : Mean numerical density of synapses ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:3a : T-tests of Mean ASp Synaptic Density of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10		Paired t-test : df=5	
	Control versus M-trained		Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.39 (ns)	0.28 (ns)	0.89 (ns)	0.66 (ns)
6	0.23 (ns)	0.41 (ns)	0.12 (ns)	0.70 (ns)
12	0.84 (ns)	2.04 (ns)	0.14 (ns)	2.10 (ns)
24	1.63 (ns)	1.86 (ns)	0.79 (ns)	0.22 (ns)
48	1.14 (ns)	1.74 (ns)	0.92 (ns)	0.82 (ns)

Results are given as t-values, see Appendix 6 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:3b : Two-way ANOVA of the Effects of Training and Hemisphere on Nv_{ASp}

Hours After Training	Hemisphere	Training	Interaction
1	1.47 (ns)	0.23 (ns)	0.02 (ns)
6	0.07 (ns)	0.01 (ns)	0.20 (ns)
12	0.59 (ns)	4.41 (*)	1.02 (ns)
24	0.63 (ns)	5.46 (*)	0.26 (ns)
48	1.66 (ns)	3.90 (ns)	0.03 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

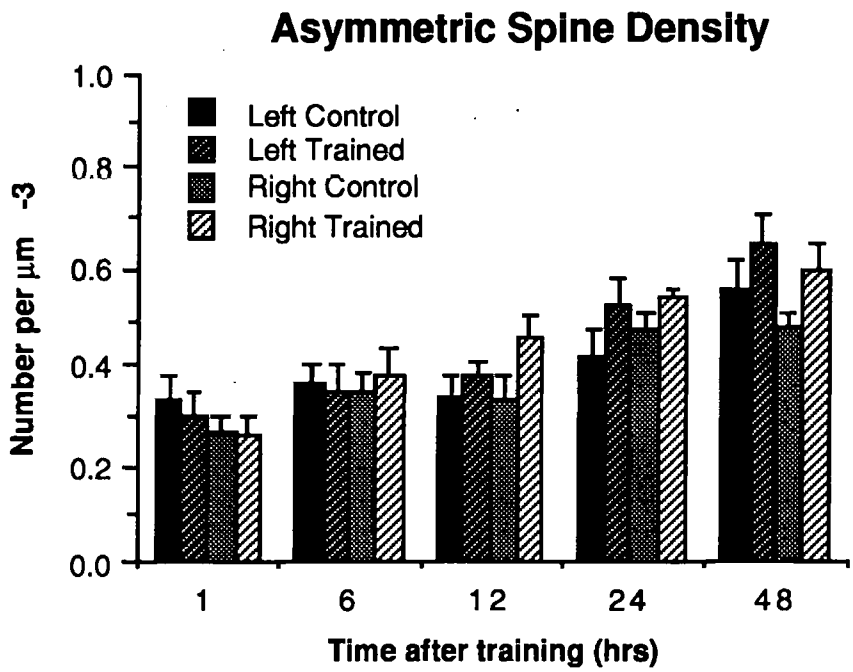


Fig.10:2 : Mean numerical density of asymmetric spine synapses ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:4a : T-tests of Mean ASh Synaptic Density of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10 Control versus M-trained		Paired t-test : df=5 Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.19 (ns)	0.16 (ns)	1.61 (ns)	0.58 (ns)
6	0.41 (ns)	0.31 (ns)	2.10 (ns)	1.42 (ns)
12	0.15 (ns)	0.48 (ns)	1.61 (ns)	0.80 (ns)
24	0.02 (ns)	0.18 (ns)	1.22 (ns)	1.07 (ns)
48	0.40 (ns)	0.76 (ns)	1.83 (ns)	2.01 (ns)

Results are given as t-vaiues, see Appendix 6 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:4b : Two-way ANOVA of the Effects of Training and Hemisphere on Nv_{ASh}

Hours After Training	Hemisphere	Training	Interaction
1	0.83 (ns)	0.01 (ns)	0.06 (ns)
6	3.78 (ns)	0.26 (ns)	0.00 (ns)
12	2.95 (ns)	0.02 (ns)	0.14 (ns)
24	2.80 (ns)	0.01 (ns)	0.02 (ns)
48	11.55 (*)	0.71 (ns)	0.11 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

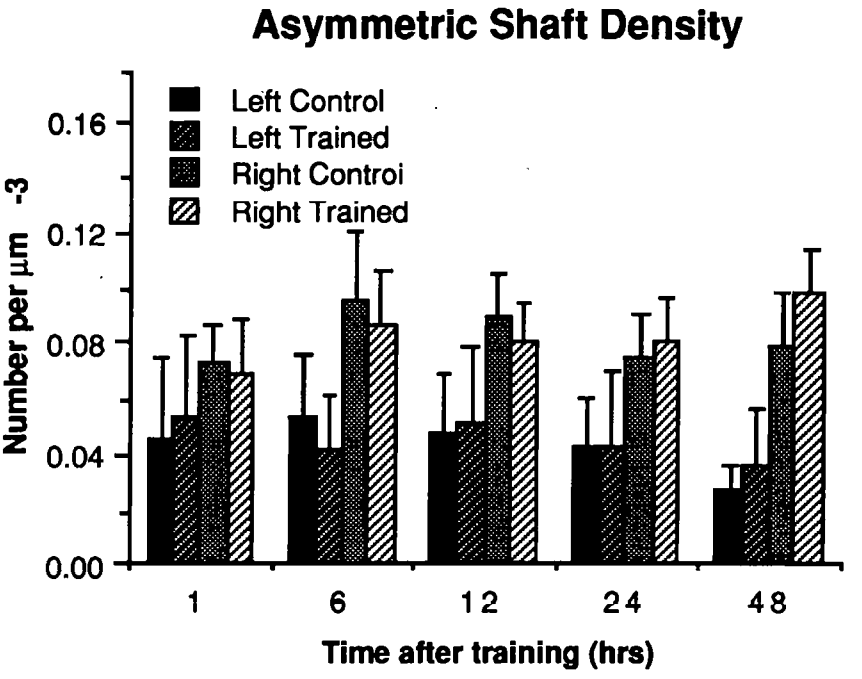


Fig.10:3 : Mean numerical density of asymmetric shaft synapses ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:5a : T-tests of Mean SSp Synaptic Density of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10		Paired t-test : df=5	
	Control versus M-trained		Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.76 (ns)	0.58 (ns)	0.30 (ns)	0.76 (ns)
6	0.19 (ns)	0.99 (ns)	0.05 (ns)	1.06 (ns)
12	0.45 (ns)	0.56 (ns)	0.18 (ns)	0.57 (ns)
24	1.31 (ns)	0.44 (ns)	0.26 (ns)	0.94 (ns)
48	0.05 (ns)	0.67 (ns)	0.17 (ns)	0.83 (ns)

Results are given as t-values, see Appendix 6 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:5b : Two-way ANOVA of the Effects of Training and Hemisphere on Nv_{SSp}

Hours After Training	Hemisphere	Training	Interaction
1	0.24 (ns)	0.03 (ns)	0.91 (ns)
6	0.49 (ns)	0.25 (ns)	0.62 (ns)
12	0.22 (ns)	0.01 (ns)	0.51 (ns)
24	0.13 (ns)	1.70 (ns)	0.59 (ns)
48	0.60 (ns)	0.16 (ns)	0.23 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

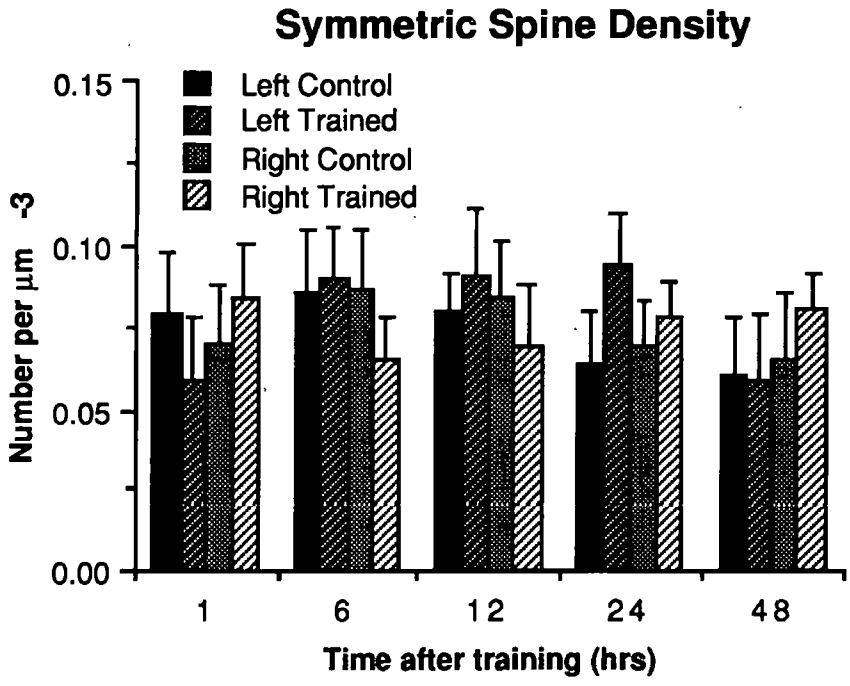


Fig.10:4 : Mean numerical density of symmetric spine synapses ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:6a : T-tests of Mean SSh Synaptic Density of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10 Control versus M-tralned		Paired t-test : df=5 Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	1.15 (ns)	1.15 (ns)	0.51 (ns)	0.51 (ns)
6	1.16 (ns)	1.88 (ns)	0.89 (ns)	1.81 (ns)
12	0.71 (ns)	1.29 (ns)	1.46 (ns)	0.86 (ns)
24	0.86 (ns)	0.84 (ns)	0.24 (ns)	0.30 (ns)
48	1.27 (ns)	0.45 (ns)	1.17 (ns)	0.68 (ns)

Results are given as t-values, see Appendix 6 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:6b : Two-way ANOVA of the Effects of Training and Hemisphere on Nv_{SSh}

Hours After Training	Hemisphere	Training	Interaction
1	0.78 (ns)	2.64 (ns)	0.01 (ns)
6	2.72 (ns)	2.04 (ns)	0.02 (ns)
12	2.18 (ns)	1.93 (ns)	0.12 (ns)
24	0.10 (ns)	1.45 (ns)	0.01 (ns)
48	2.21 (ns)	1.52 (ns)	0.37 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

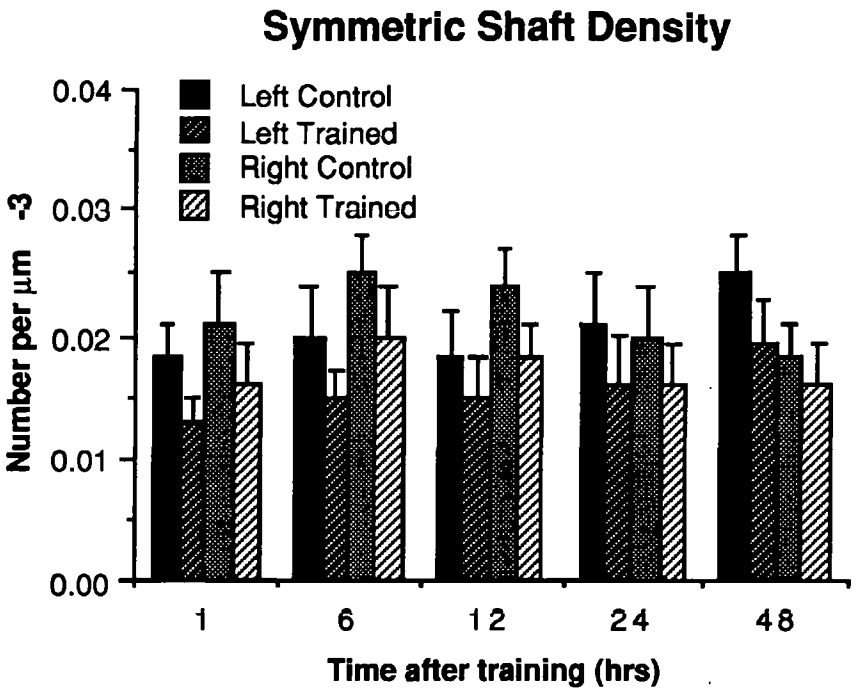


Fig.10:5 : Mean numerical density of symmetric shaft synapses ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:7 : One-way ANOVA of the Effects of Time after Training on the Numerical Density of Various Synaptic Types

	Control		M-trained	
Type	Left Hem	Right Hem	Left Hem	Right Hem
All	8.14 (*)	8.29 (*)	17.91 (*)	24.21 (*)
ASp	3.65 (*)	6.00 (*)	9.02 (*)	9.87 (*)
ASh	0.21 (ns)	0.28 (ns)	0.09 (ns)	0.35 (ns)
SSp	0.43 (ns)	0.30 (ns)	0.94 (ns)	0.29 (ns)
SSh	0.60 (ns)	0.78 (ns)	0.49 (ns)	0.47 (ns)

Results are given as F-values, df=4,25

* - significant at $p<0.05$, ns - not significant

Table 10:8a : Two-way ANOVA of the Effects of Time and Training on the Numerical Density of Various Synaptic Types

Type		Time df=4,50	Training df=1,50	Interaction df=4,50
All	Left	23.26 (*)	6.78 (*)	2.72 (*)
	Right	28.34 (*)	8.96 (*)	0.94 (ns)
ASp	Left	11.68 (*)	1.86 (ns)	0.85 (ns)
	Right	15.27 (*)	6.40 (*)	1.08 (ns)
ASh	Left	0.21 (ns)	0.01 (ns)	0.07 (ns)
	Right	0.41 (ns)	0.00 (ns)	0.23 (ns)
SSp	Left	0.89 (ns)	0.19 (ns)	0.53 (ns)
	Right	0.03 (ns)	0.00 (ns)	0.56 (ns)
SSh	Left	1.07 (ns)	5.08 (*)	0.03 (ns)
	Right	1.17 (ns)	4.25 (*)	0.10 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

Table 10:8b : Two-way ANOVA of the Effects of Time and Hemisphere on the Numerical Density of Various Synaptic Types

Type		Time df=4,50	Hemisphere df=1,50	Interaction df=4,50
All	Control	14.80 (*)	2.65 (ns)	1.64 (ns)
	M-trained	41.05 (*)	4.50 (*)	0.18 (ns)
ASp	Control	8.37 (*)	0.54 (ns)	0.67 (ns)
	M-trained	18.07 (*)	0.01 (ns)	0.72 (ns)
ASh	Control	0.38 (ns)	9.70 (*)	0.11 (ns)
	M-trained	0.03 (ns)	7.33 (*)	0.33 (ns)
SSp	Control	0.67 (ns)	0.03 (ns)	0.06 (ns)
	M-trained	0.31 (ns)	0.08 (ns)	1.08 (ns)
SSh	Control	0.21 (ns)	0.27 (ns)	1.16 (ns)
	M-trained	0.32 (ns)	0.75 (ns)	0.64 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

c) SYNAPTIC HEIGHT ($\bar{H}_{syn}$)

The data for $\bar{H}_{syn}$ estimations are summarized in Fig.10:6, and results of the statistical analysis of the data are presented in Tables 10:9a,b, 11, 12a,b. The mean values of $\bar{H}_{syn}$ ± SEM are given in Appendix 7. A comparison of the mean values of $\bar{H}_{syn}$ for Control and M-trained chicks (T-test) showed that there was a significant difference in the left hemisphere LPO at 48 hours after training ($t=2.32$ ($df=10$) $p<0.05$) (Table 10:9a). There was no statistically significant difference in the right hemisphere, at this or any earlier time. Fig.10:6 shows that this difference is due to an increase in $\bar{H}_{syn}$ in the left hemisphere of M-trained chicks. The mean synaptic height in the LPO of Control chicks is similar in both hemispheres (Table 10:9a). However, there is an asymmetry in the LPO of M-trained chicks in which the left hemisphere synapses are larger than the right (paired t-test: $t=3.36$ ($df=5$) $p<0.05$) (Table 10:9a). A two-way ANOVA did not find any significant effect of either hemisphere or training at any given time after training (Table 10:9b). When the data was pooled, and analyzed using a two-way ANOVA (Table 10:12a,b), significant effects of both time after training in left and right hemisphere LPO, and in Control and M-trained chicks. This suggests that the synaptic height does increase slightly during the period of study in the Control chicks, although no significant differences could be detected using a one-way ANOVA to investigate changes in $\bar{H}_{syn}$ with time after training (Table 10:11). The two-way ANOVA also showed that training influenced synaptic size in the left hemisphere LPO ($F_{1,50}=9.18$, $p<0.05$) (Table 10:12a), but not in the right hemisphere (Table 10:12a). This was confirmed in another two-way ANOVA, which tested the influence of time after training and hemisphere on the observed values of $\bar{H}_{syn}$ (Table 10:12b). A significant hemisphere effect was seen in the M-trained chicks only ($F_{1,50}=7.29$, $p<0.05$). There was no interaction of any of the effects stated above.

Table 10:9a : T-tests of Mean Synaptic Height of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10		Paired t-test : df=5	
	Control versus M-trained		Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.37 (ns)	0.41 (ns)	0.54 (ns)	0.16 (ns)
6	0.44 (ns)	0.36 (ns)	0.25 (ns)	1.11 (ns)
12	2.02 (ns)	0.76 (ns)	0.16 (ns)	1.18 (ns)
24	2.11 (ns)	0.48 (ns)	0.34 (ns)	1.39 (ns)
48	2.32 (*)	0.33 (ns)	0.18 (ns)	3.36 (*)

Results are given as t-values, see Appendix 7 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:9b : Two-way ANOVA of the Effects of Training and Hemisphere on $\bar{H}_{syn}$

Hours After Training	Hemisphere	Training	Interaction
1	0.06 (ns)	0.00 (ns)	0.30 (ns)
6	1.01 (ns)	0.00 (ns)	0.32 (ns)
12	0.45 (ns)	3.87 (ns)	0.79 (ns)
24	2.30 (ns)	3.29 (ns)	1.28 (ns)
48	2.36 (ns)	3.20 (ns)	1.67 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

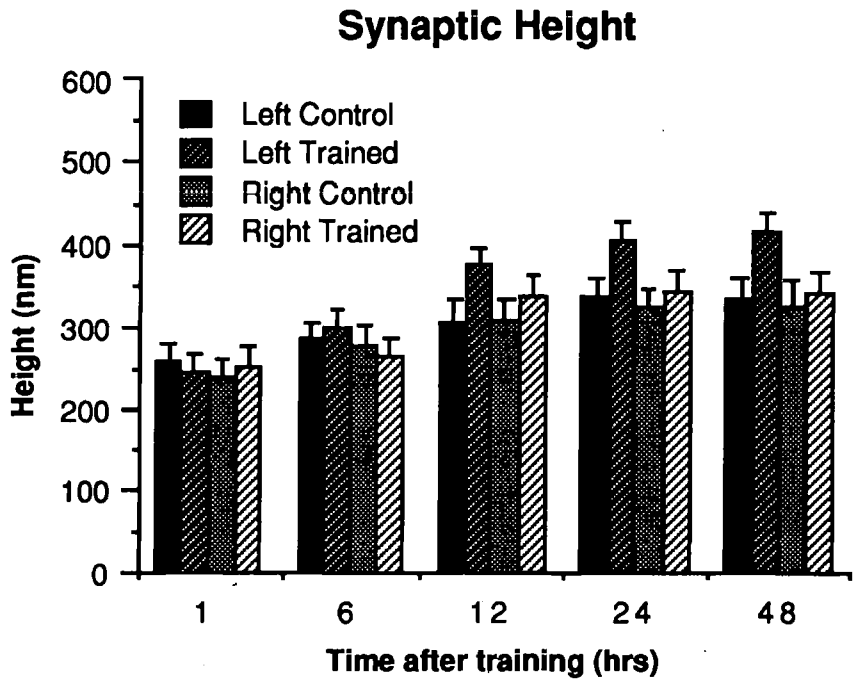


Fig.10:6 : Mean projected synaptic height ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

d) SYNAPTIC SURFACE AREA (Sa_{syn})

The mean values of synaptic contact surface area are shown in Fig.10:7, and are given, together with their SEMs in Appendix 7. Statistical analyses are summarized in Tables 10:10a,b, 11, 12a,b. A comparison of the estimated mean Sa_{syn} of Control and M-trained chicks, at various times following training, was made using a t-test. No significant differences were seen in either left or right hemisphere (Table 10:10a). Although the estimates of Sa_{syn} were similar between the hemispheres in the Control chicks, the M-trained chicks displayed a hemispheric asymmetry in Sa_{syn} at 48 hours after training ($t=5.79$ ($df=5$) $p<0.05$) (Table 10:10a). This was due to an increase in the mean value of Sa_{syn} in the left hemisphere of M-trained chicks (Fig.10:7). These results clearly mimic the pattern observed for changes in the mean $\bar{H}_{syn}$ in M-trained chicks. This is what one would expect, since both $\bar{H}_{syn}$ and Sa_{syn} are measures of

synaptic size. A two-way ANOVA was performed using the values obtained for each time after training, in order to test whether hemisphere or training had any influence on the observed values, but there were no significant effects found (Table 10:10b). The interaction of the effects was also not significant. The increase in Sa_{syn} observed in the left hemisphere of M-trained chicks at 48 hours after training, was only slight, since no detectable overall increase was seen using a one-way ANOVA to examine the effect of time on the observed values (Table 10:11). This lack of change with time was also observed using a two-way ANOVA to examine the effects of time and training (Table 10:12a) and time and hemisphere (Table 10:12b). These tests showed however, that there was a detectable effect of training in the left hemisphere LPO ($F_{1,50}$=8.01, $p<0.05$) (Table 10:12a), and that a resultant hemispheric asymmetry was present in M-trained chicks ($F_{1,50}$=4.74, $p<0.05$) (Table 10:12b).

Table 10:10a : T-tests of Mean Synaptic Surface Area of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

	Unpaired t-test : df=10 Control versus M-trained		Paired t-test : df=5 Left Hem versus Right Hem	
	Left Hem	Right Hem	Control	M-trained
1	0.43 (ns)	0.93 (ns)	0.63 (ns)	0.20 (ns)
6	0.68 (ns)	0.63 (ns)	0.33 (ns)	1.17 (ns)
12	1.39 (ns)	1.08 (ns)	0.68 (ns)	1.27 (ns)
24	1.72 (ns)	0.60 (ns)	0.72 (ns)	1.51 (ns)
48	2.10 (ns)	0.55 (ns)	0.59 (ns)	5.79 (*)

Results are given as t-values, see Appendix 7 for means ± SEM

* - significant at $p<0.05$, ns - not significant

Table 10:10b : Two-way ANOVA of the Effects of Training and Hemisphere on Sa_{syn}

Hours After Training	Hemisphere	Training	Interaction
1	0.31 (ns)	0.92 (ns)	0.11 (ns)
6	0.01 (ns)	0.37 (ns)	0.87 (ns)
12	3.06 (ns)	2.39 (ns)	0.09 (ns)
24	0.63 (ns)	0.16 (ns)	2.68 (ns)
48	3.49 (ns)	0.02 (ns)	1.15 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

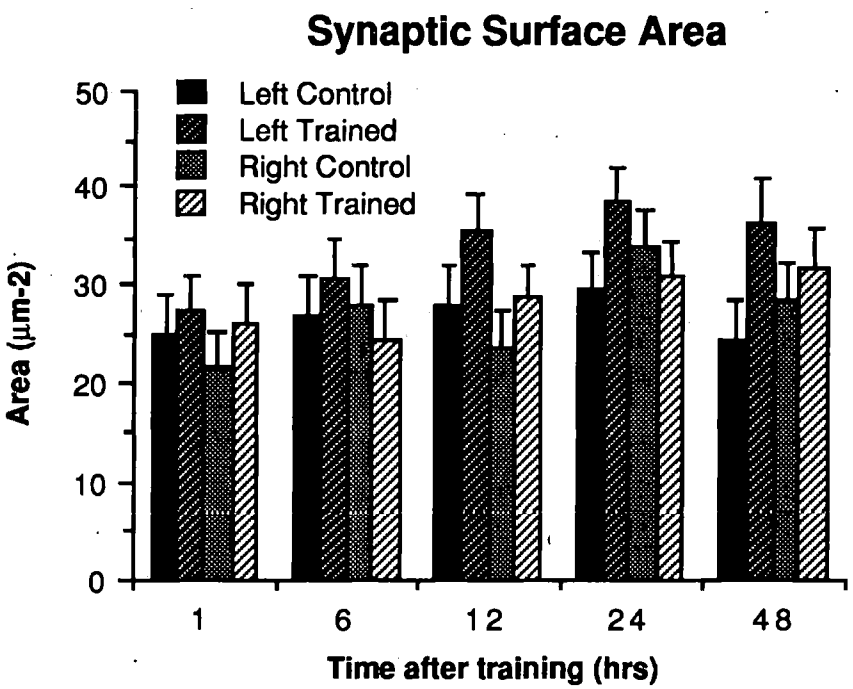


Fig.10:7 : Mean synaptic contact surface area ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:11 : One-way ANOVA of the Effects of Time after Training on Synaptic Size

	Control		M-trained	
Type	Left Hem	Right Hem	Left Hem	Right Hem
$\bar{H}_{syn}$	1.83 (ns)	2.44 (ns)	10.43 (*)	3.05 (*)
Sa_{syn}	0.31 (ns)	1.63 (ns)	1.43 (ns)	0.66 (ns)

Results are given as F-values, df=4,25

* - significant at $p<0.05$, ns - not significant

Table 10:12a : Two-way ANOVA of the Effects of Time and Training on Synaptic Size

Group		Time df=4,50	Training df=1,50	Interaction df=4,50
$\bar{H}_{syn}$	Left	10.09 (*)	9.18 (*)	1.56 (ns)
	Right	5.36 (*)	0.53 (ns)	0.17 (ns)
Sa_{syn}	Left	1.24 (ns)	8.01 (*)	0.51 (ns)
	Right	1.66 (ns)	0.32 (ns)	0.65 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

Table 10:12b : Two-way ANOVA of the Effects of Time and Hemisphere on Synaptic Size

Group		Time df=4,50	Hemisphere df=1,50	Interaction df=4,50
$\bar{H}_{syn}$	Control	4.19 (*)	0.25 (ns)	0.06 (ns)
	M-trained	11.72 (*)	7.29 (*)	0.86 (ns)
Sa_{syn}	Control	1.34 (ns)	0.02 (ns)	0.57 (ns)
	M-trained	1.89 (ns)	4.74 (*)	0.24 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

e) SYNAPTIC BOUTON VOLUME ($\bar{V}v_{bouton}$)

The data of mean volume-weighted mean volume of synaptic boutons is summarized in Fig.10:8, and is detailed in Appendix 8. Statistical analysis of the data is presented in Tables 10:13,16,17,18a,b. A comparison of the estimated mean values of $\bar{V}v_{bouton}$ between the hemispheres was made using a paired t-test. No significant differences were found between the hemispheres of either Control chicks or M-trained chicks (Table 10:16). There was no significant alteration in $\bar{V}v_{bouton}$ in either hemisphere, at any time after training, when M-trained chicks were compared with Controls (t-test) (Table 10:16). A two-way ANOVA was used to test the effect of hemisphere and training on the observed values of $\bar{V}v_{bouton}$, but did not detect any significant effects (Table 10:13). However, a one-way ANOVA did show a significant change in $\bar{V}v_{bouton}$ with time, in both left ($F_{4,25}=4.61$, $p<0.05$) and right hemisphere ($F_{4,25}=6.02$, $p<0.05$) of M-trained chicks, but not of Controls (Table 10:17), suggesting that some effect of training on $\bar{V}v_{bouton}$ was evident. A two-way ANOVA was used to analyse the effects of time and training on the observed mean values of $\bar{V}v_{bouton}$, but was unable to detect any direct effect of training (Table 10:18a). There was however, a significant effect of time after training in the left hemisphere LPO ($F_{2,30}=6.65$, $p<0.05$), but not the right (Table 10:18a). Time had an effect on M-trained chicks ($F_{2,30}=10.38$, $p<0.05$) rather than Controls (Table 10:18b). There was no evidence to support the hypothesis that there was a difference in mean $\bar{V}v_{bouton}$ between the hemispheres, or that $\bar{V}v_{bouton}$ was affected differently in the hemispheres following training (two-way ANOVA: Table 10:18b).

Table 10:13 : Two-way ANOVA of the Effects of Training and Hemisphere on $\bar{V}v_{bouton}$

Hours After Training	Hemisphere	Training	Interaction
1	0.00 (ns)	0.10 (ns)	1.11 (ns)
24	1.51 (ns)	4.16 (ns)	0.05 (ns)
48	0.92 (ns)	3.56 (ns)	0.07 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

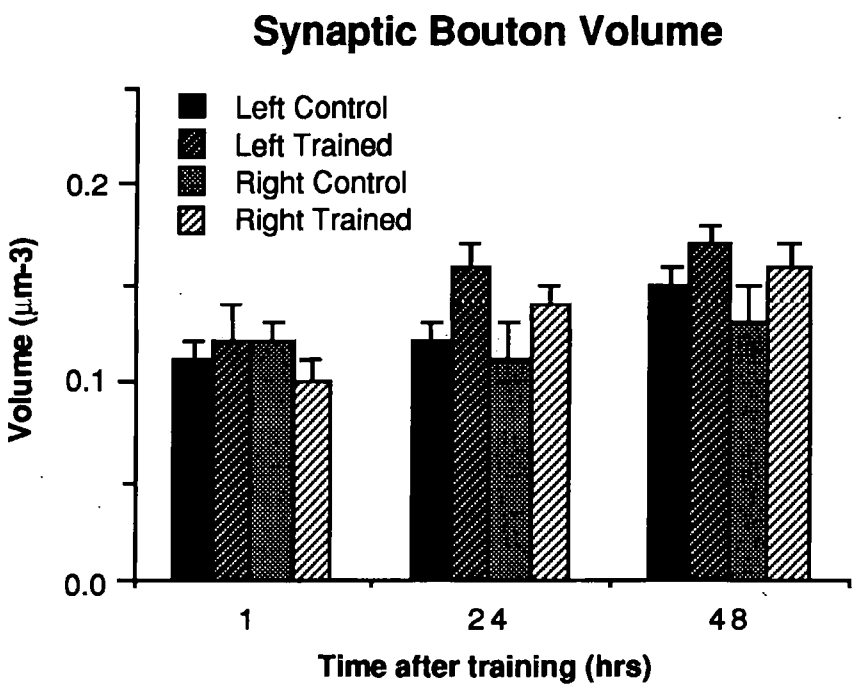


Fig.10:8 : Mean volume-weighted mean volume of synaptic boutons ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

f) DENDRITIC SPINE VOLUME ($\bar{V}v_{spine}$)

A graph of the mean ± SEM $\bar{V}v_{spine}$ for both M-trained and Control chick LPO is shown in Fig.10:9. The data can also be found in Appendix 8. The mean estimate of $\bar{V}v_{spine}$ was consistently greater in the M-trained chicks than in the Controls, although in the right hemisphere LPO, the difference was negligible. When the difference in means between the two groups was tested using a t-test, a significance was observed at 48 hours after training in the left hemisphere only ($t=2.46$ ($df=10$) $p<0.05$) (Table 10:16). A two-way ANOVA of the data from LPOs obtained 48 hours after training, did not provide evidence for a significant effect of training on the observed values of $\bar{V}v_{spine}$ at this, or any other time (Table 10:14). Nor could this test detect any difference in the hemispheres in this respect (Table 10:14). Similarly, a one-way ANOVA failed to show any significant change in the estimates of $\bar{V}v_{spine}$ with time following training, in either of the hemispheres of Control or M-trained chicks (Table 10:17). However, a two-way ANOVA which tested the effects of both time and training on the observed values, showed significance in both cases, but in the left hemisphere only (Time: $F_{2,30}=3.66$, $p<0.05$, Training: $F_{1,30}=12.97$, $p<0.05$) (Table 10:18a).

Table 10:14 : Two-way ANOVA of the Effects of Training and Hemisphere on $\overline{V}v_{Spine}$

Hours After Training	Hemisphere	Training	Interaction
1	0.22 (ns)	3.39 (ns)	0.81 (ns)
24	0.69 (ns)	2.17 (ns)	0.08 (ns)
48	0.44 (ns)	3.64 (ns)	1.94 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

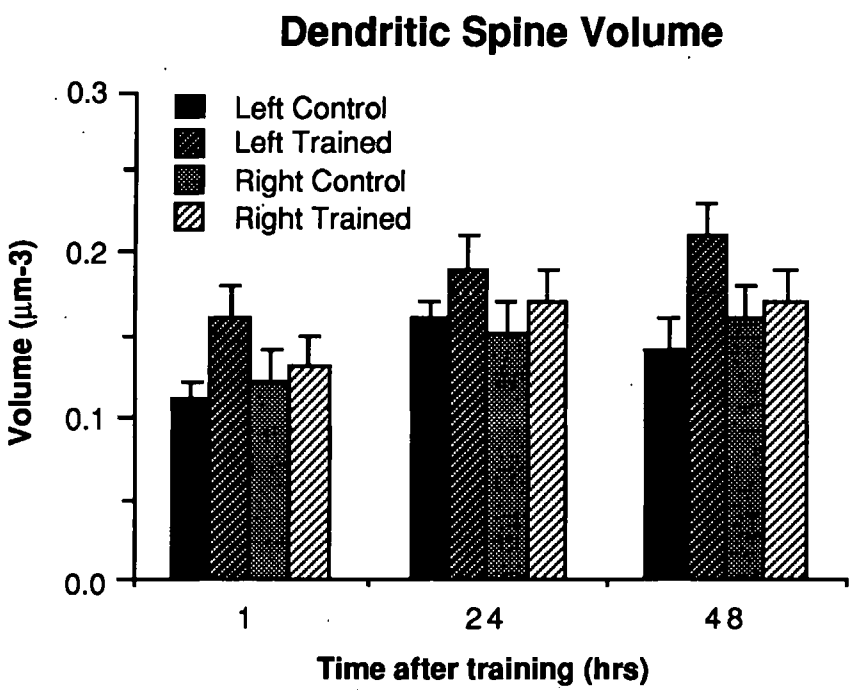


Fig.10:9 : Mean volume-weighted mean volume of dendritic spines ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

g) DENDRITIC SHAFT VOLUME ($\bar{V}v_{shaft}$)

The data for $\bar{V}v_{shaft}$ is summarized in Fig.10:10, and the means ± SEM are given in Appendix 8. T-tests were used to compare mean values from left and right hemispheres of both Control and M-trained chicks, the results of which are presented in Table 10:16. A series of one-way and two-way ANOVAs were performed, and the results are presented in Tables 10:15, 10:17,10:18a, and 10:18b. It is clear from Fig.10:10 that $\bar{V}v_{shaft}$ is not significantly affected by training, and does not appear to change much during the 48 hour period. This is confirmed using a variety of statistical tests (Tables 10:15, 10:16, 10:17, 10:18a, 10:18b), in which no significant differences or effects could be found. Hence one may conclude that $\bar{V}v_{shaft}$ is relatively stable in magnitude both during early post-hatch development and following a passive avoidance learning task.

Table 10:15 : Two-way ANOVA of the Effects of Training and Hemisphere on $\bar{V}v_{Shaft}$

Hours After Training	Hemisphere	Training	Interaction
1	2.19 (ns)	0.84 (ns)	0.00 (ns)
24	0.93 (ns)	0.93 (ns)	0.20 (ns)
48	0.45 (ns)	0.33 (ns)	1.46 (ns)

Results are given as F-values, df=1,20

* - significant at $p<0.05$, ns - not significant

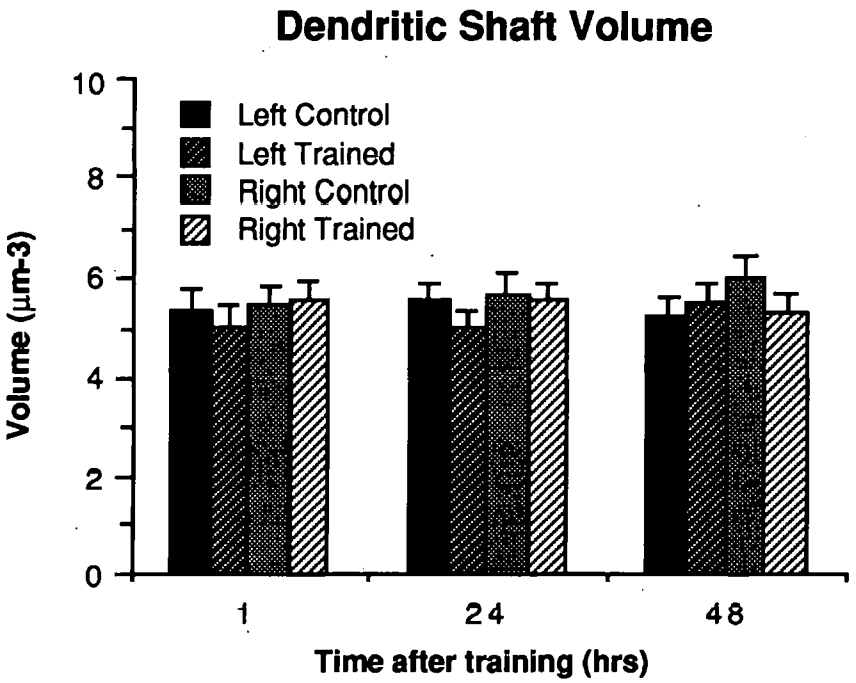


Fig.10:10 : Mean volume-weighted mean volume of dendritic shafts ± SEM of left and right hemisphere LPO, from both M-trained and control chicks at selected intervals following a passive avoidance learning procedure. Each estimate is based on results from 6 chicks.

Table 10:16 : T-tests of Mean Volume-Weighted Mean Volume of Various Neuronal Elements of Control vs M-trained and Left versus Right hemisphere LPO at Various Times following Training.

		Unpaired t-test : df=10 Control versus M-trained		Paired t-test : df=5 Left Hem versus Right Hem	
		Left Hem	Right Hem	Control	M-trained
Bouton	1	0.51 (ns)	0.81 (ns)	1.02 (ns)	0.78 (ns)
	24	1.90 (ns)	1.13 (ns)	0.82 (ns)	1.06 (ns)
	48	1.19 (ns)	1.47 (ns)	1.18 (ns)	0.50 (ns)
Spine	1	2.10 (ns)	0.62 (ns)	0.28 (ns)	0.98 (ns)
	24	1.58 (ns)	0.72 (ns)	0.37 (ns)	1.03 (ns)
	48	2.46 (*)	0.35 (ns)	0.59 (ns)	1.15 (ns)
Shaft	1	0.62 (ns)	0.67 (ns)	0.75 (ns)	1.32 (ns)
	24	1.02 (ns)	0.36 (ns)	0.32 (ns)	1.30 (ns)
	48	0.46 (ns)	1.24 (ns)	2.07 (ns)	0.53 (ns)

* - significant at $p<0.05$, ns - not significant

Results are given as t-values, see Appendix 8 for means ± SEM

Table 10:17 : One-way ANOVA of the Effects of Time after Training on Volume-Weighted Mean Volumes

	Control		M-trained	
Type	Left Hem	Right Hem	Left Hem	Right Hem
Bouton	2.41 (ns)	0.31 (ns)	4.61 (*)	6.02 (*)
Spine	2.55 (ns)	1.00 (ns)	1.91 (ns)	0.87 (ns)
Shaft	0.15 (ns)	0.16 (ns)	0.52 (ns)	0.17 (ns)

Results are given as F-values, df=4,25

* - significant at $p<0.05$, ns - not significant

Table 10:18a : Two-way ANOVA of the Effects of Time and Training on Volume-Weighted Mean Volumes

Group		Time df=2,30	Training df=1,30	Interaction df=2,30
Bouton	Left	6.65 (*)	4.12 (ns)	0.39 (ns)
	Right	2.72 (ns)	1.17 (ns)	1.67 (ns)
Spine	Left	3.66 (*)	12.97 (*)	0.71 (ns)
	Right	1.84 (ns)	0.92 (ns)	0.03 (ns)
Shaft	Left	0.10 (ns)	0.38 (ns)	0.56 (ns)
	Right	0.08 (ns)	1.80 (ns)	0.25 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

Table 10:18b : Two-way ANOVA of the Effects of Time and Hemisphere on Volume-Weighted Mean Volumes

Group		Time df=2,30	Hemisphere df=1,30	Interaction df=2,30
Bouton	Control	1.55 (ns)	0.24 (ns)	0.65 (ns)
	M-trained	10.38 (*)	2.11 (ns)	0.11 (ns)
Spine	Control	2.91 (ns)	0.10 (ns)	0.25 (ns)
	M-trained	2.42 (ns)	3.33 (ns)	0.22 (ns)
Shaft	Control	0.01 (ns)	2.70 (ns)	0.31 (ns)
	M-trained	0.04 (ns)	0.77 (ns)	0.63 (ns)

Results are given as F-values,

* - significant at $p<0.05$, ns - not significant

10.4 : DISCUSSION

This study has shown specific evidence of changes in the numerical density and size of synapses, and the volume-weighted mean volume of dendritic spines in the chick LPO following memory formation. In addition, it describes the time-course over which such changes take place. Previous studies by other workers, using a variety of animal models, have also shown that the synapse is particularly liable to such changes following sensory experience or environmental stimulation (for review, see Greenough and Chang 1985). The data presented here also show similarities to synaptic plasticity that occur as a consequence of training in both vertebrates (Vrensen and Nunes-Cardoso 1981) and invertebrates (Bailey and Chen 1990). The significance of such plastic change is uncertain, although it clearly must have a profound effect on the functioning of neuronal connectivity.

The validity of much of the previous work in this field comes into question, since the quantitative methods used by the majority of other workers, are highly biased. This is because previous numerical estimates often relied upon

assumptions regarding synaptic shape and/or size (see Chapter 7 for details). If training results in an alteration in the size or complexity of synapses, and/or changes in their shape, then this may lead to over-estimation of synaptic counts, using traditional counting procedures. In future, unbiased stereological methods will become the only acceptable ones for the estimation of number, size and volume. However, a wealth of data obtained using biased methods has already bombarded the scientific literature, and one must take many of the conclusions reached, at face value.

A second criticism of previous studies in this field, is that synapses have in general, been regarded as a single entity, and hence are assumed to have a single and unified function (Bailey and Chen 1989a, Aghajanian and Bloom 1967, DeGroot and Vrensen 1978). This is clearly not so, since the synapses may vary in both neurotransmitter content and have substantially differing targets. Thus the locality and transmitter may influence the effect on a particular post-synaptic neuron. Synapses can be classified into multiple types (Gray 1959, Hassler *et al* 1978), and these different types may constitute different functional forms. One advantage of quantifying changes in different synaptic types, as in the present study, is that one can assess changes in existing synapses, e.g. changes in the post-synaptic target from shaft to spine or *vice-versa*. This would not be possible using standard methodologies.

A criticism of the present study, is that no account has been taken of the changes in the neuronal population following training. The number of synapses per neuron may be a more important parameter than synaptic number alone. It is known that in the chick IMHV, the numbers of neurons decrease over the first few days of post-hatch life (Curtis *et al* 1989). If this is also true for the LPO,

then the synapse to neuron ratio would show even greater differences than synapse number alone. Quantification of neuronal changes following PAL in the chick LPO must however, remain a priority for future studies.

In the present study, estimations of numerical density estimations were made rather than absolute number. It was necessary therefore, to determine whether the volume in which these density estimates were made, remained stable after the memory task has been performed. This was confirmed by examining the LPO volume in both Control and M-trained chicks, at various intervals after training. The results clearly showed that there was no significant alteration in volume as a consequence of learning (Table 10:1a). The most accurate method of determining number changes in a given region, is by estimating absolute number for that brain region, either by direct counts or more usually, by sampling. This can only be achieved by including a measurement of the volume of that region, for the brain in which numerical density measurements are obtained. However, this was not possible in the present experiment, due to the desire to keep the IMHV intact. As second best therefore, volume estimations were made on brains of chicks reared and trained in identical conditions to those used for quantitative morphological analysis. Since there were no significant alterations in LPO volume of M-trained chicks compared to Controls, comparisons of numerical density are considered justified.

Total synaptic density (Nv_{syn})

The estimated mean synaptic densities of the Control group are comparable to the estimates obtained previously for the 1-day old group in the developmental study (Chapter 9, Fig.9:1). Hence, the technique is shown to yield consistent results. The first few days of post-hatch life are a period of intense synaptic

modification in the LPO. New synapses are being formed at a very high rate of synthesis, since the Control chicks display significant increases in Nv_{syn} in both left and right hemispheres (Table 10:7). Training with methyl anthranilate however, causes an even greater synthesis. It is not possible to say whether these changes are due to the process of memory formation, or due to some concomitant of the training experience, such as the taste of the bead. However, previous studies have shown that other morphological alterations in the chick IMHV are due to the memory of the taste alone (Patel *et al* 1988). Also biochemical changes in the chick brain following memory formation have been shown to be a direct consequence of memory, and not of some non-specific stimulus (Rose and Harding 1984). It has been shown for other species (e.g. rat), that non-specific environmental stimulation may cause an increase in synaptic number (Turner and Greenough 1985). Studies such as these are crucial to the present discussion, since without knowledge of non-specific effects, one cannot relate the observed changes to any given behaviour. The present results therefore, and their implied relevance to memory formation, are only valid on the assumption that the changes only occur in response to the formation of a memory. In the light of studies by Patel *et al* (1988) and Mason and Rose (1988), this assumption is reasonable. It is further given substance, by the lack of evidence of involvement of the LPO, in response to either gustatory or olfactory stimuli (see Chapter 3 (3.3 and 3.4)).

The observed increase in synaptic numerical density 24 hours following PAL has been reported previously for other models of memory processes. Synaptic number has been shown to be increased following LTP in the *dentate gyrus* of the rat hippocampus (Wenzel *et al* 1985, Desmond and Levy 1983, Desmond and Levy 1986), and in the hippocampus *in vitro* (Chang and Greenough 1984).

Similar findings have been shown to occur after a brightness discrimination task in rat hippocampus (Wenzel *et al* 1980). In this latter study, an increase in synaptic number of approximately 40% was seen in trained animals, in comparison with controls. Another large increase in synaptic number has been shown to occur in the *robustus archistriatalis* of the adult female canary following song-learning. These studies compare favourably with the large increases witnessed following PAL in the present study. The significance of the magnitude of the increase is uncertain. Clearly, each 'memory' cannot be associated with a large increase in synaptic number, otherwise total synaptic number would be of enormous magnitude following a second or future memory acquisition. Rather, the increases may be associated with generalized learning processes, which would subserve many different memories.

It is also clear from the present results, that an increase in synaptic numerical density persists until at least 48 hours after training. It is not known whether there is a subsequent reduction in number after this time. An increase in synaptic number in the invertebrate, *Aplysia* following sensitization to a tactile stimulus (Bailey and Chen 1983, Bailey and Chen 1990) was shown to be more permanent than a change in synaptic size. Indeed, synaptic number increases lasted as long as the behavioural retention of the memory, which was several weeks (Bailey and Chen 1989b).

The results from the present study and from those of other workers (Bradley *et al* 1985, Horn *et al* 1985, Stewart *et al* 1987), suggest that synaptic number increases following memory do not appear before 24 hours have elapsed since the training experience. This leads one to ask whether the synapse is capable of genesis before this time. A study by Lee *et al* (1980) showed that synapses in the rat hippocampus could be formed by 15 minutes following the

induction of LTP. Dendritic shaft and 'sessile' spine synapses were also formed within the rat hippocampus by a little as 10-15 minutes after a multi-stimulus treatment (lasting 6 minutes) prior to LTP formation (Chang and Greenough 1984). For synaptic modification to occur in such a rapid manner, initiation must take place locally along the axon, and not be directed by protein synthesis centrally.

In contrast, the IMHV of the chick brain, which has also been shown to be concerned with the process of memory formation, does not show any increase in synaptic number 24 hours following training (Stewart *et al* 1984). It is a feature therefore, that is not common to all areas of the brain concerned with memory formation. The possibility arises, that an increase in synaptic number is associated only with the long-term storage of memory. This hypothesis is supported by lesion studies which showed that the IMHV is only essential for the acquisition of memory for the avoidance task in chicks (Patterson *et al* 1989), whilst the LPO is necessary for its long-term storage (Gilbert *et al* in press). In *Aplysia* it has been shown that short-term sensitization is associated with an increase in active zone size, and synaptic vesicle complement (Bailey and Chen 1983), and long-term sensitization with an increase in synaptic number and pre-synaptic varicosities (Bailey and Chen 1989b). Thus, long-term memory as a generalized process, may be partially a consequence of lasting changes in synaptic number.

The present data show that there is no hemispheric difference in the estimated mean Nv_{syn} either between Control or M-trained chicks. Thus, the observed increases in Nv_{syn} are approximately equal in both hemispheres. This finding is in close agreement with the results of a previous investigation of

synaptic changes in the LPO following memory formation (Stewart *et al* 1987). This result is of particular interest, since it has been shown that unilateral lesions of the LPO do not result in amnesia for the taste avoidance paradigm (Gilbert *et al* in press). Only lesions placed bilaterally cause amnesia (Gilbert *et al* in press). This is in marked contrast to the chick IMHV, which displays many synaptic asymmetries (Stewart *et al* 1984). It has further been shown that lesion of the left IMHV, and not the right, causes amnesia for the taste of the bead (Patterson *et al* 1989).

The time required for significant changes in the density of synapses in the chick LPO, was shown to be 24 hours in the left hemisphere, and 48 hours in the right hemisphere (Table 10:2a). Whether the delay in the right hemisphere is of biological significance is uncertain. A previous study has shown a statistically significant increase in Nv_{syn} in the LPO in both hemispheres, 24 hours after passive avoidance training (Stewart *et al* 1987). However, the methods used in the study by Stewart *et al* were biased, since they relied on assumptions regarding synaptic shape. Despite this, their results are broadly in line with the present findings.

An early report by Stewart *et al* (1983) published data which contradicts their later findings (Stewart *et al* 1987), and hence also differs from the present findings. As stated previously (Section 4.3), these two studies make entirely opposite conclusions; namely that the former states that there is no statistically significant difference between trained and control chicks, with respect to synaptic density, whilst the latter states that there is. The former study provided estimations obtained directly on the electron microscope fluorescent screen, whilst the latter, using LPOs from a different group of birds,

employed the standard technique, in which estimations are made from electron-micrographs. Both studies used biased quantitative methods to make final estimates. There are substantial differences in the estimates between the two studies. The latter study reported a mean estimate of Nv_{syn} for the left hemisphere of Control chicks, which was 61% greater than that reported in the former study. Similarly, there was a difference of 64% in estimates of Nv_{syn} in the right hemisphere. This suggests that there was a procedural error in the first study (1983), possibly arising from inaccuracies of synaptic identification on the microscope screen. The latter study by Stewart *et al* (1987) employed a more accurate method of location of the LPO (Rose and Csillag 1986), and perhaps as a consequence, is more in line with the estimations of synaptic density made here.

Spine versus Shaft synapses

Changes in the pattern of connectivity of synapses upon the target neuron are important, in that changes in the locality of the synapse may play an important factor in achieving an alteration in synaptic efficacy (Landis 1985, Pongracz 1985). Many studies of synaptic number following learning and memory have failed to detect any changes (Vrensen and Nunes-Cardoso 1981, Stewart *et al* 1984, Horn *et al* 1985). However, since the numbers of synapses of different types were not counted, these studies were unable to comment upon the possibility of a shift in synaptic locality. Such alteration in synaptic target has been shown to occur in the primate visual cortex (Mates and Lund 1983a), and in the Zebra-Finch *ectostriatum* (Nixdorf 1989). In this latter study, axospinous synapses reduced in number from 40% of the total to 22%, whilst the numerical density of synapses remained constant. Changes in synaptic proportions may underlie important functional changes in the properties of the

action potential propagation, which may have significance for the efficacy of synaptic transmission.

There were no significant differences in the mean numerical density of asymmetric spine synapses in either the left or right hemisphere (Table 10:3a) between the LPOs of Control and M-trained chicks. The mean values correlate well with estimates made previously in the developmental study (Chapter 9, Fig.9:4a). A recent study has demonstrated an increase in spine density in the LPO of M-trained chicks in comparison to Controls (water-trained), using 3-dimensional reconstruction of Golgi impregnated tissue (Lowndes and Stewart 1990). These authors found a 13% increase in the left hemisphere and an 8% increase in the right hemisphere. Although the present results show estimated mean $Nv_{ASp}s$ for the LPOs of M-trained chicks which are 26% greater in the left, and 15% greater in the right hemisphere, these values are not statistically significant. Had this difference been significant, it would mimics the pattern observed for spine increases as shown in the study by Lowndes and Stewart, although the increased ratio of Nv_{ASp} in each hemisphere is exactly double the increase reported for dendritic spines. However, since the present estimates are not statistically significant, one cannot say with any degree of certainty whether the numerical density of synapses increases in direct correlation with that of dendritic spines. A two-way ANOVA showed the significant effects of training on the density of ASp synapses as early as 12 hours after training (Table 10:3b). This shows the capacity for rapid synaptic plasticity in response to the learning experience.

The present study could detect no significant differences between the hemispheres with respect to the estimated mean asymmetric shaft synaptic

densities. This is in keeping with the previous estimate for Nv_{ASh} in the developmental study (Fig.9:4b, Fig.9:10b). The estimates obtained for the Control chicks of this study, and for the 1-day old chicks in the developmental study (Chapter 9, Fig.9:1), are within the same order of magnitude.

The estimated mean Nv_{ASh} obtained for the left hemisphere LPO are consistently (though not significantly) lower than those obtained for the right hemisphere (Fig.10:3). This may be a real difference which I am unable to detect, given the degree of error with which the estimates are made. A similar finding was shown in the developmental study (Chapter 9), where both numerical density and absolute number estimates of ASh synapses were made. Neither of the estimates of chicks aged 1-day post-hatch, which is the nearest approximate age of chicks used in the present study, were significantly different.

One study of the rat hippocampus showed that there was an increase in the number of spine, but not shaft synapses, following LTP (Desmond and Levy 1983). A second study found exactly the opposite; ie. an increase in shaft synapses, but no overall synaptic number increase (Wenzel *et al* 1985). The results of asymmetric shaft synapses, which constitute the majority of shaft synapses in the chick LPO, provide evidence that suggests that this is not the case in the present study, since training did not have any effect on the density of asymmetric shaft synapses (Table 10:4b). However, the possibility exists that some synapses may have been transformed from shaft to spine synapses, whilst the numerical density of asymmetric shaft synapses was maintained by the synthesis of new asymmetric shaft synapses. This method of synaptic shift has been suggested (Stewart 1990) as a possible mechanism in the chick IMHV, to explain two conflicting reports of an increase in the density of dendritic spines (Patel and Stewart 1988), and a lack of change in the numerical density of

synapses (Stewart *et al* 1984). A decrease in the number of shaft synapses would result in an increase in the number of spine synapses. In a developmental study of the chick IMHV, it was shown that there was a significant increase in the density of spine synapses, but no corresponding increase or decrease in shaft synapses (Bradley 1985). Since this region is involved in memory processes (Rose and Csillag 1984), one might expect that these developmental changes would mimic the changes due to memory formation in the experimental model, using passive avoidance learning.

It is worth noting that the estimates obtained in the present study for asymmetric spine synapses, are over six times those obtained for asymmetric shaft synapses. This is in marked contrast to other regions of the chick brain, such as the IMHV, where the numerical densities of each type were approximately equal (Bradley 1985). The *hyperstriatum accessorium* was shown to have approximately twice as many spine synapses as shaft synapses (Bradley 1985). There is thus considerable differences in the ratio of shaft to spine synapses in different regions of the chick brain. In addition, the developmental study of the LPO (Chapter 9) revealed hemispheric differences in this ratio. It is not known to what function, if any, these differences relate.

Asymmetric versus Symmetric synapses

As was shown previously (Fig.9:5c), the number of symmetric spine synapses is small in comparison to the total number of synapses (<10%). Since symmetric synapses in the young avian brain lack any distinguishing features to differentiate them from asymmetric synapses, other than the appearance of the PSD (see Chapter 5 and Nixdorf 1989), an under-estimation of the true numerical density may have been made, as some symmetric synapses may have

been overlooked. However, similarly low estimates of the ratio of symmetric synapses to the total population is seen in the chick IMHV (Curtis *et al* 1989), and in the Zebra-Finch *ectostriatum* (Nixdorf 1989). Given the low number, and hence the extremely small numbers sampled using the methods described, it is perhaps difficult to detect small changes in the densities of these synapses following memory formation, should these exist. No such changes were detected in the present study. However, the above argument loses credibility when one examines the symmetric shaft density estimates. These synapses are equally low in number (Fig.9:5d), and yet a statistically significant effect of training is seen in both hemispheres, using a two-way ANOVA (Table 10:8a). These differences are not detectable however, at individual time-points using the less powerful t-test, as the statistical tool (Table 10:6a).

Mean projected synaptic height ($\bar{H}_{syn}$)

One mechanism whereby a pre-synaptic element may increase the probability of causing a post-synaptic cell to fire, is by increasing the efficacy of synaptic transmission between the two. This may be achieved in part, by increasing the size of the synaptic contact zone. This may increase the amount of neurotransmitter released at the site, since a greater number of vesicles can be accommodated at the pre-synaptic grid. Secondly, an increase in the post-synaptic density, would probably allow a greater density of receptors for that neurotransmitter to be located at the synaptic site. Such mechanisms may be involved in modifications of functioning of the synapse, and may underlie changes in synaptic efficacy that are thought to play a role in the formation of memory (Herrera *et al* 1985).

Synaptic height is an estimator of synaptic size, which makes no assumptions

about synaptic shape. Many previous estimates of synaptic size were based on biased methods which assumed that the synapse was disc-shaped (Curtis *et al* 1989, Devon and Jones 1981). Comparison of the estimations made using these two different methods is not possible, since different parameters are being measured in each case. In the present study, synaptic height refers to the mean projected height of the synapse perpendicularly between the two section planes. Its validity is dependent upon either a randomness of the synapses within the sample volume, or a randomness of the disector planes with which the sample is made. In previous studies, estimations were made of the length of the synaptic profile in the section plane, with correction for true diameter assuming a circular shape, using the unfolding method of Schwartz-Saltykov (for details, see Chapter 7, Section 2a).

Comparisons can be made however, with the estimates made previously in the developmental study (Chapter 9, Fig.9:12). The present results for Control chicks, are consistent with the results obtained for the 1-day chicks of the developmental study (Fig.9:12). Also in agreement with these results, is the lack of asymmetry in $\overline{H}_{syn}$ in Control chicks. A study by Stewart *et al* (1987) showed that synaptic length was approximately 10% greater in the right hemisphere of 2-day old Control chicks (water-trained). One possible explanation of this apparent discrepancy, is that perhaps the estimation of synaptic height, which although unbiased, is a much cruder measure of synaptic size, and is not sensitive enough to detect differences of this magnitude. An increase of $\overline{H}_{syn}$ in the order of approximately 22% by 48 hours after training in the left hemisphere of M-trained chicks, was significantly different to both the estimated mean $\overline{H}_{syn}$ of the right hemisphere of M-trained chicks, and left hemisphere of Control chicks (Table 10:9a).

The results described here show that synapses in the left hemisphere of the LPO undergo a substantial increase in size following training. This was the conclusion of Stewart *et al* (1987) comparing synaptic length measurements, although the results of this increase in size achieved a different outcome of significance. Stewart *et al* reported that there was no significant difference in synaptic length of Controls (water-trained) compared to M-trained chicks 24 hours later. This was due to an increase in synaptic length in the left hemisphere, since Control chicks had a hemispheric asymmetry of synaptic size, such that the right hemisphere was 10% larger than the left. Increases in synaptic size have also been reported following PAL (Bradley and Galal 1987) and following imprinting (Bradley *et al* 1981, Bradley *et al* 1985b) in synapses in the chick left IMHV. Other studies of the effects of imprinting on morphological characteristics of the synapse, have shown that the increase in length of the synapse in the left hemisphere of the chick IMHV, is restricted to those targeting onto dendritic spines (Horn *et al* 1985). The training paradigm which involved exposure to a flashing red light, showed that the effect of training on synaptic length, was dependent upon the time of exposure (20 minutes of training gave no significant differences, compared to dark-reared controls). The use of dark-reared chicks as a control group for this experiment gives some concern, since it has been shown that visual deprivation in young birds causes a decrease in synaptic length (Nixdorf 1990a), albeit in the *ectostriatum.* In addition, Nixdorf has shown that although both hemispheres are affected, they are so to different degrees. This may account for the significant hemispheric differences reported by Horn *et al* (1985). This is unlikely however, since other studies have reported similar findings for the chick IMHV, 2-3 days following visual imprinting (Petrova *et al* 1990).

The present study did not take any account of the post-synaptic target in measurements of $\bar{H}_{syn}$, although measurements were restricted to asymmetric synapses. An analysis of $\bar{H}_{syn}$ of particular classes of synapse in the present study may have been useful. If a similar situation to that of the chick IMHV exists in the LPO, (i.e. size increases restricted to the axospinous synapse) then a more efficient evaluation of size may have been made. Of course, one is limited by the bounds of time and other constraints.

Previous studies have shown that following imprinting there is an increase in the mean length of synapses in the left hemisphere of the IMHV of the chick (Bradley *et al* 1981, Bradley *et al* 1985b). Following passive avoidance learning, Bradley and Galal (1987) were able to demonstrate a similar increase in the mean length of synapses in the left IMHV. This increase in size was not seen in chicks who had been previously injected with anisomycin, a protein synthesis inhibitor (Bradley and Galal 1987). Protein synthesis is known to be essential for the formation of a long-term memory trace (Matthies 1982, Rosenzweig and Bennett 1984b).

In addition to work on the effects of learning and memory on synaptic size in the chick, there is evidence that synapses in other species undergo similar changes following memory formation. In the rat hippocampus, the PSD length increases following a brightness discrimination task (Wenzel *et al* 1980, Matthies 1989b). Sensitization in *Aplysia* also causes a larger mean PSD size (Bailey and Chen 1983). Further studies by these latter authors, has shown that the increase in mean size is transient, since it returned to a control level after 48 hours (Bailey and Chen 1989b). The increases in synaptic height found in the present study, persist until at least 48 hours after training, and do not show any

signs of regression at this time. However, it is not known whether there is a subsequent decline in size after this time.

Synaptic surface area (Sa_{syn})

Any increase in the size of the synapse would result in an increase in the area of membrane specialization associated with that synapse. Measurement of surface area can also be applied to investigations of changes in synaptic shape, since if a straight synapses becomes curved, its mean projected height (linear dimension) will be reduced. This can be detected by making an estimation of $\bar{H}_{syn}$. If on the other hand, the synapse maintains its linear dimension by growth whilst increasing in curvature, although measurements of $\bar{H}_{syn}$ would fail to detect such changes, measurement of the synaptic surface area would reveal substantial increases. Hence Sa_{syn} is a measure of synaptic size and synaptic shape.

In this study, an unbiased quantitative method was used to estimate the surface area available for synaptic chemical transmission. No statistically significant difference was found between the mean estimated Sa_{syn} in the LPOs of Control and M-trained chicks at any given time period following training, using a t-test as the statistical test (Table 10:10a). However, using the more powerful ANOVA, a significant effect of training was seen in the left hemisphere LPO (Table 10:12a). Comparing estimated means from both Control and M-trained chicks (Fig.10:7), one can see that this is due to an increase in the size of the synapses in the left hemisphere. This is in line with the results discussed previously for synaptic height ($\bar{H}_{syn}$), where it was shown that the mean estimated projected height of synapses in the left hemisphere was greater in M-trained chicks than in Controls, 48 hours after training.

One possible outcome of the increase in synaptic surface area in left hemisphere LPO synapses is an increase in the efficacy of those synapses (Herrera *et al* 1985). An increase in the area of membrane specialization available for synaptic transmission, is likely to result in an increase in the probability that the post-synaptic target will be depolarized beyond threshold. This particular capacity for an increase in synaptic efficacy was speculated by Hebb (1949) as being a probable mechanism of synaptic plasticity associated with learning and memory.

Only one previous study in the field of learning and memory, has chosen to investigate changes in synaptic surface area (Desmond and Levy 1988), although their data relate to total membrane apposition, and hence not specifically to the PSD. This study made an investigation of ultrastructural changes as a consequence of LTP in the rat hippocampus. They found that although there was no significant change in Sa_{syn} across the entire molecular layer of the *dentate gyrus*, there was an increase in Sa_{syn} in the activated portion, and this could be correlated with a change in shape of the spine synapses in this region from convex to concave.

Since the changes in synaptic surface area reflect changes seen in synaptic height, one may conclude that synaptic shape remained relatively stable, following the learning experience. This is contrary to many studies in other species. In rat sensorimotor cortex during development (Petit and Markus 1987), and in rat hippocampus following repetitive neuronal activation (Petit *et al* 1989), there was a progressive change in synaptic shape from convex, to straight, and then ultimately, concave. Following the onset of LTP in the hippocampus, Desmond and Levy (1983, 1986) found that there was a

significant increase in concave spine synapses, which would support this idea. However, these findings are far from universal, and many studies have failed to show evidence for a change in synaptic shape following learning (Stewart *et al* 1987), or indeed have shown the opposite to that stated above; i.e there has been a loss of synaptic concavity following learning (Wenzel *et al* 1977).

Synaptic bouton volume density ($\bar{V}v_{bouton}$)

The estimates obtained for $\bar{V}v_{bouton}$ in the present study are approximately twice the magnitude of those obtained previously (Stewart *et al* 1987). The methods used in the two studies differ considerably, since the present study used unbiased volume weighted mean volume estimations (Gündersen and Jensen 1985), whilst the study by Stewart *et al* used simple areal volume ratios, which produces highly biased estimations (see Chapter 7, Section 3 for details).

The study by Stewart *et al* (1987) concluded that there was no significant effect of training with methyl anthranilate on the size of the pre-synaptic bouton. The results of the present study are largely in agreement with this, since no statistically significant difference was found in the estimates of $\bar{V}v_{bouton}$, in either left or right hemisphere LPO, between Control and M-trained chicks (Tables 10:16). A two-way ANOVA also failed to show any significant effect of training on $\bar{V}v_{bouton}$ (Table 10:18a). However, there is an indication that a small increase in $\bar{V}v_{bouton}$ may occur following training, since M-trained chicks show a significant increase in $\bar{V}v_{bouton}$ in both left and right hemisphere (Table 10:17). This is in contrast to the Controls, who show no such trend (Table 10:17). The difference may be small enough to be masked by the associated error of estimation. The effect is also seen in the two-way ANOVA of the effects of 'time' and 'hemisphere' on the estimated $\bar{V}v_{bouton}$ (Table 10:18b), where M-trained

chicks show a significant effect of 'time', whilst the Control chicks do not.

Given that an increase in the surface area and mean projected height of the synapse has been demonstrated for the LPO in this study, one might empirically expect to see a small but corresponding increase in the volume of the pre-synaptic bouton, as a consequence. Stewart (1990) summarized the findings of several recent studies by him and his collaborators, by stating that an increase in the number of vesicles per synapse ($Nv_{ves.syn}$) was found in the left hemisphere of the chick IMHV and LPO (of approximately 60%). One might therefore expect to see an increase in the pre-synaptic bouton volume to accommodate this increase. It is of course possible that the increase in vesicle number can be contained within the existing bouton volume, or that the increase in vesicle number precedes a subsequent expansion of the bouton. Indeed, Stewart *et al* (1984) report that the bouton volume increases by approximately 23% in the left hemisphere of the IMHV following training. A significant increase in $\overline{Vv}_{bouton}$ of approximately 19%, was also seen in the left hemisphere of the *paleostriatum augmentatum* (Stewart *et al* 1987). Both regions were analysed 24 hours after the training procedure.

Previous studies of the morphological consequences of imprinting in the IMHV of young chicks, failed to detect any significant alteration in bouton volume (Bradley *et al* 1981, Bradley and Galal 1987). Synaptic bouton volume has been shown to decrease as a consequence of training in the rat hippocampus (Wenzel *et al* 1980). It is possible, that these differences in changes to bouton volume merely reflect different neuronal strategies for learning, and subsequent memory formation.

Dendritic spine volume density ($\bar{V}v_{spine}$)

The results showed that the volume density of the dendritic spines only increased significantly 48 hours after training, and that the increase was restricted to the left hemisphere (Table 10:16). A previous study has shown that the numerical density of dendritic spines in the chick LPO, increases by approximately 13% in the left hemisphere, and 8% in the right (Lowndes and Stewart 1990). Additional data has shown that the increase in spine density in the left hemisphere is approximately twice that in the right (Lowndes, personal communication). The increase in spine density in the left hemisphere was universal across all branch orders of the dendrite (branches from the soma). However, in the right hemisphere, the only significant differences in this parameter were in the 5th branch order (Lowndes, personal communication).

An increase in spine volume density may be due to either an increase in the total number of spines, or an increase in the size of existing spines. Both mechanisms may occur together. Given that the present data show an increase in the numerical density of spine synapses, then it seems probable that the number of spines would also increase, since each spine is commonly associated with a single synapse. Such increases have been shown in the left hemisphere IMHV, but not the right (Patel and Stewart 1988). In addition, Patel and Stewart have demonstrated increases of 9% in the mean diameter of the spine head, and a decrease of 17% in the mean spine stem length following training in the IMHV. Both of these size changes of the dendritic spines were restricted to the left hemisphere. Whether a similar mechanism occurs in the chick LPO is not known.

The size of the dendritic spine has previously been shown to increase in mammals following environmental enrichment (Greenough 1985), and the spine

head to increase during early experience (for review, see Coss and Perkel 1985). It has been suggested previously in this thesis (see Chapter 4) that an increase in the size (and number) of the dendritic spines, may result in an increase in the degree of electrical transfer between neighbouring neurons, via their synaptic connections. This would in effect increase the probability of propagation of a given action potential between neurones (Shepherd *et al* 1985).

Dendritic shaft volume density ($\bar{V}v_{shaft}$)

Training had no significant effect on the volume of the dendritic shaft (Tables 10:16, 17, 18a). A similar conclusion was drawn from studies of the chick IMHV after passive avoidance training, where no significant effect was seen in either dendritic diameter or dendritic length, the products of dendritic volume (Patel and Stewart 1988). Rats exposed to an enriched environment showed an increase in dendritic branching (Greenough 1985), showing that the shaft is capable of plastic change. It does not appear to do so, however, in response to a learnt task, such as that of the present study, and is therefore unlikely to be involved in the formation of memory.

Overview of the time-course of memory formation

Following a single trial passive avoidance learning task, day-old chick brains undergo a series of biochemical, physiological and morphological changes (Rose 1986). These changes are likely to be linked in a time-dependent manner, and may be a consequence of the formation of a permanent memory for the event. Behavioural tests have shown that the chick forms a long-term memory for the taste of the aversive bead 50-60 minutes after training (Gibbs and Ng 1977). The permanence of the memory has yet to be tested beyond 48 hours. This long-term memory, once formed, cannot be blocked by protein synthesis inhibitors,

which are known to cause amnesia if administered before LTM is established (Patterson *et al* 1988). Prior to LTM formation, a period known as intermediate-term memory (ITM) occurs. This begins after 10 minutes, and decays 30 minutes following training (Rosenzweig and Bennett 1984b). ITM is dependent upon Na^+/K^+ pump activity (Gibbs and Ng 1977). Short-term memory therefore lasts no more than 10 minutes following the training experience (Gibbs and Ng 1977). It is thought that these 3 phases are sequentially linked (Rosenzweig and Bennett 1984b). Hence, a series of identifiable stages of memory formation can be correlated with known cellular changes at various times following training. It is clear however, that changes in the cellular or chemical environment associated with memory formation and storage in the chick brain, may not necessarily reflect the stages of memory indicated above. It is possible that certain events may be concerned with 'priming' of the system, in order that a subsequent stage may be attained. It is perhaps more useful to view the steps taken in the process of memory formation, as a continuum. These events are summarized below.

The initial cellular events following training with the aversive substance are unlikely to occur in the LPO, since it has no known involvement with gustatory sensation, and little involvement with the olfactory sense (see Chapter 3). However, within 30 minutes of training, the LPO shows enhanced utilization of glucose (Kossut and Rose 1984, Csillag and Rose 1985), perhaps indicating an increase in synaptic activity, and neuronal firing. There have been no studies of biochemical, physiological or morphological changes in the LPO prior to this time, perhaps largely as a consequence of the focus of research on the IMHV. The STM phase of memory formation in the chick can be inhibited by administration of LiCl, KCl or glutamate (Gibbs and Ng 1977), which cause membrane

depolarization. It has hence been suggested that STM is characterized by neuronal hyperpolarization, perhaps due to an increased membrane conductance to K^+ (Gibbs and Ng 1977). This is contrary to the situation in the invertebrate model, *Hermissenda*, where K^+ currents were shown to be reduced by approximately 30% in type B photoreceptors, following phototaxic training (Alkon *et al* 1982). This finding was thought to explain the increased excitability of the photoreceptors of conditioned animals (Alkon 1983). However, Alkon's group have also shown that there is a cellular influx of Ca^{2+} (Alkon *et al* 1982), which is known to cause an increase in K^+ conductance (Grossman *et al* 1981). The connection between altered ion movements and the correlates of memory, is far from clear.

The LPO, together with the IMHV and paleostriatum augmentatum, show enhanced glucose utilization during short- and intermediate-term memory formation (Kossut and Rose 1984, Csillag and Rose 1985). In the LPO, chicks injected with ^{14}C-2-deoxyglucose 5 minutes prior to training, and 10 minutes (but not 30 minutes) after training, showed elevated intra-cellular accumulation of its breakdown product, ^{14}C-2-deoxyglucose-6-phosphate, which is unable to be metabolized further (Csillag and Rose 1985). ITM is characterized by its dependence upon the activity of the Na^+/K^+ pump (Gibbs and Ng 1977, Rosenzweig and Bennett 1984b). This may partially explain the need for an increase in glucose utilization during this time. The left IMHV demonstrates enhanced metabolic activity in the time-period between 30 and 60 minutes following training (Rose and Csillag 1985), and hence may exhibit a different time-course of memory formation, than that of the LPO.

During the period of ITM following PAL, there is a transient increase in

muscarinic cholinergic receptors in the chick forebrain (Rose *et al* 1980). These increased by 21% at 30 minutes following training, and returned to control levels by 3 hours (Rose *et al* 1980). This elevation in cholinergic receptor density and/or availability, may be an initial stage in modification of the post-synaptic density, resulting in an increase in the efficacy of synaptic transmission. Changes in the biochemistry of pre-synaptic membrane fractions of trained chicks have also been demonstrated. These include protein kinase-C induced phosphorylation of a synaptic membrane protein, with a molecular weight of approximately 52kD (Ali *et al* 1988a, Ali *et al* 1988b).

At the end of the ITM phase of memory formation for the taste avoidance task, there is a period of protein synthesis, since protein synthesis inhibitors administered at this time prevent long-term memory formation (Gibbs and Ng 1977, Patterson *et al* 1989, Rosenzweig and Bennett 1984b). Indeed, 30 minutes after imprinting, chicks display an increase in RNA synthesis (Haywood *et al* 1975, Rose *et al* 1970), and an increase in protein synthesis after 2 hours (Bateson *et al* 1983). Protein synthesis inhibitors can be shown to take effect as early as 30 minutes following passive avoidance training (Gibbs and Ng 1977). There is currently interest in an oncogene which expresses a protein known as c-fos. It is thought that this 'early gene' may be activated in response to some of the biochemical changes outlined above, such as conductivity changes in synaptic membranes and phosphorylation of presynaptic proteins (Rose 1991). There is an increase in c-fos in both the IMHV and the LPO, 30 minutes following PAL (Anokhin *et al* in press). This would indicate that it is a likely candidate in early activation of RNA synthesis.

One group of proteins which show marked changes following PAL, are the glycoproteins. There is an increase in the incorporation of ^{3}H-fucose into

synaptic glycoproteins (Burgoyne and Rose 1980, Sukumar *et al* 1980, Rose and Harding 1984). Fucose is used as a marker for newly synthesized glycoproteins, since it is not metabolized by any other biochemical pathway. The increase was detected by 30 minutes and lasted up to 24 hours post-training (Sukumar *et al* 1980). A similar time-course of the incorporation of ^{14}C-leucine into tubulin was also demonstrated (Mileusnic *et al* 1980). As tubulin is a protein involved in the cellular cytoskeleton, one may hypothesize that cellular reconstruction and re-organization is initiated at least as early as 30 minutes post-training, and lasts up to 24 hours. These events may be precursors for the morphological changes seen in the IMHV (Stewart *et al* 1984), and in the LPO in the present studies.

3-7 hours after avoidance training in the chick, there is an episode of neuronal 'bursting' in both the IMHV (Mason and Rose 1987) and LPO (Gigg 1991) of M-trained chicks but not in controls. 'Bursts' are high frequency, large amplitude, action potential spikes of short duration. The onset in the LPO is delayed by approximately 1 hour (i.e. activity is restricted between 4 to 7 hours post training). The significance of these 'bursting' patterns is not known, although it has been suggested that they are a result of increased excitability of the neuronal population in the presence of increased extracellular K^+, and also as a result of a modification of synaptic inputs into individual neurons (Gigg 1991). This latter feature may involve a reduction in tonic inhibitory input from GABAergic synapses (Gigg 1991). STM is thought to involve an increase in K^+ intra-cellularly, through a change in K^+ membrane conductance, and ITM involves the Na^+/K^+ pump (Gibbs and Ng 1977). This may cause an increase in extracellular K^+ at this time, but does not explain the delay in bursting several hours later.

The early sequence of events subsequent to the experience of the bitter-tasting bead, may be a pre-requisite for the emergence of long-lasting synaptic morphological changes, in the IMHV and LPO. These changes are not observed before 24 hours in the LPO (Stewart *et al* 1987, and present study), and 12 hours in the IMHV (Bradley and Galal 1987). Imprinting studies using chicks have demonstrated an increase in the length of the PSD, 6.5 hours post training (Bradley 1985). It is not known whether the time-course of synaptic plasticity differs between the IMHV and LPO. However, there is some indication that time-courses of the pharmacologically defined stages of memory (Gibbs and Ng 1977) differ in chicks who have received injections into the IMHV, and those who have received injections into the right *lateral neostriatum* (Patterson *et al* 1988). This would suggest that at least for these two regions of the chick brain, there are differences in the time-course of memory formation. There is also some evidence that the memory for the avoidance task is acquired by the IMHV, but is subsequently transferred out of the IMHV after approximately 1 hour, and is then stored elsewhere (Patterson *et al* 1989, Davies *et al* 1988). Since post-training lesions of the LPO cause amnesia, it has been argued that the LPO may be the permanent store for the memory (Gilbert *et al* in press). This model of 'memory flow' between IMHV and LPO has a potential flaw, since no direct neuroanatomical link exists between these two nuclei. An intermediate route may exist however, through the *dorsal archistriatum*. It is therefore important to identify whether any trace of the memory can be found in this nucleus, and to establish whether lesions to it have any specific effects on memory retention.

It is clear that a time-delay of several hours is required for the establishment of morphological changes following PAL. It is not yet known

whether these changes are indeed permanent, or at least as permanent as the memory itself. Other model systems have suggested that some morphological parameters may be longer-lasting than others. In studies of sensitization in *Aplysia*, it has been shown that increases in the size of the PSD are transient, whilst increases in the number of synapses are as permanent as the memory of the stimulus (Bailey and Chen 1990). The results of the present study have shown that there are alterations in both the size and numerical density of synapses in the LPO as a consequence of PAL, and that these changes last at least as long as the 48 hour period, which was the maximum time included in the study. Clearly, the present study opens the door for further experiments to map the longer-term effects on synaptic morphology, and to correlate these more closely with the duration of the memory.

CONCLUSIONS AND FUTURE DIRECTIONS

The capacity of the brain to modify its cellular connections as a consequence of experience, suggests that this feature is a feature and possible mechanism of memory. The present study is not the first to show synaptic morphological changes as a consequence of memory formation. A wealth of evidence is accumulating that the synapse undergoes a variety of structural changes as a direct consequence of training (for review, see Stewart 1990, Greenough 1985, Bailey and Chen 1989a, Petit 1988, Bailey and Chen 1990). In addition, these changes are similar in a wide variety of animals, both vertebrate (Thompson 1985, Greenough 1985, Stewart 1990) and invertebrate (Squire 1987, Kandel and Schwartz 1982, Matthies 1989b). Indeed, much knowledge has now been acquired on the role of particular nuclei in the chick brain for memory tasks (for review, see Stewart 1990). The present study is unique however, in that it maps the time-course of changes in the LPO, a region of the chick brain thought to be involved in long-term memory formation (Patterson *et al* 1989, Gilbert *et al* in press, Stewart 1990). The permanence of these changes has not been fully investigated, although some changes were still shown to be significantly different to Controls, 48 hours after the training experience. It is important to map these changes into adulthood, in order to correlate these with long-term memory retention.

Synaptogenesis is one feature of the brain's remarkable plasticity, in response to learning. This is a process which is specific. It does not occur in all brain regions following training (Greenough *et al* 1990), and is a consistent feature in a variety of species, in forms of learning that differ from one another substantially (Horn 1985b, Desmond and Levy 1983, Greenough 1985, DeVoogd *et al* 1985, Greenough and Bailey 1988). Size and volume changes of the

synaptic environment represent a second feature of neuronal plasticity, and are likely to result in changes in synaptic efficacy (Herrera *et al* 1985) that may also underlie the formation of memory.

The data obtained in the present study partially relate to changes in synaptic number and / or synaptic density. It may have been more prudent to measure the ratio of synapses to neurones, since this is perhaps more meaningful in a functional sense. However, the time required to make counts of neurons, in addition to synapses, would be prohibitive for completion in the present study. This would be of great benefit in further work, for clarification of the extent of neuronal versus synaptic plasticity, in response to the learning task.

The results of the present study are of major interest in the light of another recent experiment on the chick LPO, which showed that this nucleus is essential for the formation of a long-term memory trace, but not for its acquisition (Gilbert *et al* in press). It is clearly important to establish the role played by the various nuclei in the chick brain. Similarities (and differences) in biochemistry, physiology and morphology of these regions following the training procedure, will help our understanding of the onset of the memory trace, and to map its possible alteration in locality with time. The studies presented here, only briefly describe the morphological changes of synapses, and of some pre- and post-synaptic features within the LPO. It is important to correlate these changes more fully with others in the chick brain, in order to have a clearer understanding of the plastic change. Future investigations should perhaps concentrate on particular neurotransmitter systems, since this may be an important factor in regulating which particular synapses are involved in the formation of memory.

APPENDIX 1

TISSUE PROCESSING SCHEDULE

Buffer wash	Two changes.	30 mins
Post-fixation	in 2% osmium tetroxide	120 mins
Buffer wash		10 mins
Dehydration	30% ethanol	5 mins
	50% ethanol	15 mins
	70% ethanol	15 mins
	80% ethanol	10 mins
	95% ethanol	5 mins
	100% ethanol	20 mins
	100% ethanol (dry)	20 mins
Embedding	Spurr's resin (see Appendix 2)	
	50% absolute ethanol	
	50% Spurr's resin	120 mins
	100% Spurr's resin	overnight
	100% Spurr's resin	120 mins
Polymerization	in embedding mould @ 60°C	24 hrs

APPENDIX 2

BUFFER COMPOSITION

Solution A :	5.98% Sodium Cacodylate
Solution B :	0.97 ml 1N HCl

Solution B was added to solution A to produce 0.1M Cacodylate Buffer at pH 7.3, (approximately 385 mOsm)

SALINE COMPOSITION : 0.9% NaCl 20 ml

RESIN : Spurr's resin was chosen for its rapid infiltration of the tissues and its ease of use. The standard embedding medium was prepared as follows;

ERL 4206	10.6g
DER 736	6.0g
NSA	26.0g
S-1	0.4g

These components were thoroughly mixed for several hours before use, using a motorized rotating paddle. Resin was only used if prepared fresh.

APPENDIX 3

TABLE OF MEAN ± SEM SYNAPTIC DENSITIES DURING DEVELOPMENT

Type \ Age		E16 n=5	P1 n=4	P9 n=5	P22 n=4
Nv_{syn}	L	0.23 ± 0.12	0.57 ± 0.09	1.72 ± 0.11	1.10 ± 0.10
	R	0.11 ± 0.07	0.64 ± 0.06	1.06 ± 0.11	1.06 ± 0.10
Nv_{Asymm}	L	0.19 ± 0.09	0.48 ± 0.08	1.54 ± 0.09	1.01 ± 0.09
	R	0.09 ± 0.05	0.54 ± 0.06	0.94 ± 0.10	0.98 ± 0.08
Nv_{Symm}	L	0.03 ± 0.02	0.07 ± 0.01	0.15 ± 0.02	0.08 ± 0.01
	R	0.01 ± 0.01	0.07 ± 0.01	0.13 ± 0.02	0.06 ± 0.02
Nv_{Spine}	L	0.15 ± 0.08	0.50 ± 0.08	1.47 ± 0.09	0.96 ± 0.08
	R	0.08 ± 0.06	0.52 ± 0.04	0.85 ± 0.12	0.87 ± 0.06
Nv_{Shaft}	L	0.06 ± 0.04	0.06 ± 0.02	0.19 ± 0.02	0.12 ± 0.02
	R	0.04 ± 0.02	0.10 ± 0.02	0.17 ± 0.03	0.17 ± 0.03
Nv_{ASp}	L	0.15 ± 0.07	0.47 ± 0.07	1.45 ± 0.09	0.95 ± 0.08
	R	0.08 ± 0.05	0.49 ± 0.05	0.79 ± 0.11	0.85 ± 0.07
Nv_{ASh}	L	0.05 ± 0.02	0.07 ± 0.01	0.14 ± 0.02	0.10 ± 0.02
	R	0.02 ± 0.01	0.08 ± 0.01	0.16 ± 0.02	0.14 ± 0.03
Nv_{SSp}	L	0.02 ± 0.01	0.04 ± 0.01	0.08 ± 0.01	0.04 ± 0.01
	R	0.01 ± 0.01	0.05 ± 0.01	0.09 ± 0.02	0.03 ± 0.01
Nv_{SSh}	L	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.01
	R	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

Results are displayed in μm^{-3}

E16 - 16 days in ovo, P1 - 1 day post-hatch,
P9 - 9 days post-hatch, P22 - 22 days post-hatch,
L - left hemisphere LPO, R - right hemisphere LPO

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

APPENDIX 4

TABLE OF MEAN ± SEM SYNAPTIC NUMBER DURING DEVELOPMENT

Type \ Age		E16 n=5	P1 n=4	P9 n=5	P22 n=4
N_{syn}	L	1.31 ± 0.65	4.41 ± 0.71	12.90 ± 0.85	8.34 ± 0.74
	R	0.49 ± 0.30	3.82 ± 0.36	10.00 ± 1.08	7.46 ± 0.67
N_{Asymm}	L	1.08 ± 0.53	3.73 ± 0.64	11.50 ± 0.66	7.69 ± 0.69
	R	0.39 ± 0.24	3.28 ± 0.34	8.99 ± 0.93	6.91 ± 0.59
N_{Symm}	L	0.18 ± 0.10	0.56 ± 0.09	1.13 ± 0.14	0.57 ± 0.10
	R	0.06 ± 0.04	0.42 ± 0.07	1.00 ± 0.18	0.39 ± 0.07
N_{Spine}	L	0.87 ± 0.44	3.88 ± 0.64	11.00 ± 0.68	7.43 ± 0.60
	R	0.35 ± 0.24	3.14 ± 0.25	8.11 ± 1.13	6.06 ± 0.44
N_{Shaft}	L	0.36 ± 0.20	0.38 ± 0.10	1.40 ± 0.15	0.82 ± 0.10
	R	0.09 ± 0.04	0.59 ± 0.11	1.75 ± 0.13	1.17 ± 0.24
N_{ASp}	L	0.84 ± 0.40	3.65 ± 0.57	10.90 ± 0.68	7.22 ± 0.60
	R	0.34 ± 0.22	2.92 ± 0.28	7.52 ± 1.05	5.96 ± 0.49
N_{ASh}	L	0.27 ± 0.13	0.22 ± 0.06	1.03 ± 0.12	0.73 ± 0.12
	R	0.08 ± 0.04	0.48 ± 0.08	1.48 ± 0.15	1.03 ± 0.19
N_{SSp}	L	0.11 ± 0.04	0.36 ± 0.05	0.60 ± 0.11	0.27 ± 0.04
	R	0.06 ± 0.05	0.30 ± 0.06	0.72 ± 0.12	0.26 ± 0.03
N_{SSh}	L	0.13 ± 0.08	0.18 ± 0.05	0.41 ± 0.08	0.13 ± 0.04
	R	0.04 ± 0.02	0.12 ± 0.04	0.32 ± 0.11	0.18 ± 0.05

Results are expressed as number X 10^8

E16 - 16 days in ovo, P1 - 1 day post-hatch,
P9 - 9 days post-hatch, P22 - 22 days post-hatch,
L - left hemisphere LPO, R - right hemisphere LPO

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

APPENDIX 5

TABLE OF MEAN ± SEM SYNAPTIC HEIGHT DURING DEVELOPMENT

Type \ Age		E16 n=5	P1 n=4	P9 n=5	P22 n=4
$\bar{H}_{syn}$	L	198 ± 17	217 ± 30	317 ± 30	359 ± 17
	R	212 ± 12	203 ± 20	349 ± 22	353 ± 23

Results are displayed in nm

E16 - 16 days in ovo,
P1 - 1 day post-hatch,
P9 - 9 days post-hatch,
P22 - 22 days post-hatch,
L - left hemisphere LPO,
R - right hemisphere LPO

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

APPENDIX 6

TABLE OF MEAN ± SEM SYNAPTIC DENSITIES AT VARIOUS TIMES FOLLOWING TRAINING

Type \ Group	hours	CONTROL Left Hem n=6	CONTROL Right Hem n=6	M-TRAINED Left Hem n=6	M-TRAINED Right Hem n=6
Nv_{syn}	1	0.44 ± 0.02	0.42 ± 0.03	0.41 ± 0.04	0.43 ± 0.02
	6	0.49 ± 0.03	0.53 ± 0.03	0.49 ± 0.04	0.54 ± 0.02
	12	0.45 ± 0.04	0.52 ± 0.04	0.54 ± 0.05	0.60 ± 0.04
	24	0.53 ± 0.05	0.65 ± 0.05	0.69 ± 0.03	0.75 ± 0.03
	48	0.68 ± 0.02	0.66 ± 0.03	0.75 ± 0.02	0.78 ± 0.03
Nv_{ASp}	1	0.33 ± 0.05	0.27 ± 0.03	0.30 ± 0.05	0.26 ± 0.04
	6	0.36 ± 0.04	0.35 ± 0.04	0.35 ± 0.05	0.38 ± 0.05
	12	0.34 ± 0.04	0.33 ± 0.05	0.38 ± 0.03	0.46 ± 0.04
	24	0.42 ± 0.05	0.47 ± 0.04	0.53 ± 0.05	0.54 ± 0.02
	48	0.56 ± 0.06	0.48 ± 0.03	0.65 ± 0.06	0.60 ± 0.05
Nv_{ASh}	1	0.05 ± 0.03	0.07 ± 0.01	0.05 ± 0.03	0.07 ± 0.02
	6	0.05 ± 0.02	0.10 ± 0.03	0.04 ± 0.02	0.09 ± 0.02
	12	0.05 ± 0.02	0.09 ± 0.02	0.05 ± 0.03	0.08 ± 0.01
	24	0.04 ± 0.02	0.08 ± 0.02	0.04 ± 0.03	0.08 ± 0.02
	48	0.03 ± 0.01	0.08 ± 0.02	0.04 ± 0.02	0.10 ± 0.02
Nv_{SSp}	1	0.08 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.02
	6	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.01
	12	0.08 ± 0.01	0.08 ± 0.02	0.09 ± 0.02	0.07 ± 0.02
	24	0.06 ± 0.02	0.07 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
	48	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	0.08 ± 0.01
Nv_{SSh}	1	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
	6	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
	12	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
	24	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
	48	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

Results are displayed in μm^{-3}

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

APPENDIX 7

TABLE OF MEAN ± SEM SYNAPTIC HEIGHT AND SURFACE AREA AT VARIOUS TIMES FOLLOWING TRAINING

Type \ Group	hours	CONTROL Left Hem n=6	CONTROL Right Hem n=6	M-TRAINED Left Hem n=6	M-TRAINED Right Hem n=6
$\bar{H}_{syn}$	1	256 ± 23	238 ± 22	244 ± 22	251 ± 24
	6	286 ± 21	276 ± 25	299 ± 22	263 ± 24
	12	304 ± 28	310 ± 22	375 ± 22	336 ± 27
	24	336 ± 24	326 ± 23	408 ± 24	343 ± 26
	48	332 ± 26	326 ± 29	415 ± 24	339 ± 28
Sa_{syn}	1	24.8 ± 4.1	21.5 ± 3.6	27.0 ± 3.3	26.2 ± 3.6
	6	26.7 ± 4.1	28.0 ± 4.0	30.7 ± 4.3	24.6 ± 3.7
	12	28.1 ± 3.7	23.5 ± 3.8	35.6 ± 3.9	28.8 ± 3.2
	24	29.5 ± 3.8	34.1 ± 3.8	38.5 ± 3.6	31.0 ± 3.6
	48	24.5 ± 3.8	28.3 ± 4.0	36.6 ± 4.4	31.6 ± 4.4

Results of $\bar{H}_{syn}$ are displayed in nm

Results of Sa_{syn} are displayed in μm^2

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

APPENDIX 8

TABLE OF MEAN ± SEM VOLUME-WEIGHTED MEAN VOLUMES AT VARIOUS TIMES FOLLOWING TRAINING

Group / Type / hours	CONTROL		M-TRAINED	
	Left Hem n=6	Right Hem n=6	Left Hem n=6	Right Hem n=6
$\bar{V}v_{bouton}$ 1	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.02	0.01 ± 0.01
$\bar{V}v_{bouton}$ 24	0.12 ± 0.01	0.11 ± 0.02	0.16 ± 0.01	0.14 ± 0.01
$\bar{V}v_{bouton}$ 48	0.15 ± 0.01	0.13 ± 0.02	0.17 ± 0.01	0.16 ± 0.01
$\bar{V}v_{spine}$ 1	0.11 ± 0.01	0.12 ± 0.02	0.16 ± 0.02	0.13 ± 0.02
$\bar{V}v_{spine}$ 24	0.16 ± 0.01	0.15 ± 0.02	0.19 ± 0.02	0.17 ± 0.02
$\bar{V}v_{spine}$ 48	0.14 ± 0.02	0.16 ± 0.02	0.21 ± 0.02	0.17 ± 0.02
$\bar{V}v_{shaft}$ 1	5.35 ± 0.37	5.93 ± 0.42	5.03 ± 0.36	5.56 ± 0.35
$\bar{V}v_{shaft}$ 24	5.52 ± 0.33	5.69 ± 0.34	5.04 ± 0.33	5.52 ± 0.36
$\bar{V}v_{shaft}$ 48	5.23 ± 0.39	5.98 ± 0.38	5.48 ± 0.39	5.27 ± 0.42

Results are displayed in μm^{-3}

N.B. Values given above are rounded up or down, and are not those used in the statistical analysis. Statistical tests were performed using 'raw' data.

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